Developmental aspects of midazolam metabolism

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Ontwikkelingsaspecten van het metabolisme van midazolam

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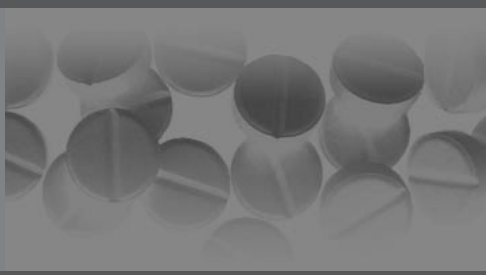
Introduction

Chapter 1



Drug therapy in children

From fetal life through adolescence, dramatic changes in pharmacokinetics and pharmacodynamics occur as a consequence of organ maturation and changes in body composition associated with normal development. 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).

Pharmacokinetics

For those drug molecules that have the ability to be altered via biotransformation, metabolism is the major determinant of drug clearance. Although relative hepatic size and liver blood flow may affect the rate of drug metabolism during development, the maturation of drug-metabolizing enzymes is the predominant factor accounting for age-associated changes in nonrenal drug clearance (2).

In man, the quantitatively most important and well-studied group of drug metabolizing enzymes is the cytochrome P450 (CYP) superfamily. The CYP3A subfamily, the most abundant subfamily of the CYP isoforms in liver, kidney and intestine, consists of at least four isoforms: CYP3A4, CYP3A5, CYP3A7 and CYP3A43 (3). The individual CYP3A isoforms demonstrate a different ontogenic pattern of expression and activity. Accordingly, the pharmacokinetics of specific substrates for CYP3A isoforms may change as a function of developmental changes in enzyme activity (4).

In contrast to the cytochrome P450s, much less is known of phase II drug metabolizing enzymes [e.g., enzymes capable of catalyzing conjugation reactions such as sulfotransferases and uridine 5'-disphosphate (UDP)-glucuronosyltransferases (UGTs)] and how normal growth and development produces functional regulation of their activity. Collectively, phase II drug metabolizing enzymes play important roles in drug clearance by increasing the polarity of drug molecules and/or their metabolites produced by phase I drug metabolizing enzymes (e.g., cytochrome P450s). In humans, many drug conjugation reactions are catalyzed by the different isoforms of UGT (5) and hence, these particular phase II enzymes are quantitatively important as determinants of drug clearance. Failure to recognize the impact of ontogeny on UGT activity has produced therapeutic tragedies in pediatrics such as the 'grey-baby' syndrome associated with administration of chloramphenicol (an UGT substrate) to neonates and young infants (4).

Individual differences in drug metabolism throughout infancy, childhood and adolescence are only partially explained by the ontogeny of drug-metabolizing enzymes. Differences in diet, exposure to non-pharmacologic xenobiotics (e.g., environmental chemicals) and concomitant administration of other therapeutic drugs may contribute substantially to interindividual differences in drug metabolizing enzyme activity. It is now recognized that genetic variation directs the expression of many drug-metabolizing enzymes and therefore also contributes to interindividual differences in drug disposition and action (6).

In many instances, pharmacokinetic data (e.g., drug clearance, elimination half life) obtained in selected pediatric subpopulations for substrates of specific drug metabolizing enzymes have provided valuable insights as to developmental influences in their

activity and in some cases, the demonstration of clear, age-associated "break points". Despite this information, the impact of ontogeny on the regulation of CYP3A and the UGTs at the level of the gene (e.g., regulatory regions) is currently not known (6). However, in view of pharmacogenetic data now available for these and other drug metabolizing enzymes, it is reasonable to suspect that allelic variants of the CYP3A and UGT genes may contribute to the activity of these and other drug metabolizing enzymes.

Midazolam as probe of CYP3A activity.

CYP3A activity can be assessed *in vivo* with the use of pharmacological probes. According to the validation criteria for non-invasive probes for CYP3A activity, midazolam clearance is currently one of the best methods to assess CYP3A activity in adults (7). Midazolam, a short acting benzodiazepine routinely used in both pediatric and adult patients as a sedative and anticonvulsant agent, is metabolized by CYP3A4, CYP3A5 and, to a much lesser extent, by CYP3A7 to its main metabolite 1-OH-midazolam, a compound that also exhibits pharmacologic activity (8). This hydroxylated metabolite of midazolam is subsequently conjugated by one or more UGT isoforms to 1-OH-midazolam-glucuronide, a polar compound that is primarily excreted by renal mechanisms (9).

The careful study of midazolam disposition in neonates and infants, as reflected by its biotransformation and pharmacokinetics, will enable the study of this proven pharmacologic probe for CYP3A4. Specifically, a study of the impact of growth and development on the formation and elimination of 1-OH midazolam and its glucuronide metabolite provides a "window" through which to examine the ontogeny of both CYP3A4/5 and of the UGT isoforms responsible for the conjugation of 1-OH midazolam.

Pharmacodynamics

Despite known differences in drug-receptor interaction that are associated with development, clinicians rarely consider this information in the context of interpreting drug effect, be it therapeutic or toxic, in neonates and young infants. Extrapolation of adult pharmacodynamic data to neonates and infants must be done with care and with the knowledge that development may define the dose *vs*. concentration *vs*. effect relationship. The therapeutic plasma concentration range of drugs is not necessarily the same in children and adults and for most therapeutic drugs, has not been strictly defined for pediatric patients. Maturation processes of receptors and/or other cellular targets responsible for the determination of drug effects may alter the pharmacodynamic response in the developing human. As well, the large interindividual variability observed for pharmacokinetics of many drugs in neonates and infants renders the *a priori* definition of an age-appropriate dosage regimen from adult data difficult if not impossible. The only appropriate alternative is to have a thorough understanding of developmental differences in both pharmacokinetics and pharmacodynamics which is wrought only through the careful investigation of drugs in the pediatric subjects where they are intended for use and in turn, expected to improve health.

Midazolam for sedation in neonates, infants and children

Midazolam exerts its effects through potentiation of the effects of γ-amino butyric acid (GABA), an inhibitory neurotransmitter in the central nervous system. Through this effect, midazolam possesses sedative, anxiolytic, anticonvulsive, muscle relaxant and amnesic effects in both adults and children (10). While midazolam pharmacodynamics have been studied widely in adults, validated tools necessary to quantitate and characterize its sedative effects have scarcely been applied to pediatric intensive care patients (11). In the absence of accurate pharmacodynamic data for midazolam in neonates, current dosing recommendations of this drug for neonates and young infants are based upon prior therapeutic experience and the notion that these patients can be "titrated" to effect as is currently done for older children and adults. In the absence of specific pharmacodynamic data for midazolam in neonates, the therapeutic utility of this potentially useful agent may be compromised by the risks of either over- or underdosing.

In view of the profound age-related differences in pharmacokinetics and pharmacodynamics, physicians who prescribe drugs for neonates, infants and children must be aware of the interindividual differences that often result from developmental patterns of pharmacokinetic and pharmacodynamic processes. The research represented by this thesis provides an example of an integrated approach to critically examine the pharmacokinetics and pharmacodynamics of midazolam, an important and commonly used drug, in a population of neonatal and pediatric intensive care patients. The experimental approaches used were driven by an overall goal of translational science – namely, the discovery of new information that translates the discovery of new scientific information into that which can be used clinically.

The goals the research described in this thesis was to:

1. To review the current knowledge on the ontogeny and genetics of CYP3A and UGTs (Chapter 2 and 3)
2. To determine the pharmacokinetics of midazolam, in children from birth through to adolescence, as a surrogate measure of the ontogeny of CYP3A and UGT activity *in vivo* (chapter 4, 5 and 6)
3. To gain insight into the pharmacogenetics of CYP3A4 and its impact on the developmental expression of CYP3A4 *in vivo* (chapter 7, 8 and 9)
4. To evaluate the pharmacodynamics of midazolam in neonatal and pediatric intensive care patients (chapter 10 and 11)

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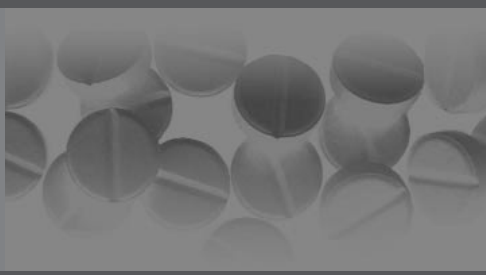
Cytochrome P450 3A. Ontogeny and drug disposition

Chapter 2



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Summary

Maturation of organ systems during fetal life and childhood exerts a profound effect on drug disposition. The maturation of drug-metabolizing enzymes is probably the predominant factor accounting for age-associated changes in non-renal drug clearance. The group of drug-metabolizing enzymes most studied is the cytochrome P450 superfamily. The CYP3A subfamily is the most abundant group of cytochrome P450 enzymes in the liver and consists of at least three isoforms, CYP3A4 , CYP3A5 and CYP3A7. Many, frequently described drugs are mainly metabolized by the CYP3A subfamily. Therefore, maturational changes in CYP3A ontogeny may impact on the pharmacokinetics of many of these drugs.

CYP3A4 is the most abundantly expressed CYP and accounts for approximately 30-40 % of the total CYP content in human adult liver and small intestine. CYP3A5 is 83% homologous to CYP3A4, it is expressed at a much lower level than CYP3A4 in the liver, but is the main CYP3A isoform in the kidney. CYP3A7 is the major CYP isoform detected in human embryonic, fetal and newborn liver, but is also detected in adult liver, although at a much lower level than CYP3A4. Many drugs have been identified as important substrates for the CYP3A family. However, substrate specificity for the individual isoforms has not been fully elucidated. Because of large interindividual differences in CYP3A4 and CYP3A5 expression and activity genetic polymorphisms have been suggested. However, although some gene mutations have been identified, the impact of these mutations on pharmacokinetics of CYP3A substrates has to be established.

Ontogeny of CYP3A activity has been studied in vitro and in vivo. CYP3A7 activity is high during embryonic and fetal life and decreases rapidly during the first week of life. On the contrary, CYP3A4 is very low before birth, but increases rapidly thereafter reaching 50% of adult levels between 6 and 12 months of age. During infancy CYP3A4 activity appears to be slightly higher than in adults. Large interindividual variation in CYP3A5 expression and activity was observed during all stages of development, but no apparent developmental pattern of CYP3A5 activity has been identified to date.

Concluding, profound developmental changes occur in the activity of CYP3A isoforms during all stages of development. These changes have, in many instances, proven to be of clinical significance with respect to treatment, using substrates, inhibitors or inducers of CYP3A. Investigators and clinicians should consider the impact of ontogeny on CYP3A in both pharmacokinetic study design and data interpretation, as well as when prescribing drugs to pediatric patients

Introduction

Maturation of organ systems during fetal life and in the neonatal and paediatric period exerts a profound effect on drug disposition. From fetal life through adolescence, dramatic changes in pharmacokinetics occur. Accordingly, effective and safe drug therapy in newborns, infants and children requires a thorough understanding of human developmental biology and of the dynamic ontogeny of drug absorption, distribution, metabolism and drug excretion (1).

Drug metabolism is one of the major determinants of drug clearance in a given individual. Although the relative size of the liver and of hepatic blood flow may affect the rate of drug metabolism during development, the maturation of the drug-metabolising enzymes is probably the predominant factor accounting for age-associated changes in nonrenal drug clearance (2).

The group of drug-metabolizing enzymes most studied includes the cytochrome P450 (CYP) superfamily. The CYP3A subfamily, the most abundant subfamily of cytochrome P450 isoforms in the liver, consists of at least three isoforms: CYP3A4, 3A5 and 3A7 (3, 4). Recently, developmental changes in CYP3A expression and catalytic activity have been studied (5-9) demonstrating a different ontogenic pattern for the individual CYP3A isoforms. Accordingly, the pharmacokinetics of CYP3A substrates may change as a function of developmental changes in CYP3A activity (10).

In view of the profound age-related differences in CYP3A activity, physicians who prescribe drugs for neonates, infants and children should be aware of the interindividual differences that often result from developmental patterns of enzyme expression and activity. The goal of this review is to provide the practitioners with insight into the developmental aspects of CYP3A activity and their possible impact on drug therapy.

Cytochrome 3A, structure and function

Cytochrome P450 superfamily

The cytochrome P450 superfamily represent a superfamily of heamcontaining proteins that catalyze the metabolism of many lipophilic endogenous substances and exogenous substrates. The biological and pharmacological relevance of this gene family has been the subject of several recent publications (11-13).

The nomenclature for the P450 superfamily is based on divergent evolution of the genes. CYPs that share at least 40% homology, are grouped in a family, which is annotated by an Arabic number (e.g. CYP3). A subfamily, indicated by a letter represents highly related genes (e.g. CYP3A). Another Arabic numeral (e.g. CYP3A4) sequentially numbers the individual genes. In humans, 17 CYP gene families have been described to date; the first three families are mostly involved in the biotransformation of pharmaceuticals and xenobiotics, wheras the other gene families represent genes responsible for the biotransformation of endogenous compounds (4). The most abundant and most involved in drug metabolism are the isoforms of the CYP3A subfamily (11).

The CYP3A subfamily

The CYP isoforms belonging to the 3A subfamily account for the majority of drug metabolising enzymes present in adult human liver and intestine (14-17). The CYP3A

subfamily consists of at least three functional genes: CYP3A4, CYP3A5 and CYP3A7 (4) which are located on chromosome 7 (18). The enzymes compromising the CYP3A subfamily share at least 85% amino acid sequence homology. However, they have been shown to differ substantially in substrate specificity and expression (11). The existence of many additional CYP3A genes is not likely, since a single CYP3A gene is approximately 30 kbp in length and only 90 kbp of human genomic DNA hybridize with CYP3A complementary DNA under low-stringency conditions (19).

CYP3A4

CYP3A4 is the most abundantly expressed CYP in both the human liver and in the small intestine and accounts for approximately 30%-40% of total CYP content in both liver and intestine (14, 20). In adult human liver, CYP3A immunoreactivity was detected in midzonal and centrilobular regions (21), wheras intestinal CYP3A occurs in the enterocytes lining the lumen of the small intestine (14, 20). Inoue et al. (18) assigned the CYP3A4 gene to chromosome 7 at band q22.1. The gene is divided in 13 exons and 12 introns with a length of approximately 27 kbp (22).

The catalytic properties of CYP3A4 have been studied extensively using expressed enzyme, immunoinhibition experiments and the correlation of metabolic activity and immmunoreactive CYP3A4 content in microsomal samples (11). The list of exogenous substrates of CYP3A4 is growing fast (table I) and includes a large number of therapeutically important drugs such as erythromycin, midazolam, cyclosporin, lidocaine (lignocaine) and nifedipine (10). Endogenous substrates include steroids as testosterone, cortisol, progesterone, androstanediol, dehydroepiandrosterone 3-sulfate (DHEA-S) and estradiol (11). CYP3A4 also metabolizes procarcinogens as sterigmatocystin and aflatoxin B1 (23).

CYP3A5

CYP3A5 is 83% homologous to CYP3A4 and is also found in hepatic tissue, although at a lower level than CYP3A4. Large interindividual differences for CYP3A5 expression are found. Initially, CYP3A5 mRNA and protein were detected in 10 to 30% of adult human liver samples (15, 24-27). More recently, however, Jouanaidi et al. (28) detected CYP3A5 mRNA in all livers and CYP3A5 protein in 74% (14 out of 19) of all livers examined . This apparent difference in expression of CYP3A5 may be explained by the use of more sensitive analytical methods (28). It should also be noted that expression of CYP3A5 mRNA differed widely between individuals, ranging from little CYP3A5 mRNA in some individuals, to CYP3A5 being the only CYP3A expressed in others (28).

The substrate specificity of CYP3A5 appears to be similar to that of CYP3A4. However, some differences in catalytic properties have been found. In a reconstituted system, the formation rate of 1-OH-midazolam is considerably higher with CYP3A5 than with CYP3A4. In contrast, the rate of formation of 4-hydroxy-midazolam with CYP3A4 and CYP3A5 is similar (29). No CYP3A5 catalytic activity was found towards quinidine, 17α-ethinylestradiol and aflatoxins (30), all substrates for CYP3A4. However, Gillam et al. (31) did find considerable catalytic activity of CYP3A5 towards both erythromycin (about six times higher when compared to CYP3A4) and ethylmorphine. Interestingly, Gorski et al. (29) found a much better correlation between midazolam hydroxylation and

Table 1 *List of important CYP3A substrates, adapted from (67, 116).*

Drugs			Xenobiotics	Endogenous substrates
Antihistamines	**Anti-fungals**	**Anesthesia/analgetics**	Aflatoxin B1	DHEA-S
Astemizole	Ketoconazole	Alfentanyl	Sterigmatocystin	DHEA
Mizostaline	Miconazole	Fentanyl	Benzphetamine	Testosterone (6-β-OH)
Terfenadine	**Immunosuppressants**	Lidocain	Heterocyclic amines	Testorsterone (2-β-OH)
Anti-reflux	Cyclosporin (M17 formation)	Ethylmorphine	Benzopyrene activation	Testosterone (15-β-OH)
Cisapride	Cyclosporin (M1 formation)	**Antihypertensives**		Cortisol (6-β-OH)
Anti-emetic	Tacrolimus (FK506)	Amlodipine		Progesterone (6-β-OH)
Ondansetron	**Chemotherapeutics**	Felodipine		Androstanedione (6-β-OH)
Anti-epileptic	Busulfan	Isradipine		Estradiol
Carbamazepine	Doxorubicin	Nicardipine		17-α-ethinylestradiol
Clonazepam	Etoposide	Nifedipine		
Ethoxisumide	Tamoxifen (also 2D6)	**Anti-arrhythmics**		
Zonisamide	Vinblastine	Verapamil		
Anti-HIV	Vincristine	Quinidine		
Indinavir	**Benzodiazepines**	**Antidepressants**		
Ritonavir	Alprazolam	Imipramine		
Saquinavir	Diazepam (minor)	Nafzadone		
Antibiotics	Midazolam (1-OH formation)	Sertraline		
Clindamycin	Midazolam (4-OH formation)	**Miscellaneous**		
Erythromycin	Temazepam	Dextromethorphan		

erythromycin *N*-demethylation when livers containing both CYP3A4 and CYP3A5 were excluded from analysis; a finding which supports the different isoform specificity for these drugs. Wrighton et al. also reported that erythromycin is not metabolized by CYP3A5 (30).

The formation of carbamazepine 10,11-epoxide from carbamazepine and 2-sulphamoylacetylphenol (SMAP) from zonisamide catalyzed by CYP3A5 was approximately 33% and 10% , respectively, as compared with CYP3A4 activity (32).

CYP3A5 actively metabolizes estradiol, DHEA-S and cortisol (30). However, Ohmori et al. (32) did not find any activity towards DHEA-S by heterologously expressed CYP3A5. The 6β-hydroxylation of testosterone by CYP3A5 occurred at about 10% of CYP3A4 activity in one study (32), but was similar in another (31).

In summary, the specificity of CYP3A4 and CYP3A5 for biotransformation of many substrates appears to be similar although the extent and rate of metabolic conversion by the individual isoforms may be quite different for a given substrate. The discrepant results of *in vitro* studies probably reflect the sensitivity of CYP3A metabolic activities to incubation conditions.

CYP3A7

CYP3A7 is the major CYP isoform detected in embryonic, fetal and newborn liver, but is also detected in adult liver, although at much lower levels than CYP3A4 (7, 9, 33). The CYP3A7 gene is also located on chromosome 7 and its nucleotide sequence is nearly 90% homologous to that of CYP3A4 (18, 19).

Only a few substrates have been studied with regard to a role for CYP3A7 in their biotransformation. Formation of 1-hydroxy-midazolam and carbamazepine-10,11-epoxide are only marginally supported by CYP3A7 as compared to CYP3A4 (5). The metabolism of zonisamide by CYP3A7 was approximately 70% of that by CYP3A4 activity (32). In contrast, the biotransformation of cisapride to either nor-cisapride or its two primary ring-hydroxylated metabolites by CYP3A7 is at least 10-fold less than that observed with CYP3A4 under the same experimental conditions (34).

CYP3A7 also plays an important role in the biotransformation of endogenous compounds. CYP3A7 catalyzes the 16α-hydroxylation of DHEA-S, a physiologically important reaction for the formation of estriol in pregnancy with a higher affinity and maximal velocity than CYP3A4 (32, 35). CYP3A7 is minimally involved in the 6β-hydroxylation of testosterone, although this conversion is essentially supported by CYP3A4 (5). Finally CYP3A7 is capable of metabolising potential environmental pollutants (e.g. aflatoxin B1) (5, 36). Data derived from fetal liver microsomes may provide additional information on the role of CYP3A7 in the biotransformation of several substrates, since it is the main CYP isoform present in the fetal liver (33).

Interindividual and intraindividual variation of CYP3A activity

Interindividual variation in CYP3A expression (3, 21, 37) is reflected by large interindividual differences (e.g. 4- to 13-fold) in plasma clearance of CYP3A substrates (38-43). Using a human liver bank (24 adult Caucasian men and women), Transon et al. (27) found a 3.8 fold (1.9 to 7.2 µl/L) difference in the Michaelis-Menton constant (Km) and a 13 fold (9.5 to 123.4 nmol/mg protein/h) difference in the maximum rate of metabolism (V_{max}) for the 1'-hydroxylation of midazolam. Thummel et al. (44), using a human liver bank assembled from organ donors (n = 21) found a 29 fold variation of V_{max} for midazolam 1-OH hydroxylation, whereas Kronbach et al. (45) only found a 5 fold variation. Thummel et al. (46) determined CYP3A content in intestinal microsomes from 15 donors and found an interindividual variation exceeding 18-fold for each region of the gut.

Intraindividual variation in midazolam plasma clearance [CV% as a measure of CYP3A activity *in vivo*] ranged from approximately 5 to 20%, which is considerably smaller than interindividual variation (47). Ten adult patients, not receiving known CYP3A inducers, underwent two erythromycin breath tests within two weeks which showed a <27 % change in test results (43). As reviewed by Fahr (38), the pharmacokinetics cyclosporin exhibit up to two-fold intraindividual variability, which may partially be attributed to variability in CYP3A activity.

In contrast, the interindividual variation in CYP3A7 mRNA in fetal liver was only 2.5-fold, which is considerably lower when compared with the 10-fold variation in CYP3A4 expression levels found in adult liver (37).

Genetic variation in CYP3A expression

Racial and gender influences, which may explain part of the variability in CYP3A activity; however, study results remain inconclusive. In one study, no differences in hepatic CYP3A mRNA and protein were found between Caucasians and Japanese, but CYP3A dependent activities appear to be higher in Caucasians when compared to Japanese (3). Others could not detect differences in catalytic activity towards nifedipine, a well-known CYP3A substrate (48). Recently, Chavez-Teyes et al. (49) have suggested that Mexican (Mestizo) males may have reduced CYP3A4 activity as compared with Caucasian males based upon higher midazolam bioavailability and reduced plasma clearance of midazolam in a small population (n=11) of adults.

CYP3A activity measured *in vivo* by means of different probe drugs (50) was slightly higher in women when compared to men in two studies (39, 43), but other investigators could not confirm this gender difference (47, 51). Christians et al. (52) did not find a

gender difference in the area under the concentration-time curve (AUC) after oral cyclosporin administration, but when co-administered with the CYP3A inhibitor diltiazem, the AUC of cyclosporine was significantly more increased in women than in men. *In vitro* studies, using human liver microsomes, failed to support a gender difference in CYP3A activity(3, 37), but when using human intestinal microsomes, cyclosporine was metabolized significantly faster by microsomes from female than from male patients (51). Finally, the menstrual cycle phase does not appear to influence CYP3A4 activity evaluated using midazolam in adults (47). No correlation has been found between age or gender and heterogeneous CYP3A5 expression (26, 30).

To date, the large interindividual variation in CYP3A4 activity and expression could not be attributed to a genetic polymorphism of the CYP3A4 gene (39). Recently, however, Rebbeck et al. (53, 54) and Felix et al (54) identified a mutation in the CYP3A4 promoter region which was associated with an increased severity of prostate cancer at diagnosis compared to patients without the mutation. The authors speculated that this may be because of CYP3A4 mediated differences in testosterone metabolism. They also reported a strong correlation between the incidence of secondary tumors after acute lymphocytic leukemia in children and this CYP3A4 promoter region mutation. However the consequence of allelic variation in the CYP3A4 promoter region for CYP3A4 activity *in vivo* has not been addressed.

CYP3A5 cDNA sequencing revealed a point mutation, which was detected in two out of five individuals with absent CYP3A5 protein (28). The authors suggest that one or more mutations may explain the heterogeneous expression of CYP3A5, but no additional studies have pursued this possibility.

Extrahepatic sites of CYP3A expression

The presence of CYP3A in other organs may also contribute to interindividual variability in the disposition of CYP3A4 substrates. CYP3A4 is expressed in esophagus, duodenum, small intestine and colon, but not the stomach, with the upper small intestine serving as the major drug metabolising site of the gastrointestinal tract (15-17, 55). In adults, approximately 40% of total CYP3A4 content is thought to reside in the small intestine (46). Significant biotransformation of selected CYP3A4 substrates has been observed in the intestinal wall, considerably affecting the oral bioavailability of these drugs (14, 42, 56-58). For example, when midazolam was given to healthy volunteers, the extraction ratio for intestine and liver were comparable (approximately 40%) resulting in an overall oral availability of 30% (46, 58). Consequently, disease states that affect the intestinal epithelium may reduce CYP3A4 activity, resulting in an increased bioavailability of CYP3A4 substrates (59). In contrast, when midazolam was administered parenterally and delivered by arterial blood to the intestinal mucosa, only less than 10% of the delivered dose was extracted in the intestine, compared with almost 60% of the dose when delivered, after oral administration, to the luminal side of the intestine (46).

Although intestinal CYP3A4 activity plays an important role in the first pass-extraction of many CYP3A4 substrates, interindividual variability in the activity of the intestinal transporter P-glycoprotein (P-gp) may be an equally significant determinant of oral bioavailability, at least for cyclosporin (46). Moreover, overlapping substrate specificity exists for intestinal CYP3A and P- glycoprotein, which should also be

accounted for when comparing disposition of CYP3A substrates after intravenous and oral administration.

CYP3A5 appears to be the main CYP3A isoform in the stomach and esophagus, although data are conflicting regarding the expression pattern of CYP3A5 in the remainder of the gastrointestinal tract. In general, depending on the analytical method used, CYP3A5 appears to be polymorphically expressed in the human intestinal tract, with considerably lower activities, when compared to CYP3A4. (15, 16, 55, 60). CYP3A5 is the major CYP3A isoform detected in human kidney, lungs, blood and pituitary gland. Haehner et al. (61) detected CYP3A5 protein and mRNA in 100% of kidney samples. Interestingly, the investigators found a bimodal distribution in CYP3A5 content and activity. Renal CYP3A5 content and activity were 100- to 1000-fold lower than that found for hepatic CYP3A4. (61). Renal CYP3A4 mRNA was detected in 40% of kidney samples and in 70% of these samples, catalytically active CYP3A4 protein could be measured (61).

CYP3A is expressed in neutrophils and B-lymphocytes, but not T-lymphocytes, although the analytical method was unable to distinguish between CYP3A4 and CYP3A5 (62). Since CYP3A content could not be induced by rifampicin (rifampin), it was suggested that the CYP3A isoform detected in human blood cells was more likely to be CYP3A5 than CYP3A4 (63) (64).

Interestingly, CYP3A5 mRNA and protein were detected in the human pituitary gland and were located in growth hormone containing cells. This finding suggests that CYP3A may be involved in the regulation of growth hormone production (65). Finally, low or negligible CYP3A7 and CYP3A5 expression was found in extrahepatic embryonic and fetal tissue (7, 61, 66).

CYP3A drug interactions

The vast majority of drug interactions mediated via CYP3A are the result of either induction or inhibition of this enzyme (67).

The effect of induction is to increase CYP3A4 content and to enhance drug clearance by this route (68). CYP3A4 activity can be induced *in vitro* and *in vivo* by corticosteroids (e.g. dexamethasone), anticonvulsants (e.g. phenobarbital, phenytoin, carbamazepine) and several antimicrobials (e.g. rifampin, rifapentine) (69-75). The molecular mechanism of CYP3A induction has not been fully elucidated. Only recently, Lehmann et al. (76) identified a specific nuclear receptor in the CYP3A promoter region, which may explain the induction mechanism of CYP3A activity at least for some compounds. Inhibition of CYP3A4 activity *in vitro* and *in vivo* has been reported for several compounds such as imidazole derivatives, erythromycin, clarithromycin, troleandomycin, gestodene, ritonavir, fluvoxamine and grapefruit juice (71, 77-81). Known inducers of CYP3A4, such as dexamethasone, rifampicin or phenobarbital, do not appear to induce CYP3A5 activity *in vitro* (24, 25). Nevertheless, Schuetz et al. (82) showed that the CYP3A5 gene contains the sequence information (encoding a dexamethasone responsive element in the promoter region) needed for corticosteroid regulation of transcription. *In vitro* inhibition of CYP3A5 activity has been demonstrated for triazolam (32) and gestodene (30). Ketoconazole and fluconazole are only weak inhibitors for CYP3A5 activity in human hepatocytes relative to their inhibiting effect on CYP3A4 activity(83). Pretreatment of HepG2 cells expressing human CYP3A7, with dexamethasone,

rifampicin, troleandomycin, erytromycin, phenobarbital, phenobarbital-like inducers or lovastatin produced dose-dependent increases of CYP3A7 mRNA and immunoreactive protein (24). Finally, rifampicin has been shown to induce CYP3A7 expression in adult human hepatocytes (84), while gestodene inhibits its activity (36).

Development of CYP3A activity

Although several exogenous factors are important determinants of interindividual variation in CYP3A activity, the impact of ontogeny on the activity of CYP3A isoforms contributes significantly from birth through adolescence. *In vitro* techniques have been used to assess the impact of development on CYP3A activity, but the majority of information currently available is derived from either pharmacokinetic studies of CYP3A substrates or the *in vivo* examination of biotransformation of CYP3A pharmacological 'probe' compounds.

Ontogeny of CYP3A activity assessed in vitro

The benefits and problems of the different methods used to determine CYP3A expression and activity *in vitro* have been discussed in recent reviews (8, 85). Briefly, problems with sensitivity or quantitation may arise, depending on the methods used to detect CYP expression. Specifically, studies which have determined total amounts of immunoquantifiable CYP3A protein from liver may not have used assays capable of differentiating the respective CYP3A isoforms (48). Such limitations may explain conflicting results concerning the assessment of ontogeny of CYP3A activity *in vitro*. The limitations may now be overcome with the use of antibodies raised against isoform-specific peptide sequences or individual isoforms expressed in cells (5).

Total CYP content

Total hepatic CYP content appears to be fairly stable from early fetal life to the first year after birth with reported levels between 30% and 60% of adult levels (86-88). Other authors, (21, 89) however, reported levels in neonates and infants similar to those in adults.

CYP3A content

Fetal CYP3A content (immunoquantitated from liver microsomes) ranges from 30% to 100% of adult CYP3A content (88, 90). An early report showed that total CYP3A content represents 85% of total CYP content in fetal liver, increasing in parallel with the total amount of CYP during development (86). However, another study specifically measuring CYP3A7 showed that its content represents only around 30% of total fetal CYP content (88). During the transition from fetal to neonatal life total CYP3A content appears to be relatively stable (5).

CYP3A4

In embryonic hepatic tissue (6-12 weeks gestational age) CYP3A4 mRNA could not be detected (37). In contrast, CYP3A4 mRNA was detected in fetal liver microsomes (at between 11 and 30 weeks gestational age) at 10% of adult levels, increasing immediately after birth and reaching approximately 50% of adult levels between 6 and 12 months

of age (5, 84, 91). As was shown by Lacroix et al. (5) testosterone β-hydroxylase activity in human liver microsomes, mainly an activity of CYP3A4, was extremely low in the fetus with an increase after birth reaching 30-40% of adult levels from 3 months to one year of age and 120% of adult levels after the age of one year. Gestational age at birth (25 to 40 weeks) did not have an influence on this ontogenic pattern of CYP3A4 activity (5).

CYP3A5

CYP3A5 is consistently demonstrated in embryonic liver (37). However, CYP3A5 protein could be detected in only 10% of fetal livers (30). Gender does not appear to affect fetal expression of CYP3A5 (37). Wrighton et al.(30) showed that CYP3A5 was expressed in nearly 50% of all infant livers studied, while in the same study expression of CYP3A5 was only found in 29% of adult livers. CYP3A5 protein was detected in the livers of a 2-month and a 14-year-old patient in another study (25). Given the dependence of CYP3A5 quantification on various (i.e. different) analytical methods, the aforementioned results provide a useful, albeit limited, picture of CYP3A5 ontogeny.

CYP3A7

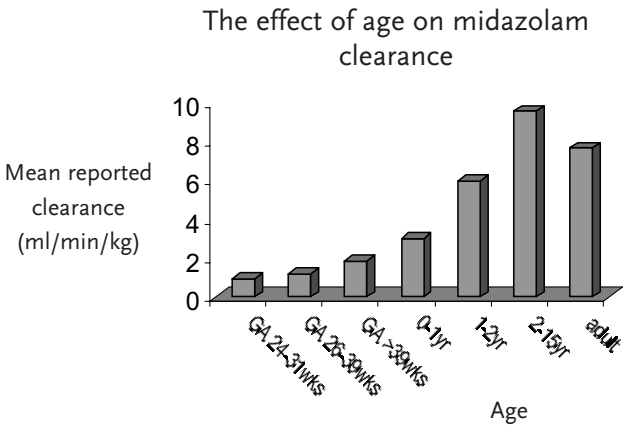
CYP3A7 constitutes about 32% of total CYP content in the human fetal liver (88) and is not detected in other organs during embryogenesis (days 50 to 60) (7). The hydroxylation of DHEA-S, which is mainly catalyzed by CYP3A7 and to a much lesser extent by CYP3A4, was considerably higher in liver samples from 10- to 20 weeks fetuses, when compared with a liver sample from a term infant (5, 35). Immediately after birth, DHEA-S hydroxylation more than doubled, with the highest activity being reached between postnatal day 1 and 7. The activity decreased dramatically after the first week of life to only 10% of newborn levels between 3 and 12 months of age (5). Reported CYP3A7 mRNA expression in human adult livers ranges from 54% to almost 90% of samples (37, 91). However, the amount of CYP3A7 mRNA detected was only 1.7% to 10% in adults when compared to the fetus (37, 84).

CYP3A7 mRNA and protein are also detected in adult endometrium and placenta and in higher amounts in the endometrium of pregnant women. The placental and endometrial CYP3A7 content appears to increase significantly from the first to the second trimester of pregnancy (92). CYP3A7 content in placenta and endometrium per gram of tissue is between 0.6 and 5.5 % of CYP3A7 content in fetal liver. During gestation, the contribution of CYP3A7 in endometrium and placenta to the metabolism of substrates for this isoform should not be underestimated since placenta weight is approximately five times more than total fetal liver weight.

In summary, before birth CYP3A7 activity is high whereas CYP3A4 activity is very low. Directly after birth, a transition from predominantly CYP3A7 activity to mainly CYP3A4 activity occurs (5).

Finally, Table II summarises *in vitro* studies on CYP3A-mediated metabolism during development.

Figure 1 *Mean midazolam clearance (corrected for body size using kilogram bodyweight) after intravenous midazolam administration using data from: (100, 102, 104-106, 163-167).*



GA = gestational age, postnatal age <15 days

Figure 2 *The ontogeny of CYP3A4 and CYP3A7 activity expressed as activity measured using isoform specific probes in human liver microsomes. Due to a lack of information on CYP3A5 activity during development we did not include an ontogenic pattern for CYP3A5 in the figure.*
For references see text.

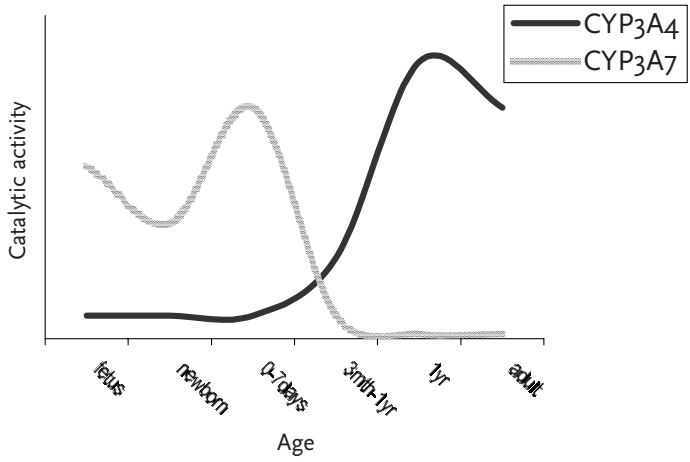


Table II CYP3A Substrates

Substrates	Isoforms expressed in cells			Catalytic activity towards CYP3A substrates in human liver microsomes7				References (67, 116)	Comments
	CYP3A4	CYP3A5	CYP3A7	Adult (%)	Child (%)	Newborn (%)	Fetus (%)		
Drugs									
Carbamazepine	+++	++	+	Present			CBZ-E formation (?)	(32)(155)	age-dependent change in metabolites formed CBZ-E present in stillborn fetus of mother receiving CBZ
Zonisamide	+++	+	++					(32)	
Indinavir				100	67 (6-11yrs)		32	(90)	
Erythromycin	+++	ND/++++						(30)(31)	
Cyclosporin (M17 formation)	+++	ND						(26)	3A5, less metabolites formed
Cyclosporin (M1 formation)	+++	+++						(26)	
Diazepam (minor)				100	140 (3-12 months)	15 (<24h pp), 40-50 (1-7days pp)	<5	(142)	Temazepam formation rate (CYP3A mediated)
Midazolam (1-OH formation)	+	+++	+/-					(5)(29)	
Midazolam (4-OH formation)	++	+++						(29)(156)	
Lidocaine	++	+++	?					(117)	
Ethylmorphine	+	++		100			100	(31)(66)	
Dextromethorphan				100			30	(157)(158)	3 MM formation (CYP3A)
Nifedipine	+++		+++	100	44		18	(26)(30)(90)	
Quinidine	+++		ND					(30)	
Endogenous substrates									
DHEA-S	++	++	+++					(30)(32)	
DHEA (16α-OH)	+		+++	+			+++	(5)(30)(35) (86)	
DHEA (16β-OH)				+++			+	(86)	
Testosterone (6-β-OH)	+++	++	+	100	30-40%	30-40	2-10	(88)(159)(160)	
Testosterone (2-β-OH)	+++	++	++ / ++++	<1% or 100	12-38		12	(5)(25)(32)(90)	
Testosterone (15-β-OH)	+++	+/-						(26)	
Cortisol	+++	++	_					(30)(112)	
Progesterone (6-β-OH)	+++	++						(26)(37)	
Androstanedione (6-β-OH)	+++	++	++					(26)(159)	
Xenobiotics									
Aflatoxin B1	+++		ND	100			200	(23)(36)(88)(161)	activation of afb1 in vitro
Sterigmatocystin	+++		+++	100			50	(36)(88)	
Benzphetamine	+++		+++					(31)(33)	
Heterocyclic amines	+	++	+++					(162)	(activation of IQ and MeIQ, from cooked meat and fish and tabacco)
Benzopyrene activation	Activity?		+++	100			600	(88)	

Isoforms expressed in cells: *Catalytic activity measured using CYP3A isoforms expressed in cells, +(little)-++++(extensive): catalytic activity in relation to the other CYP3A substrates, ND: Not detected*

Catalytic activity towards CYP3A substrates: *percentage catalytic activity measured in pediatric liver microsomes/hepatocytes when compared to activity in human adult liver microsomes/hepatocytes*

Ontogeny of CYP3A activity in vivo

Methods to measure CYP3A activity in vivo

As reviewed by Watkins (50), CYP3A activity can be assessed *in vivo* with the use of different pharmacologic probes. According to the validation criteria for non-invasive probes for CYP3A4 activity as defined by Watkins (50), the erythromycin breath test (ERMBT) is currently one of the best validated method to assess CYP3A activity *in vivo* (93). The ERMBT results correlate significantly with the plasma clearance of orally given cyclosporin (94) and intravenously administered midazolam (93), the latter also correlates with *in vitro* CYP3A4 content and catalytic activity (44, 58). Furthermore, the plasma clearance of midazolam significantly correlates with that of nifedipine (95) and nifedipine oxidation activity *in vitro* is significantly correlated with CYP3A4 protein levels (48). Another important CYP3A probe is the urinary 6β-hydroxy-cortisol:cortisol (6βOHF:C) ratio which, however, does not correlate with either ERBMT or midazolam clearance (96, 97). As discussed by Watkins (50) a possible explanation for this lack of correlation may be that renal CYP3A5 also catalyzes the metabolism of cortisol and therefore, urine based assays, such as the 6βOHF:C ratio, may not correctly reflect hepatic CYP3A activity. Nevertheless, cortisol is frequently used as a probe to assess CYP3A induction (72).

Because of the intravenous use of [N-methyl^{14}-C]-erythromycin, the ERBMT has formidable ethical obstacles to overcome before it can be used in children. An attractive alternative is the use of oral ^{13}C-labeled (stable isotope) erythromycin which, because of technical difficulties has not yet been used to study CYP3A activity in this population. In contrast, the plasma clearance of erythromycin does not provide information on CYP3A activity since the drug is mainly cleared unchanged into bile and only a small part proceeds by hepatic N-demethylation (98).

The plasma clearance of midazolam has also been used to assess CYP3A activity. Jacqz-Aigrain et al. (99-101) studied the pharmacokinetics of midazolam in premature and term neonates. The plasma clearance of midazolam was significantly reduced in newborn infants (1.8 - 2.2 ml/kg/min) when compared with that of adults (6.3-11 ml/kg/min) (100, 102) and was even lower in preterm infants younger than 39 weeks gestational age (1.2 – 1.6 ml/kg/min) (5, 100, 103). Since midazolam is only slightly metabolized by CYP3A7 (29), the reduced clearance of midazolam in the newborn may be explained by developmentally low CYP3A4 activity following birth [5]. A marked increase in midazolam clearance (per kg bodyweight) in the first weeks of life, as may be expected to occur in parallel with a developmentally regulated surge in CYP3A4 activity, is not, however, observed. Conflicting data exist on midazolam clearance after the newborn period. In one study, clearance was similar in older infants, children (i.e., 8.0 ml/kg/min) (104) and adults (i.e., 6.3 –11.1 ml/kg/min) (102, 105). While in another, Hughes et al. (106) found lower clearances in children (2.3 and 3 ml/kg/min) until two years of age. In older children, however, (3 to 13 years old) midazolam clearances were higher (13ml/kg/min) than those observed in adults (6.3-11 ml/kg/min). These changes are summarised in figure 1.

The oral clearance of midazolam has been proposed as a measure of combined hepatic and intestinal CYP3A activity in adults (46, 50) Although the cDNA of hepatic and intestinal CYP3A4 is similar (56), they appear not to be regulated in concert (107). Therefore, it is unclear if intestinal CYP3A activity changes in parallel with hepatic

CYP3A activity during ontogeny. Oral midazolam bioavailability is approximately 36% in adults (108), somewhat higher than that found in children where bioavailability ranged from 15% to 27% with doses ranging from 0.15 to 1.0 mg/kg (109). No data are currently available on the oral clearance of midazolam during the first weeks of life.

Cortisol is hydroxylated by CYP3A4 and CYP3A5 (110, 111) and to a much lesser extent by CYP3A7 (112). The 6β-OHF : C ratio has been used to study CYP3A activity in neonates up to one year of age. Nakamura et al. (113) measured the 6β-OHF : C ratio at different time points during the first two weeks of life. Within 24 hours of after delivery, the ratio was significantly larger compared to adults and declined to adult levels within 3 days after birth. A similar pattern of urinary 6βOHF : C ratios was found by Vauzelle-Kervoedan et al. (114) in term infants at a single occasion between 1 and 15 days after birth. In infants from one month to one year of age, the mean 6β-OHF : C ratio was lower compared with that of neinates and adults. No correlation could be found between the high 6βOHF : C ratio of term neonates on the day of birth and the 6βOHF : C ratio of their mothers, measured two hours after delivery (111). The authors speculate that, given their observation that cortisol is also metabolised by CYP3A7 and that CYP3A7 activity is high directly after birth (5), the observed elevated 6βOHF : C ratio seen on the first day of life may be caused by age-related changes in CYP3A7 activity. This is, however, in marked contrast to the low testosterone 6β-hydroxylase activity observed *in vitro* in fetal livers, CYP3A7 has a higher catalytic activity towards testosterone than cortisol *in vitro* (5).

The 6βOHF : C ratio in premature infants was significantly lower directly after birth, than in term infants, with no change during the first 2 weeks of life (113). It is unclear if these differences in 6βOHF: C ratio between preterm and term infants accurately reflect the differences in CYP3A4 activity during the first two weeks of life. Lacroix et al. (5) did not find a gestational age related (25 to 40 weeks) difference in postnatal development of CYP3A7 and CYP3A4 activity in human liver microsomes. Since the reduced renal function seen in preterm infants may reduce the renal clearance of cortisol and its metabolite, gestational age and postnatal age related changes in CYP3A activity may be obscured.

Nifedipine is a well-defined CYP3A substrate and its plasma clearance may therefore be associated with CYP3A activity (50). Pharmacokinetics of oral nifedipine were studied in children with bronchopulmonary disease aged 5 to 68 months. The elimination half-life ($t^{1}/_{2}\beta$) of nifedipine was lower in children (1.8 h.) when compared with the previous data in adults (2.4 to 3.4 h) (115). As was pointed out by Kearns (116), the plasma clearances varied considerably, which obscured age-dependent changes (from 5 to 68 months of age) in clearance.

Lidocaine is also metabolized by CYP3A4 (117) to its main metabolite monoethylglycinexylodide (MEGX). The MEGX formation rate has been proposed as a general test for hepatic dysfunction (as was recently reviewed by Tanaka (118)). However, the use of MEGX formation rate as a measure of CYP3A4 activity *in vivo* seems less appropriate, since only few of the criteria for a CYP3A probe, as discussed by Watkins et al. [48] have been met. Moreover, lidocaine is a high extraction drug, and variability in its clearance may therefore be more importantly explained by hepatic blood flow differences than by variation in CYP3A activity (50).

Pharmacokinetics of important CYP3A substrates in children

Cyclosporin

Cyclosporin is a widely used immunomodifier in peadiatric transplant recipients. In their comprehensive review, Cooney et al. (119) discussed the age-related differences in cyclosporin pharmacokinetics in transplant recipients. Akthough the apparent volume of distribution does not appear to change as a function of age, cyclosporin plasma clearance (on a per kg or body surface area basis) is considerably higher in paediatric patients younger than 8 years when compared to adults. As a result age-specific cyclosporin dosage requirements are necessary to prevent allograft rejection (120, 121).

This increased dose requirement for cyclosporin in children is suggestive of increased hepatic CYP3A activity in this age-group. Furthermore, as cyclosporin is also metabolized by intestinal CYP3A (122), developmental changes in intestinal CYP3A activity may also contribute to the observed lower oral bioavailability in paediatric patients as opposed to in adults (119). Differences in surgical approach to liver transplantation between children and adults may also contribute to higher dose requirements in children (119). Moreover, it is possible that age-dependent expression and activity of transporter protein P-glycoprotein which is also located in the intestinal wall may contribute significantly to the presystemic clearance of cyclosporin (123). As illustrated by a recent case-report where CYP3A activity was induced following rifampin administration (75), the altered activity of CYP3A, which occurs consequent to development, may be of significant magnitude to impact upon the toxicity profile of this drug.

Tacrolimus

Tacrolimus (FK 506) is also an immunosuppressant used in paediatric patients to prevent post-transplant allograft rejection. As with cyclosporin, a correlation appears to exist between whole blood concentration of tacrolimus and both efficacy (i.e. no rejection) and toxicity in adults and children (124). Yasuhara et al. (125) found marked interindividual variability in the pharmacokinetics of tacrolimus in children aged between 3 months to 15 years after liver transplantations. Part of this variability was explained by a decrease in clearance (per kg bodyweight) of tacrolimus with increasing bodyweight and an increase in clearance with postoperative days. This finding supports the observation, that children younger than 3 years old need 2 to 3 times higher oral doses per kg bodyweight of tacrolimus to maintain the same plasma trough concentrations as older children and adults (125).

Filler et al.(126), who studied the disposition of tacrolimus in renal transplant patients, also found considerable interindividual variation in AUC values after oral administration of tacrolimus. Therefore, they recommend therapeutic drug monitoring of tacrolimus in paediatric patients. Interestingly, they did not find substantial pharmacokinetic differences in tacrolimus disposition in this group of patients when compared with adult data, which may be explained by the older age of the children studied and the inclusion of young adults (age range was to 23 years).

Etoposide

After oral administration of the antineoplastic agent etoposide (VP-16) in paediatric patients between 1.1 and 17 years of age, the AUC and half-life values were only slighter lower than those reported for adults (127). However, the systemic clearance of etoposide

was not significantly altered by the age in contrast with disease-related changes in serum albumin, which accounted for 46% of the variability of etoposide clearance(127). Likewise, Boos et al. (128) did not observe any age-related changes in the clearance of etoposide, in children and young adults between 3 months and 28 years of age when corrected for bodyweight or body surface area(128).

Carbamazepine

CYP3A4, and to a lesser extent CYP2C8, catalyse the biotransformation of carbamazepine to its main metabolite carbamazepine-10,11-epoxide (73). The observation of this metabolite at very low concentrations when compared to carbamazepine concentrations in the circulation of stillborn fetuses of mothers receiving carbamazepine may be interpreted as evidence that carbamazepine is metabolised by CYP3A7. (129). A significant correlation was found between age and carbamazepine dose ratio (reciprocal of apparent oral clearance) in patients with epilepsy, ranging between 3 months and 29 years of age (130). In addition, Korinthenberg et al.(131) found an inverse relationship between the ratio of the epoxide metabolite to carbamazepine and age in children ranging in age from 2 weeks to 15 years who were at steady-state, either on carbamazepine monotherapy or with concomitant administration of other aromatic anticonvulsants capable of inducing CYP.

Astemizole

Shortly after its introduction, the histamine H_1- receptor antagonist astemizole, which is a CYP3A4 substrate (67), was associated with cardiotoxic effects in children following accidental overdosing of this drug. Several investigators reported a prolonged QT interval, ventricular dysrrhythmia, atroventricular block and brief episodes of ventricular tachycardia and fibrillation (132-134). However, no data are available on the pharmacokinetics of astemizole in children, so the effect of coadministration of a CYP3A inhibitor or predisposition for a prolonged QT-interval cannot be determined at this moment.

Cisapride

The prokinetic drug cisapride is widely used in young children for the treatment of gastro-oesofageal reflux. The biotransformation of cisapride is mediated by CYP3A4 with little contribution of the other CYP3A isoforms (34). Reports of prolonged QTc interval and in some cases serious ventricular arrhythmias, when cisapride was ingested with known CYP3A4 inhibitors (135-137) suggest that reduced CYP3A4 activity present in young infants (5) may predispose them to concentration-related adverse effects. Despite reduced CYP3A4 activity in the first month of life, an increased clearance of cisapride (normalized to bodyweight) was seen in premature infants with postnatal ages from 15 to 199 days when compared to the dose requirements with adults. There was no evidence of significant increases in the plasma cisapride concentrations (137). However, further clinical studies will be needed to clarify the association between the pharmacokinetics of cisapride and the consequences of a prolonged QT-interval in preterm infants.

Caffeine

In the neonate, CYP1A2 activity is severely reduced, while CYP3A isoforms are present. Cazeneuve et al. (138) demonstrated that the biotransformation of caffeine in the newborn

is primarily dependent on CYP3A and not on CYP1A2, as is the case in older infants and adults. Moreover, in contrast with the adult, 85% of a caffeine dose is excreted unchanged by the kidney in neonates (139). Consequently, reduction in the activity of CYP1A2 and CYP3A activity early in life is less important than renal function with respect to caffeine pharmacokinetics in the neonate (138-140).

Inhibition and induction of CYP3A activity during development

In vitro inhibition studies with known CYP3A substrates (midazolam, DHEA en progesterone) have revealed significant inhibition of ethylmorphine metabolism (66). Significant inhibition of CYP3A7 expressed in COS cells was seen for triazolam, but not for troleandomycin (32).

Tateishi et al.(9) detected CYP3A7 protein in two out of 10 livers of patients aged 2 years and older (8 and 10 year of age, respectively). The authors speculated that the long-term treatment of these children with drugs known to induce CYP3A activity may have induced their CYP3A7 expression. However, CYP3A7 mRNA but not functional protein was induced by rifampicin in human adult hepatocytes.

No controlled studies have been performed studying the effect of inhibitors or inducers on CYP3A activity in children. Some limited information can, however, be gained from examination of relevant case-reports. Sereni et al. (141) reported that in neonates who received phenobarbital during fetal life or early after birth, the urinary excretion of diazepam metabolites was significantly higher than in neonates who did not. Treluyer et al. (142), showed that in fetal liver microsomes from the offspring of mothers who received a CYP3A inducer (e.g. phenobarbital, prednisone), concomitedly with diazepam, the formation of both temazepam (CYP3A-catalyzed) and N-desmethyldiazepam (CYP2C-catalyzed) were dramatically increased when compared with controls.

Hiller et al. (143) described the case of an 8 year old boy who received an oral doze of midazolam (0.5mg/kg orally) as premedication, followed one hour later by an erythromycin infusion. Shortly thereafter he lost consciousness which was subsequently regained after discontinuation of the erythromycin, a known CYP3A4 inhibitor. As expected, the plasma concentrations of midazolam were significantly elevated when erythromycin was coadministered. Hughes et al. (106) reported a similar inhibitory effect of erythromycin on the kinetics of midazolam, resulting in reduced consciousness in a child.

Cyclosporin produced a two-fold increase in the AUC of the CYP3A substrate etoposide when given as combination chemotherapy for solid tumors in children (144, 145). This inhibitory effect of cyclosporin on clearance of etoposide is similar in children when compared with adults (67) and suggestive of competitive inhibition of hepatic CYP3A by cyclosporin. The authors advised reducing etoposide dose by 50% when given to paediatric patients in combination with cyclosporin (145).

The $t^{1}/_{2}\beta$ of carbamazepine in neonates exposed to the drug *in utero* was comparable with that seen in adults (146). Given the fact that CYP3A4 activity is considerably lower in neonates (5) and that all mothers in this study also received phenytoin, it would appear that CYP3A isoforms in the fetus and neonate are inducible (146). Detection of carbamazepine-10,11-epoxide in fetuses of mothers who received carbamazepine before delivery not only suggest a role for CYP3A7 in the epoxidation of carbamazepine, but

also the inducibility of this process. However, it should be noted, however, that -10,11-epoxide concentrations are also determined by the activity of the drug-metabolising enzyme epoxide hydrolase which further metabolises epoxide, and that the activity of this enzyme may also be altered by drugs such as phenytoin (147).

Finally, carbamazepine concentration : dose ratios were decreased, and formation of the 10,11-epoxide metabolite was significantly increased, in paediatric patients who received phenytoin in addition to phenobarbital (148). Despite clear evidence which demonstrates that CYP3A isoforms are inducible during development, the impact of ontogeny on the rate and/or extent of such interactions is not generally known.

Regulation of CYP3A activity during ontogeny

Little is known about the factors that govern CYP3A7 expression in the fetus and the transition from CYP3A7 to CYP3A4 activity after birth. Hashimoto et al. (22) showed that both CYP3A7 and CYP3A4 have characteristic sequences in the 5'-flanking region with an identity of 91%. The 5' flanking region of CYP3A5 is only 74% related to both CYP3A4 and CYP3A7 (149). Several consensus sequences for transcription factors (e.g. estradiol response element, CAAT box) were found in all three 5'flanking regions. Moreover, the 5'flanking region of CYP3A has specific deletions and/or sequences which seem characteristic for each gene. However, whether these sequences and/or deletions are involved in the regulation and differential expression of the individual CYP3A genes during development remains, however, to be established.

Given the ability of human growth hormone to modulate the effect of many general transcription factors, and the effect of human growth hormone to enhance CYP3A mediated drug metabolism, growth hormone may be an important factor in the transcription of CYP3A, as discussed by Leeder et Kearns (10). Interestingly, Liddle et al. (150) showed recently that human growth hormone and also dexamethasone regulate CYP3A4 gene expression, possibly at the pretranslational level, in cultured human hepatocytes. Moreover, they demonstrated an inhibitory effect of tri-iodothyronine on the transcription of the CYP3A4 gene. The role of these hormonal factors in the regulation of CYP3A4 expression during development needs, however, to established.

Ontogeny of CYP3A activity and the impact on drug development

The CYP3A family is involved in the biotransformation of numerous therapeutically important drugs. Clearly, distinct ontogenic patterns exist for the three isoforms of this subfamily.

Because of the difficulty in obtaining liver and other tissue material from children, scarce data are available on the ontogeny of the individual CYP3A isoforms in the neonatal period, infancy and childhood. Moreover, data on the disposition of individual substrates and their specific metabolic profile at different phases of ontogeny are lacking for many CYP3A substrates.

Accordingly, application of existing data to modify the design of pharmacokinetic studies of CYP3A substrates, inducers or inhibitors is limited in most instances to inferential information gleaned from individual published studies. Given the apparent

lack of correlation between liver size and hepatic microsomal enzyme activity (151), and between hepatic CYP activity and the amount of hepatic microsomal protein (152) caution must be exercised when plasma drug clearance is used to assess the impact of development on CYP3A activity. More specifically, when extrapolating pharmacokinetic data for CYP3A substrates in relation to CYP3A activity, it is important to note that corrections made for bodyweight or body surface area may not accurately reflect age-related differences in drug metabolism (153). *In vitro* (5) and *in vivo* (116) data clearly support a marked reduction in the activity of CYP3A4/5 during the first one to two months of postnatal life. However, during infancy, and for the first two to three years of life, CYP3A4 activity appears to exceed adult values as reflected by clearance of midazolam (106), cyclosporin (119) and tacrolimus (125). When these data are converted using the allometric model (2), the age-dependent changes in clearance persist, which is suggestive of elevated CYP3A activity in this age group. Accordingly, pharmacokinetic data would appear to support at least two developmental 'breakpoints' for CYP3A activity during the first three years of life. It must be noted, however, that these apparent developmental 'patterns' of CYP3A activity, may be significantly influenced by both exogenous (e.g. drugs capable of altering CYP3A expression, activity and /or the access of a substrate to the enzyme) and endogenous (e.g. intrinsic hepatic disease, or the effect of cytokines associated with systemic infection on CYP3A activity (118, 154)). Such factors, depending upon the direction and magnitude of effect, could well obscure the apparent impact of ontogeny (10). Nonetheless, the design of pharmacokinetic studies and ultimately of dosing regimens for CYP3A substrates during the first two to three years of life must capture the developmental pattern of enzyme function so as to ensure adequate characterisation of the disposition characteristics of a drug and the translation of this information into the provision of safe drug therapy.

Future directions

Clearly, several critical information gaps exist with regard to the overall impact of ontogeny on CYP3A activity. First, the pharmacokinetically important CYP3A substrates should be completely characterized over the first 3 to 5 years of life. Also, other important drugs used in the newborn period should be studied in order to clarify a possible 'compensatory' role for CYP3A when the CYP isoforms may be markedly reduced.

Second, the factors governing the transition from CYP3A7 to CYP3A4 around birth should be elucidated since this may provide us insight into the genetic and possibly, neurohumoral factors, that upregulate the activity of this and possibly other CYPs.

Thirdly, the role of both hepatic and renal CYP3A5 on xenobiotic disposition must be better characterised. Several authors suggest that the lack of correlation between different probe drugs to assess CYP3A activity may be because of the differential contribution of hepatic and renal CYP3A5 to their metabolism. This is a potentially important consideration when CYP3A4 activity is low.

Finally, the role of intestinal CYP3A and the impact of ontogeny on its activity must be studied because of its potential importance in determining presystemic drug clearance. It is quite possible that age-dependent differences in the rate and/or extent of oral bioavailability for CYP3A substrates may be more a function of changes in the activity of intestinal CYP3A than age-associated differences in gastrointestinal motility.

Conclusion

Profound developmental differences in the activity of CYP3A isoforms occur *in utero*, in the neonatal period, through infancy and early childhood and to adolescence. In many instances these changes have proven to be of clinical significance with respect to treatment using substrates, inhibitors or inducers of CYP3A. Investigators and clinicians must consider the impact of ontogeny on CYP3A in both the design of studies and the interpretation of pharmacokinetic data, as well as in the provision of therapy to paediatric patients.

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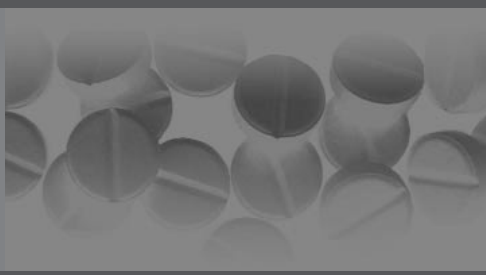
Glucuronidation in humans: Pharmacogenetic and developmental aspects

Chapter 3



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Summary

During human development impressive changes in drug disposition occur. An important determinant of drug clearance is metabolism, something that is not only determined by ontogenic regulation but also by genetic processes which add to the variability of drug metabolism during different stages of childhood. Therefore, and understanding of the developmental regulation of different metabolic pathways, together with information on the genetic determinants of drug metabolism, will increase the knowledge of inter- and intraindividual variability in drug disposition during childhood.

Conjugation has historically received less attention than cytochrome P450 metabolism. An important group of conjugation reactions are catalysed by the uridine 5'-diphosphate(UDP)-glucuronosyltransferases (UGTs); to date at least 10 different UGT isoforms have been identified. The UGTs are not only involved in the metabolism of many drugs [e.g. morphine, paracetamol (acetaminophen)] but also capable of the biotransformation of important endogenous substrates (e.g. bilirubin, ethinlyestradiol) and several xenobiotics. Isoform specificity for these substrates has, however, not been fully characterised.

Serious adverse effects associated with chloramphenicol toxicity in the neonate have highlighted the importance of developmental changes in UGT activity. However, isoform-specific differences preclude the generalisation of a simple developmental pattern for UGT activity. UGT 2B7 is the only UGT isoform for which ontogeny has been characterized both in vitro and in vivo, using morphine as the probe drug. However, no general developmental pattern for the individual UGT isoforms which might be of value for the clinician is currently available.

Genetic polymorphisms have been identified for the UGT family. Not only for the UGT1A gene, which is associated with impaired bilirubin conjugation, leading to genetic hyperbilirubinemia (the Crigler Najjar and Gilbert's syndromes), but also for 3 other UGT isoforms. However, the impact of these genetic differences on drug metabolism remains to be established because overlapping isoform specificity of the drugs studied, as well as a lack of specific probe substrates to test the activity of individual UGT isoforms in relation to these gene mutations.

Clearly, an information gap exists regarding the developmental and genetic aspects of UGT regulation and its potential impact on therapy. More research is needed on the pharmacogenetics and ontogeny of the UGTs for effective translation of scientific information into clinically applicable knowledge.

Introduction

Maturation of organ systems during fetal life and in the neonatal and paediatric period exerts a profound effect on drug disposition. From fetal life through adolescence, dramatic changes in the activity of drug-metabolising enzymes occur. Accordingly, effective and safe drug therapy in newborns, infants and children requires a thorough understanding of human developmental biology and of the dynamic ontogeny of drug metabolism and drug excretion (1).

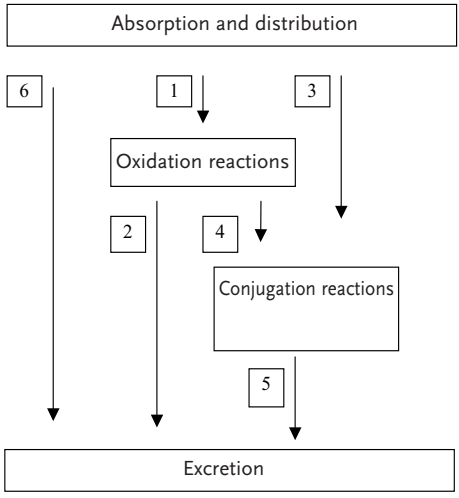
Drug biotransformation reactions by the cytochrome P450 (CYP) family have been subject to extensive research. Historically, less attention has been given to the discussion of conjugation reactions [e.g. by sulfotransferases or uridine 5'-diphosphate (UDP)-glucuronosyltransferases (UGTs)]. Nevertheless, enzyme conjugation reactions play an important role in drug metabolism and detoxification. In humans, a large group of conjugation enzymes is represented by the UGTs (fig. 1).

Failure to recognise the impact of ontogeny on UGT activity has produced therapeutic tragedies in paediatrics such as the 'grey-baby' syndrome associated with administration of chloramphenicol to neonates (2).

Individual differences in drug metabolism during childhood are only partially explained by the developmental changes involving drug-metabolising enzyme(s). They may also be because of differences in diet, exposure to xenobiotic compounds and concomitant administration of other drugs. It is now recognised that genetic variation directs the expression of many drug-metabolising enzymes and therefore, also contributes to interindividual differences in drug metabolism (3).

Consequently, physicians who prescribe drugs to neonates, infants and children should be aware of the interindividual and intraindividual differences that might result from the interaction between developmental and genetic factors. The goal of this review is to provide the practitioner with insight concerning pharmacogenetic and developmental aspects of the UGTs.

Figure 1 *Biotransformation pathways*



The concept of biotransformation via oxidation and conjugation pathways (see bracketed numbers in text)

Hydrophilic compounds as drugs and other xenobiotics are transformed by adding a functional group (oxygen, sulfur, carbon) to form more water-soluble compounds [1]. They can either directly be excreted in bile or urine [2], or serve as substrates for conjugation [4]. The cytochrome P450 system constitutes the largest group of these oxidative enzymes. Subsequently a substrate [3] [4] is conjugated to an endogenous compound, such as glucuronic acid, sulfate acetate, glutathione, thereby further enhancing water solubility with subsequent excretion [5]. In addition, some drugs are not subject to any drug metabolism and are excreted unchanged in urine and/or bile [6].

Uridine 5'-diphosphate (UDP)-glucuronosyltransferases (UGTs)

Structure and function

The glucuronosyltransferases have been previously characterized with respect to their activity and also their physiologic and pharmacological importance. (4) (5) (6) (7) (8) (9)

Mammalian UGTs are part of a gene superfamily consisting of enzymes that catalyze the addition of the glycosyl group from a nucleotide sugar to a small hydrophobic molecule (aglycone). (10) Homologous enzymes are found in plants, animals and bacteria.

Hydrophobic compounds that have been metabolised by phase I enzymes, providing them with an essential functional group (e.g. carbon, sulfur, and oxygen) are acceptor substrates for glucuronidation. Glucuronic acid is associated with the substrate molecule through the catalytic activity of an UGT (Figure 2).

The mammalian UGTs are responsible for the glucuronidation of hundreds of hydrophobic endogenous and xenobiotic compounds. Endogenous substrates are bilirubin, bile acids, thyroxin and steroids. (11) Numerous therapeutic agents [e.g. morphine, paracetamol (acetaminophen), Non-steroidal anti-inflammatory drugs (NSAIDs)] are also glucuronidated. Finally, the UGTs are responsible for detoxifying an extensive group of potentially carcinogenic or teratogenic xenobiotics that enter the body as components of the diet or as airborne pollutants (6).

Although UGTs do enable drug detoxification by enhancing renal excretion of hydrophilic intermediates, glucuronide metabolites are not necessarily inactive and in some instances may be pharmacologically active or toxicologically reactive. For example, morphine-6-glucuronide is approximately 100 times more potent as an analgesic than is morphine. Reduced clearance of this metabolite may therefore lead to a prolonged analgesic effect, with an increased risk of side effects (12). Drug bioactivation may be involved in the pathogenesis of drug hypersensitivity reactions. For example, acylglucuronides of NSAIDs can interact with mammalian host proteins, leading to immune-mediated anaphylaxis and other adverse effects (13) (14).

The nomenclature for the UGT superfamily is based on divergent evolution of the genes that control their expression. The root symbol UGT is commonly used. UGTs that share at least 50% homology are grouped in a family which is annotated by an Arabic number, followed by a letter, indicating a subfamily and an Arabic numeral representing the individual gene (e.g. UGT1A1 or UGT2B4). (15) To date, at least 18 different human UGT isoforms have been identified by gene sequencing and cDNA cloning (15). These advances have enabled the evaluation of a broad variety of possible substrates.

The determination of substrate specificity for the different UGTs is complicated by overlapping substrate activities (i.e. one substrate is metabolised by more than one isoform) and broad substrate specificity (i.e. one isoform glucuronidates a wide range of substrates). Representative substrates for different UGT isoforms are contained in table I.

UGT Gene Structure and Genetic Polymorphisms

The human UGT1 gene is located on chromosome 2 at locus 2q.37. (16) The UGT1 gene consists of at least 13 unique varieties of exon 1 and four common exons 2 to 5. A role for alternative splicing was suggested when cDNA sequencing showed significant homology between the different UGT cDNAs for the C-terminal region, but a lack of overlap for the N-terminal coding region (17). Each exon 1 is preceded by its own promoter region

Table I *Glucuronosyltransferase isoform specificity: representative substrates*

	Endogenous substrates	Exogenous substrates	References
UGT isoform			
UGT1A1	Bilirubin	SN-38 (irinotecan metabolite) Ethinylestradiol	(47) (91) (79) (44)
UGT1A3	Estrone	Norbuprenorphine	(61)
UGT1A4	Androstanediol	Imipramine Amitriptyline	(95)
UGT1A6	?	Acetaminophen Naftazone Naproxen 1-Napthol 2-Napthol	(91) (90) (92) (76) (85)
UGT1A9	Estrone	Propofol Acetaminophen*	(80) (90) (76) (47)
UGT1A10	?	Mycophenolic acid	(96)
UGT2B4	Hyodeoxylicholic acid	?	(97)(98)
UGT2B7	Androsterone Epitestosterone	Morphine Codeine Buprenorphine Naloxone (and other morphinan derivatives) Lorazepam NSAIDs: Naproxen Salicylate Ketoprofen Ibuprofen Valproic acid Menthol Propanolol	(85) (99) (40) (100) (62)
UGT2B15	Androgen steroids #	Eugenol 4-Hydroxy-biphenyl	(21)(101)
UGT2B17	Androsterone Testosterone Dihydrotestosterone		(63)

Vmax = maximum enzyme activity; ? = unknown

Table II *Known polymorphisms of human UGT genes*

Gene	Mutation	Distribution	Expression	In vitro activity and substrate specificity	In vivo activity	Reference
UGT1*	In exons 1 to 5	Different	Absent (CNI) Reduced (CNII)	Absent or Reduced	Leading to CN I and II	Cf. ref (31)
UGT1A1	Promoter region TA replicate of TATA-box	Homo- (up to 13%) and heterozygous (45 %)	Reduced	Reduced	Leading to Gilbert's syndrome	Cf. ref (31)
UGT1A6	Double mutation in exon 1	Heterozygous in 33% of population	Protein expression similar	Lower for salicylic acids	?	(102)
UGT2B15	?	Heterozygous and homozygous	25% of mRNA and protein expression	Substrate specificity is similar	?	(103)
UGT2B7	Single amino acid substitution	?	Similar?	Different substrate specificity	?	(62)

mRNA= messenger RNA; ? = unknown

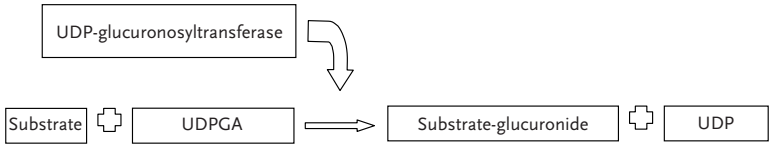
and encodes a unique UGT isoform. The messenger RNA encoding each UGT isoform is formed by the fusion of one of the exons 1 to the four exons 5 (17) (18, 19)(fig 2).

Consequently, gene mutations in the common exon 2 to 5 region can lead to changes in activity and/or expression of additional isoforms, while gene mutations in the unique exon 1 or promoter region may only affect the unique isoform involved. In contrast to the UGT1 family, molecular comparison of the different UGT2B family members suggests that they are encoded by independent genes, probably all located on the human chromosome 4 (20-22). The consequences of a genetic polymorphism for a drug-metabolising enzyme are generally most pronounced when a given pharmacological substrate has a narrow therapeutic range or when the genetic defect leads to a complete lack of enzyme activity. To date, a genetic polymorphism for the UGTs (by the definition of Meyer (3)) has only been observed in association with bilirubin glucuronidation (23, 24).

Genetic Hyperbilirubinaemia

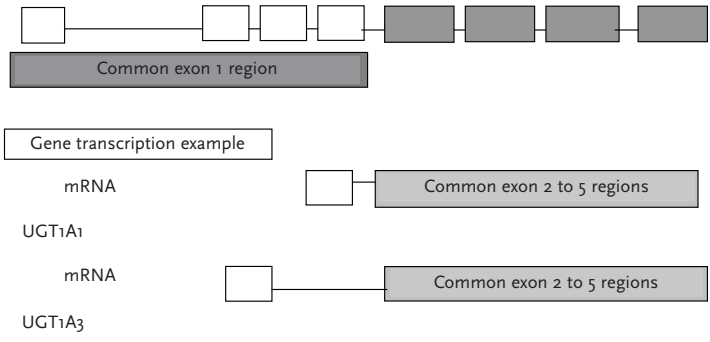
Unconjugated hyperbilirubinaemia may result from a defect in the UGT1 gene responsible for the glucuronidation of bilirubin (25). The clinical manifestation of this genetic hyperbilirubinaemias is very heterogeneous, which can partially be explained by the complicated regulation of UGT1 gene expression. Some individuals have a defect in the bilirubin specific exon 1 region, which results in reduced activity of the UGT1A1 isoform. (26) (27) (28) (29) Other individuals with defects in the common exon 2 to 5

Figure 2 *Mechanism of glucuronidation.*



Abbreviations: UDP = uridine 5'- diphosphate; UDPGA = uridine 5'diphosphoglucuronic acid

Figure 3 *UGT1 gene structure*



An example of alternative splicing leading to different mRNAs with subsequent different UGT proteins
Adapted from (31).

region (26) (30) may also have changes in activity of the other UGT1 isoforms sufficient to alter the biotransformation of endogenous or exogenous substrates (e.g. drugs and environmental chemicals).

Defects in the UGT1A gene (which occur in less than 1% of the population) lead to serious and often lethal hyperbilirubinaemia, a condition known as the Crigler Najjar syndrome type 1 (CNI) or type 2 (CNII). CN1 and CNII can be differentiated by absent and reduced enzyme (UGT1A1) activity, respectively (26). To date, over 30 mutations leading to Crigler Najjar syndrome have been reported (31).

Gilbert's syndrome constitutes a milder form of congenital unconjugated hyperbilirubinaemia, which may be considered a genetic polymorphism in the UGT1 gene. Recent studies (32) (33) have shown that a mutation in the promoter region of the UGT1 gene is associated with Gilbert's syndrome. This genotype could be correlated with the Gilbert's syndrome (phenotype) in healthy volunteers who fasted and abstained from alcohol and drug use. Genotyping performed in three different populations (i.e. Japanese, Eastern Scottish and Inuit Indians from Canada) revealed that about 3, 12 and 17%, of these populations, respectively, were homozygous for the promoter region mutation while approximately 20% of the Japanese and 50% of the other populations were heterozygous. (34) (32) (35) Three additional mutations have been identified in causal association with Gilbert's syndrome, one of them producing a clinically more serious phenotype. (31) (29) (36)

Other UGT Genetic Polymorphisms

Since the availability of heterologously expressed UGT isoforms, mutations in both the UGT1 and UGT2 gene have been found (Table II). With exception of the genetic hyperbilirubinaemias, to date a genetic polymorphism caused by these mutations has not been identified, in large part due to the absence of isoform-specific probe substrates. Consequently, polymorphic drug glucuronidation has not been extensively studied in humans. Earlier investigation of fenofibrate, paracetamol, dextromethorphan and clofibrate were difficult to interpret since these drugs are known to be metabolised by more than one UGT isoform. However, for none of these drugs could a genetic polymorphism be found. (37) (38) (39)

Extrinsic factors such as diet, exposure to pollutants and the concomitant use of other drugs may also cause a bimodal distribution of pharmacokinetic data suggestive of a genetic polymorphism. For example, dietary differences of Chinese compared to Caucasians were suggested to explain a 50% higher urinary excretion of codeine glucuronides in the Chinese group. The opposite was found for morphine glucuronidation. Given the fact that the most important UGT isoform for the metabolism of both morphine and codeine is UGT2B7, the assertion that factors other than UGT2B7 activity may explain these interethnic differences in pharmacokinetics appears to be supported. (40-42)

Impact of Genetic Defects in UGT Activity on Drug Metabolism

Reduced or absent UGT1A1 activity may not only impair the metabolism of bilirubin, but also that of exogenous compounds that are metabolised by UGT1A1. Genetic defects in the common exon 2 to 5 region of the UGT1 gene, in the case of Crigler Najjar or Gilbert's syndrome, may also lead to absent or decreased UGT activity towards

substrates of the other UGT1 gene products. Accordingly, these patients may be at an increased risk for drug toxicity from treatment (or exposure) to substrates of UGT1.

Little research has been performed on the impact of the genetic hyperbilirubinaemias on drug glucuronidation. Recently, Wasserman et al. (43) reported that the administration of the cytostatic drug irinotecan (with the active metabolite SN-38, which is metabolised by UGT1A1) led to severe toxicity in oncology patients with Gilbert's syndrome. This toxicity appeared to be related to accumulation of SN-38, as may be expected when UGT1A1 activity is reduced (44).

Conflicting results were found when paracetamol disposition was examined in patients with Gilbert's syndrome. Some researchers found a decrease in paracetamol clearance and an increase in plasma concentrations of bioactivated paracetamol metabolites, the latter being attributed to a deficient glucuronidation pathway. Ullrich et al. (45) could not confirm this change in paracetamol metabolite formation or clearance when using urinary metabolite/paracetamol ratios after oral administration. Different routes of administration as well as different observations (plasma *vs* urinary data) used to determine glucuronidation activity may explain these conflicting results. Interestingly, Esteban and Perez-Mateo (46) studied 6 patients with Gilbert's syndrome, 4 of whom did not show a difference in paracetamol glucuronidation when compared with controls. Nonetheless, 2 patients in this study had a significant lower formation of paracetamol glucuronide. This is a potentially interesting finding given the fact that paracetamol is mainly metabolised by UGT1A6 and to a lesser extent by UGT1A9, as opposed to UGT1A1, which has reduced activity in Gilbert's syndrome. A possible explanation may be that other UGT1 gene defects located in the common exon 2 to 5 region may also lead to the clinical manifestation of Gilbert's syndrome, as was recently suggested by Maruo et al. (36)

Finally, ethinylestradiol is also a substrate for UGT1A1 (47). Therefore, in women using ethinylestradiol as component of an oral contraceptive and who have Gilbert's or Crigler Najjar's syndrome, an increase in the severity of their hyperbilirubinaemia and/or estrogen-related adverse effects may occur.

In conclusion, the consequences of genetic hyperbilirubinaemias on drug glucuronidation are not clearly defined. This can be partially be attributed to the diversity in gene defects leading to Crigler Najjar and Gilbert's syndrome, and issues concerning specificity and/or overlap in the UGT isoforms responsible for the biotransformation of widely used drugs.

Developmental Changes in UGT Activity

The clinical importance of developmental reductions in UGT activity has been acknowledged since the occurrence of serious adverse events associated with chloramphenicol toxicity in neonates (2) (48). Other, less dramatic, changes in the pharmacokinetics of widely used drugs which are also UGT substrates (e.g. morphine, paracetamol and lorazepam (49) (50) (51)) highlight the dependence of the activity of this enzyme on development. Finally, developmentally regulated changes in UGT activity have also been demonstrated *in vitro*.

Glucuronidation During Embryogenesis

Immunoreactivity studies (52) (53) performed with an antibody shown to cross-react with multiple human UGT isoforms found in human and fetal livers, document the

presence of this enzyme during development. UGT immunoreactivity was found early in gestation in liver, spleen and kidney tissue. As early as 32 days post-ovulation, UGT immunoreactivity could be shown in red blood cells, mainly in the fetal precursor cells. After 12 weeks of gestation, the number of immunopositive red blood cells decreases to nearly undetectable levels. Early in gestation, UGT immunoreactivity in liver and kidney tissue is considerably lower when compared with the fetal red blood cells. Therefore, it may be possible that UGTs in circulating red blood cells prevent the fetus from possible detrimental effects of xenobiotics at a time when the liver and kidney are not fully developed to perform this task.

Endogenous Substrates and Ontogeny of UGT Activity

Fetal and neonatal UGT activity has been studied *in vitro* during the fetal and neonatal period. For example, bilirubin has been proposed as probe substrate for UGT1A1 (25) (11). Immunoreactivity and catalytic UGT activity towards bilirubin are nearly undetectable in fetal liver and the activity increases directly after birth, reaching adult levels around 3 to 6 months of age. (54) In addition, enzyme activity develops in parallel with immunodetectable protein levels. This finding suggests that the decreased activity of UGT1A1 in the fetus and neonate is related to regulation at the level of transcription or translation and not to the existence of a 'fetal' or otherwise inactive UGT1A1 isoform. Finally, the increase in catalytic activity directly after birth is not dependent upon gestational age, suggesting that birth-related events play a role in the expression and activation of the UGT1A1 gene. (55) (56) (57) (54) (58).

The impact of reduced UGT1A1 activity in the development of neonatal hyperbilirubinaemia has not, as yet, been clarified. Only recently have investigators studied the impact of the promoter region defect associated with Gilbert's syndrome on the development of icterus during the neonatal period. Gilbert's syndrome appears to accelerate the development of neonatal jaundice, although other researchers could not find a relationship between Gilbert's syndrome and neonatal hyperbilirubinaemia with plasma levels above 275 μmol/l. (2) (59) (60).

Estrone represents an endogenous substrate for UGT1A3 (61). This hormone is glucuronidated by fetal and neonatal human liver microsomes at a level of approximately 30% of adult activity. (55) While the ontogeny of UGT1A3 remains to be characterised, these data suggest that a developmental pattern for activity does exist.

UGT2B17 plays an important role in the metabolism of androgen steroids. The catalytic activity of fetal and neonatal liver microsomes towards testosterone to form glucuronides is 3% and 13% of adult levels, respectively (57). Androsterone, which is, among other biotransformation pathways, glucuronidated by both UGT2B7 (62) and UGT2B17 (63) is similarly metabolised in fetal and neonatal livers (i.e. 8 and 11% of adult UGT activity). (57)

In contrast, serotonin (5-hydroxytryptamine) is glucuronidated by fetal and liver microsomes at the same level as in the adult (57). Although the UGT isoform responsible for the glucuronidation of serotonin has not been determined to date, its identification could have important clinical implications regarding prenatal and postnatal drug exposure and possibly, the neurobehavioral effects modulated by this neurotransmitter.

Exogenous Substrates and Ontogeny of UGT Activity

The most extensive data on developmental changes of UGT activity for a single drug that can be linked with one specific UGT isoform are available from *in vitro* and *in vivo* data from morphine. Recently it was shown that morphine is largely metabolised by UGT2B7 to morphine-6-glucuronide and morphine-3-glucuronide. (40) Therefore, morphine has been proposed as a probe substrate for this UGT isoform. (19) In contrast to most other drugs, morphine undergoes considerable glucuronidation by the fetus.

In vitro studies using liver microsomes from fetuses aged 15-27 weeks indicated that the rate of morphine glucuronidation was approximately 10 to 20 % of that seen with adult microsomes. (64) (65) No correlation was found between gestational age (i.e. 15 to 27 weeks) and the rate of glucuronidation. The mean rate of morphine glucuronidation was 2-fold higher in fetal livers obtained after hysterectomy as compared to those after induced abortion (65) which suggests a regulatory mechanism for UGT activity related to labor and/or the birth process.

Morphine metabolism in premature neonates and older infants has also been studied *in vivo* (66). Morphine glucuronidation has been demonstrated in premature infants as young as 24 weeks of gestation. The mean plasma clearance of morphine was 5 fold lower in neonates (gestational age 24 to 37 weeks, postnatal age 2 to 12 days) when compared to children aged 1 to 16 years. Plasma morphine clearance from studies of continuous intravenous infusion increases slowly after birth, reaching adult values between 6 months and 30 months of age, when using the per-kg size model. Anderson et al. (67) corrected available clearance data from different age groups, using the $^3/_4$ power model. This correction showed that adult levels are reached at an earlier age between 2 and 6 months of age (67). Given the predominance of glucuronidation in the biotransformation of morphine, the maturation of morphine clearance appears to be related to developmental differences in UGT activity. (51)

The ratios of morphine-3-glucuronide : morphine and morphine-6-glucuronide : morphine in urine were both found to be significantly lower in the premature infants as compared to older infants. However, the ratio morphine-3-glucuronide : morphine-6-glucuronide was not different between the neonates and the older children, suggesting that both metabolic pathways develop in parallel (68). This knowledge has potentially important clinical implications given that morphine-6-glucuronide has more potent analgesic effects than morphine. (12) Accordingly, the clinical effect of morphine is thus not only dependent upon the clearance of morphine but also on the subsequent formation of its glucuronide metabolite. (12)

Naloxone, an opiate antagonist, was recently shown to be mainly metabolised by UGT2B7 (other pathways are N-dealkylation and 6-ketone reduction). (51, 69) The elimination half-life of naloxone is approximately 3-4 times longer in neonates than in adults, a finding which may be suggestive of reduced UGT2B7 activity in the neonate. (69)

The benzodiazepines and NSAIDs are, at least partially, glucuronidated by UGT2B7. The rate of elimination of lorazepam in the human neonate, when prenatally administered to the mother is slow when compared with that of the adult. (70) The reduced clearance of lorazepam was similar in term and preterm infants as young as 32 weeks gestational age. In a 33-week gestational age neonate, severe hypotonia necessitating prolonged assisted ventilation was associated with a toxic lorazepam concentration after administration of 1.5 mg/kg lorazepam over 27 hours, suggesting

reduced glucuronidation of the drug early in life. (50) In children aged 2.3 to 17.8 years, lorazepam plasma clearance normalised to bodyweight is similar to values reported in adults. (71) However, when lorazepam clearance was normalised for body surface area, it was lower in children. (71) When compared to healthy adults, the $t^{1}/_{2}ß$ of oxazepam is only slightly reduced in the neonate after administration to the mothers before delivery. (72) However, the isoform specificity of oxazepam remains controversial, as some authors suggest a role for UGT2B7 (73) and others could not confirm this finding. (62) Finally, the elimination of salicylate is also reduced in neonates, and, as suggested by previous investigators, appears to be partially attributable to reduced UGT activity. (74) (75)

Summarising, data pertaining to the disposition of UGT2B7 substrates suggest that activity is present in the fetus from week 15 of gestational age, with an increase in activity to approximately 10% of adult activity around birth. Adult levels appear to be attained between 2 months and 3 years of age.

Paracetamol (acetaminophen) is mainly metabolised by UGT1A6, but also to a much lesser extent by UGT1A9 (76). The rate of paracetamol glucuronide formation is negligible in the fetus (77) and rather low after birth, not reaching adult values before 10 years of age (49, 78). This apparent lack of UGT activity is, however, compensated for by higher sulfotransferase activity in infants and young children. (49) Formation of salicylamide glucuronide is similarly reduced in older children when compared to adults. (78)

Propofol is mainly glucuronidated by UGT1A9 (79, 80) and has been proposed as probe for UGT1A9 activity. (19) The clearance of this anesthetic agent, corrected for body weight, is 20 to 55% higher in children (aged 1 to 11 years) than in adults. (81, 82) However other investigators failed to detect a correlation between pharmacokinetic parameters of propofol and age. (83) Given that propofol is a high-extraction drug, which makes its clearance primarily dependent upon liver blood flow and to a lesser extent on enzyme activity (81, 84), it may not represent a suitable *in vivo* probe for UGT1A9. The pharmacokinetic data of propofol can thus not be used to predict UGT activity.

Although the reduced glucuronidation of chloramphenicol in neonates, and especially the resulting toxicity, have been well known for decades, the UGT isoforms responsible for chloramphenicol glucuronidation have not been clarified. (2) UGT2B7 glucuronidates chloramphenicol, but at negligible low levels when compared to the UGT2B7 probe morphine (85). Hence, the polyfunctionality of chloramphenicol glucuronidation does not lead to its use as probe for UGT2B7 activity.

Zidovudine, the first drug to be approved for the treatment of HIV disease, is mainly metabolised via glucuronidation, although the isoform specificity for zidovudine conjugation has not been characterised to date (86). In the first 2 weeks of life zidovudine clearance is 10.9 ml/kg/min, considerably lower than that (19.0 ml/kg/min) in children 14 days and older (range 14 to 99 days). (87) In older children, 5.4 ± 3.23 years old, the pharmacokinetics of zidovudine did not appear to be altered by age, using weight or body surface area. (88)

Ritodrine is a ß2-agonist used for management of preterm labor. Catalytic activity towards ritodrine is barely detectable in fetal liver samples. Ritodrine glucuronides account for 38% and 23% of total urinary ritodrine conjugates excreted in mothers and neonates, respectively. (89) In a manner similar to that observed for paracetamol, (49) reduced glucuronidation of ritodrine is compensated for by increased sulfation, thereby protecting the neonate from potentially toxic ritodrine plasma concentrations. (89)

More specific information on the ontogeny of the activity for the individual UGT isoforms can be deducted from *in vitro* studies with xenobiotic compounds of which the substrate specificity has been elicited. 1-Naphthol has been proposed as probe substrate for UGT1A6. (90) (91) (92) (11) In fetal, premature and neonatal liver microsomes glucuronidation of 1-naphthol occurs at 5 to 10% of adult activity, which is considerably higher than the rate of bilirubin conjugation in the fetus and neonate. (56) UGT activity towards 1-naphthol increases slowly after birth, with approximately 50 % of adult activity being reached by 6 months of age. The developmental pattern for 1-naphtol glucuronidation was also not found to be different for premature and term infants. (55)

Finally, the adult : fetal ratio of the glucuronidation activity towards 2-naphthol, which is also metabolised by UGT1A6, is more than 100 (7.89 *vs* 0.07 nmol/min/mg protein). (64) The lower enzymatic activity when compared to 1-naphthol may be explained by different analytical methods used or the overlapping substrate specificity of UGT1A6.

Implications of UGT Ontogeny in Drug Development

Isoform specific differences preclude the generalisation of a simple developmental pattern for UGT activity. Given the heterogeneity of the different UGT isoforms, developmental differences in activity for the individual UGTs may be expected. In table III we summarised *in vitro* and *in vivo* (pharmacokinetic) developmental data for various UGT substrates in order to illustrate possible developmental patterns.

Table III *Postulated ontogeny of UGT activity*

	Activity	
	In vitro[a]	**In vivo[b]**
UGT isoform		
1A1	Nearly undetectable during fetal life, increasing directly after birth, reaching adult levels 3 to 6 months after births	NA
1A3	During fetal and neonatal period around 30% of adult activity	NA
1A6	Glucuronidation during fetal life at 1 to 10% of adult levels, increasing slowly after birth, at 6 months 50% of adult activity. Independent of gestational age	Not reaching adult levels before 10 years of age
1A9	NA	NA
1A10	NA	NA
2B4	NA	NA
2B7	15 to 27 weeks of fetal life: 10 to 20% of adult levels. Change in activity regulated by birth related events?	First two weeks of life 20% of levels of older children (1 to 16 years). No apparent change during adolescence
2B15	NA	NA
2B17	Reduced activity during fetal (<10%) and neonatal (around 10%) period when compared to adults	NA

A *data from immunoreactivity or catalytic studies with fetal, neonatal or adult human liver microsomes using UGT substrates that are mainly metabolized by 1 specific UGT isoform*

B *data from pharmacokinetic studies using UGT substrates that are mainly metabolized by 1 specific UGT isoform*

NA *no information available*

These developmental patterns should however be interpreted with care, as the complete ontogeny for those UGT isoforms important in human drug metabolism is not known. A lack of complete knowledge on substrate specificity and isoform specificity complicates the interpretation of both *in vitro* and *in vivo* data with respect to the individual UGT isoforms. Only recently, 6 probe substrates for different isoforms were proposed (e.g. bilirubin for UGT1A1) by Burchell and Coughtrie. (19) Most *in vitro* data are limited to the fetal and neonatal period, resulting in a lack of data during the first year of life and thereafter.

The use of clearance expressed in L/kg is a weak size model when used to address developmental issues. The use of the per-kg model will lead to an underestimation of clearance by up to 200% in children with body weight under 3.4kg. As discussed by Holford and colleagues, (67, 93) the application of the $^3/_4$ power model, instead of the per-kg model, will generate the following equation for clearance:

$CLi = CLstd\ (Wi/Wstd)^{3/4}$

where CLi is clearance in the individual, CLstd is clearance in a standardised individual, Wi is the individual's bodyweight and Wstd is the bodyweight of the standardised individual. The use of this model will be more accurately correct for size, and will thus be more useful to detect the influence of age on differences in pharmacokinetics. Since the pharmacokinetic data used, are expressed in L/kg and not using the 3/4 power model, the interpretation of these data with respect to UGT activity should be undertaken with care and is merely speculative.

Moreover, considering that other organs are also capable of glucuronidation (53, 84, 94) with organ-specific activity and possibly an organ-specific developmental patterns, further complicates the accurate assessment of UGT ontogeny derived from data generally obtained from experiments using human liver microsomes.

Overlapping isoform specificity, the availability of alternative metabolic pathways or differences in the other factors which may change pharmacokinetics (e.g. renal function) should be considered before translating plasma clearance or drug : metabolite ratios as surrogate marker for UGT activity. At present, morphine is the only drug primarily metabolised by glucuronidation with known single-isoform specificity (i.e.UG2B7). The pharmacokinetic data for morphine may, therefore, be reliably used to reflect the ontogeny of UGT2B7.

Future directions

In contrast to the cytochrome P450, knowledge concerning the impact of development on UGT activity is lagging. Due to the importance of UGTs in human drug metabolism, more complete characterisation of these critical enzymes is important and deserves increased attention.

In vitro studies using human UGT isoforms should be performed with probe substrates in order to characterise specificity and determine the impact of ontogeny. Moreover, for drugs that are widely used in paediatric practice, and are extensively glucuronidated, the metabolic profile should be determined at different stages of development in order to search for 'breakpoints' in UGT activity of a magnitude that might warrant age-specific modifications in drug therapy.

Pharmacokinetic studies of glucuronidated drugs are also needed to supplement the *in vitro* information on the impact of ontogeny on the activity of the different UGT

isoforms. When combining these *in vitro* and *in vivo* data, a developmental 'road map' could be constructed for the individual UGT isoforms that would allow prediction of the impact of age on enzyme activity.

Patients with genetic hyperbilirubinaemia are at increased risk for drug toxicity or drug failure caused by reduced UGT activity. This group may also benefit from combined *in vitro* and *in vivo* studies, combining their specific gene defect and isoform specific *in vitro* UGT activity with pharmacokinetic data. Moreover, the impact of ontogeny on development of UGT activity in this patient population should also be studied.

Conclusion

Clearly, an information gap exists regarding the developmental and genetic aspects of UGT regulation and its potential impact on therapy. Case reports illustrate the danger of drug accumulation due to developmentally reduced UGT activity. However, no general developmental pattern for the individual UGT isoforms that might be of value for the clinician is currently available. More research on the pharmacogenetics and ontogeny of the UGTs is necessary for effective translation of scientific information into clinically applicable knowledge. As has been shown with the cytochrome P450 enzymes, coupling of basic and clinical science will be necessary to continually improve our understanding of the UGTs.

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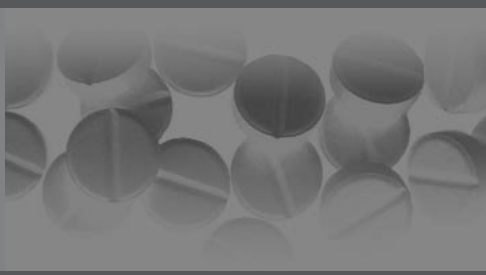
Pharmacokinetics and metabolism of intravenous midazolam in preterm infants

Chapter 4



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Submitted

Summary

Background Midazolam is a benzodiazepine, finding expanded use in neonatal intensive care units. We studied the pharmacokinetics and metabolism of midazolam after a single intravenous dose in preterm infants.

Methods The pharmacokinetics of midazolam and 1-OH-midazolam following a single 0.1 mg/kg intravenous dose of midazolam were determined in 24 preterm infants (gestational age: 26 to 34 weeks, postnatal age: 3 to 11 days). Blood was drawn prior to drug administration and at 0.5, 1, 2, 4, 6, 12 and 24 after the start of the infusion. Midazolam and 1-OH-midazolam concentrations were determined by GC-MS.

Results Total body clearance, apparent volume of distribution, and plasma half-life of midazolam (M) were [median (range)]: 1.8 (0.7-6.7) ml/kg/min, 1.1 (0.4-4.2) L/kg and 6.3 (2.6-17.7) h, respectively. In 19 out of 24 preterm infants, 1-OH-midazolam concentrations could be detected. 1-OH-midazolam (1-OH-M) C_{max}, T_{max} and 1-OH-M/M AUC ratio were [median (range)]: 8.2 (<0.5-68.2) ng/ml, 6 (1-12) h and 0.09 (<0.001- 1), respectively. Elimination half life could be calculated in 13 patients and was found to be [median (range)]: 9.8 (4.9 to 62.2) h. Midazolam plasma clearance was increased in those infants who had indomethacin exposure.

Conclusion Consequent to immature hepatic CYP3A4 activity, midazolam clearance and 1-OH-midazolam concentrations are markedly reduced in preterm infants as compared to previous reports from studies in older children and adults. Indomethacin exposure and its apparent impact on midazolam clearance supports alteration of drug disposition produced by a patent ductus arteriosus.

Introduction

Midazolam, a short-acting benzodiazepine, is used for sedation in newborn infants, requiring prolonged mechanical ventilation and prior to invasive procedures (1,2). Despite the use of the drug in neonatal intensive care units, few data are available on its pharmacokinetics in preterm infants less than 34 weeks of gestation. Moreover, the data describing the pharmacokinetics of intravenous midazolam in preterm infants show marked interpatient variability (3,4).

Midazolam undergoes extensive metabolism by members of the cytochrome P450 3A subfamily (e.g., CYP3A4 and CYP3A5) to a major hydroxylated metabolite (1-OH-midazolam) and several minor metabolites (4-OH, 1,4-OH) (5). In adults, plasma clearance of midazolam is significantly correlated with hepatic CYP3A4/5 activity (6). Cytochrome P450 3A4 (CYP3A4) is the most abundantly expressed cytochrome P450 isoform in adult liver and is responsible for catalyzing the biotransformation of over 50 currently prescribed drugs (7). Hepatic CYP3A5 expression shows large interindividual differences and displays partially overlapping substrate specificity with CYP3A4. In contrast, CYP3A7 is the major isoform expressed in human fetal liver and does not appreciably catalyze the biotransformation of midazolam (7). Lacroix et al. (8) showed that, irrespective of gestational age at birth, CYP3A4 expression is activated during the first weeks after birth, which is accompanied by a simultaneous decrease in CYP3A7 activity. The developmental pattern of CYP3A5 activity is as yet unknown. As one might expect consequent to the impact of ontogeny on CYP3A activity, midazolam plasma clearance is reduced in critically ill newborn infants (3,4,9).

Reduced CYP3A4/5 activity after birth will not only limit midazolam elimination but also, the formation of 1-OH-midazolam, an active metabolite (10). In preterm infants, 1-OH-midazolam concentrations following therapeutic midazolam administration demonstrate large interindividual variability (4). However, the pharmacokinetics of this metabolite in preterm infants has not been determined to date.

In order to investigate the pharmacokinetics of midazolam and 1-OH-midazolam in the first two weeks of life, we evaluated its disposition in preterm infants with gestational ages between 26 and 34 weeks following a single intravenous dose of midazolam.

Methods

Patient population

The study was conducted in 24 preterm infants; gestational age: 26 to 34 weeks and postnatal age: 3 to 11 days. The infants were recruited from the Neonatal Intensive Care Unit of the Sophia Children's Hospital. All children received midazolam prior to a stressful procedure (e.g. tracheal tube suction, elective nasopharyngeal intubation) and had a preexisting indwelling arterial catheter placed for purposes of medical care. Patients were excluded if they received morphine, dobutamine, dopamine or a drug known to affect CYP3A activity. In addition, patients were excluded from the study if they had significant underlying hemodynamic, renal, hepatic or neurologic dysfunction. This research protocol was approved by the Human Ethical Committee of the Sophia Children's Hospital. Written, informed consent was obtained from parents or legal guardians prior to enrollment of subjects in the study.

Drug administration and sample collection

Midazolam (Dormicum® injection, Roche Laboratories, The Netherlands) was administered as a single 0.1 mg/kg dose in a 5% glucose solution (0.03 mg/ml) infused by syringe pump over 30 minutes through microbore tubing into a peripheral vein or into a central venous catheter. Serial arterial blood samples (0.2 ml) were obtained at baseline and at 0.5, 1, 2, 4, 6, 12 and 24 hours from the time of dosing. Plasma was separated from whole blood by centrifugation (1000 X g for 10 minutes) and then stored at –80°C until analysis. The subjects were observed during the infusion for adverse reactions, with vital signs checked prior to infusion and at the time of blood samplings.

Analytical methods

Plasma samples were analyzed for midazolam and 1-OH-midazolam by gas chromatography with mass spectrometric detection (Hewlett Packard 6890, Agilent Technologies Inc, Palo Alto, CA). The column used was a J&W Scientific DB-17 EVDX [0.2 micron, 25 meters (J&W Scientific, Folsom, CA)]. Diazepam (Elkins Sinn, Cherry Hill, NJ), 5µl of 500 ng/ml solution, was added to each sample as an internal standard and solid phase extraction was performed using a Varian Bond Elut Column (Varian Inc, Palo Alto, CA). The inter-day coefficient of variation for the low standard (2 ng/ml) was consistently less than 10% for both midazolam and 1-OH midazolam. The intra-day coefficients of variation were also less than 10% for both midazolam and 1-OH-midazolam at a concentration of 2 ng/ml. The lower limit of quantitation was 1 ng/ml for midazolam and 0.5 ng/ml for 1-OH-midazolam using 0.2 ml sample volume. All samples were analyzed in duplicate with the resultant mean concentration used in the pharmacokinetic analysis.

Pharmacokinetic analysis

The maximal concentration of drug in plasma (C_{max}) and time to reach C_{max} (T_{max}) were determined by visual inspection of the plasma concentration vs. time curve. The apparent terminal elimination rate constant (λ_z) was estimated by curve fitting using a nonlinear, least-squares regression analysis with reciprocal (i.e., $1/Y^2$) weighting. Area under the concentration-time curve from time zero to the last sampling time point (AUC_{ot}) was calculated using the log-linear trapezoidal rule. Extrapolation of the AUC to infinity ($AUC_{o-\infty}$) was calculated by the summation of $AUC_{ot} + C_{pt}/ \lambda_z$, where C_{pt} represents the plasma concentration at the last sampling time (t) predicted from the fitted terminal elimination curve. The individual $t_{1/2}$ was calculated as $0.693/\lambda_z$. The apparent steady state volume of distribution (V_{ss}) and total plasma clearance (CL) were calculated using standard noncompartmental techniques. 1-OH-midazolam pharmacokinetic parameters (with the exception of V_{ss} and CL) were determined as described above for midazolam. The 1-OH-midazolam AUC_{ot} /midazolam AUC_{ot} ratio (AUC ratio) was used as a "surrogate" marker of CYP3A activity. All pharmacokinetic analyses were performed using the Kinetica (version 2.0, Innaphase, Inc, Philadelphia, PA, USA) software package.

Statistical analysis

Results are expressed as means ± standard deviation unless stated otherwise. Because most calculated pharmacokinetic parameters did not show a normal distribution, these

are expressed as median (range). Comparison of groups of patients (which were defined according to the following dichotomous co-variates: partus (cesarean section/spontaneous) feeding (parenteral/enteral), prenatal indomethacin exposure (yes/no), prenatal betamethasone exposure (yes/no), postnatal indomethacin exposure (yes/no), mechanically ventilated (yes/no), caffeine therapy (yes/no) and detectable 1-OH-midazolam concentrations (yes/no) with respect to calculated pharmacokinetic parameters was performed using the Mann-Whitney test. Association of continuous co-variates (i.e. postnatal age, gestational age, postconceptual age, Apgar score) and calculated pharmacokinetic parameters are given as Spearman's (r_s) correlation coefficients. These statistical analyses were obtained using the SPSS software (version 9.0.0, SPSS Inc., Chicago, Ill). The level of significance accepted for all statistical analysis was $\alpha = 0.05$.

Results

Clinical results

Twenty-four preterm infants (16 female, 8 male) with a median gestational age of 29 (range 26-34) weeks and a median postnatal age of 5.5 (range 3-11) days, participated in the study (Table 1). Median (range) FiO_2 was 0.21 (range 0.21-0.29) in patients (n=13) who were mechanically ventilated and 0.21 (range 0.21-0.28) in patients (n=10) who received continuous positive airway pressure (CPAP) by nasopharyngeal tube. Twenty patients were antenatally exposed to indomethacin (to prevent preterm labor) and/or betamethasone (to induce lung maturation). Three patients received both drugs, and 17 only betamethasone. Of the eleven patients who were postnatally exposed to indomethacin, four patients had a patent ductus arteriosus, for which they received indomethacin during the study and 7 patients had received their last dose of indomethacin at least 24 hours before start of the study. Thirteen patients received caffeine prior to or during the study for weaning of the ventilatory support or for treatment of neonatal apnea. Antibiotics, in most cases beta-lactams and aminoglycosides, were required before or during the study in all patients for suspected or proven infection. Additional drug

Table 1 *Patient characteristics*

Parameters		
Male / Female	15 / 9	
GA (weeks)	29.1 ± 2.3#	(26.3-33.6)*
PNA (days)	5.8 ± 2.6#	(3-11) *
Birth weight (g)	1092 ± 233#	(745-1630) *
Study weight (g)	1105 ± 230#	(770-1645) *
Postnatal indomethacin	11+, 13-	

*# mean ±SD, * range, GA: Gestational age, PNA: postnatal age*

therapy included surfactant (n=17), morphine (n=11, >12h before midazolam administration) and furosemide (n=3). No serious adverse events due to midazolam were reported throughout the course of the study.

Midazolam and 1-OH-midazolam pharmacokinetics

The mean plasma concentration-time curves for midazolam and 1-OH-midazolam are depicted in Figure 1. Midazolam clearance was [median (range)]: 1.8 (0.7-6.7) ml/kg/min, volume of distribution: 1.1 (0.4-4.2) L/kg and elimination half-life: 6.3 (2.6-17.7) hours (Table 2). In 19 out of 24 patients, 1-OH-midazolam could be quantitated over the sampling interval. Of these patients, median 1-OH-midazolam C_{max} was 8.2 (<0.5-68.2) ng/ml with a median T_{max} reached at 6 (1-12) h. The median 1-OH-midazolam AUC_{ot}/midazolam AUC_{ot} ratio was low (0.09) with large interindividual variation (range: <0.001 – 1, CV 191%). For only 13 patients sufficient data were available to calculate 1-OH-midazolam elimination half-life which was significantly longer than that observed for midazolam [median $t_{1/2}$: 9.8 (range 4.9 - 62.2) vs. 6.3 (range 2.6 - 17.7) h for midazolam, p=0.046].

The effect of co-variates on midazolam and 1-OH-midazolam pharmacokinetics

No significant relationship was detected between age (gestational, postnatal or postconceptional age) and midazolam CL, V_{ss} or $t_{1/2}$. Newborn infants exposed postnatally

Table 2 *Calculated midazolam and 1-OH-midazolam pharmacokinetic parameters in preterm infants*

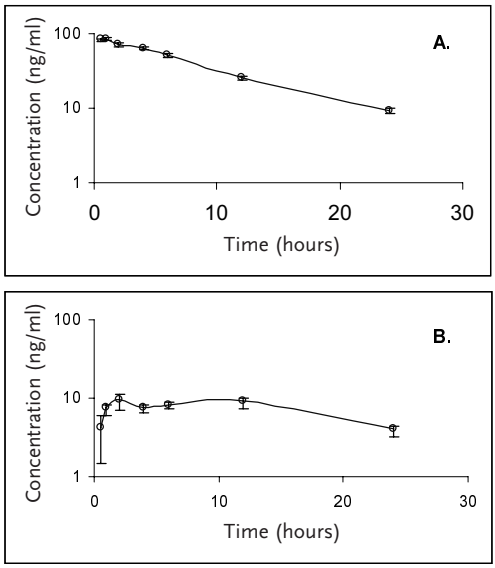
	Midazolam	**1-OH-midazolam**
Parameters		
AUC_{0t} (ng/ml.h)	804 (153 - 2118)	66.7 (<6-997.87)
$AUC_{0-\infty}$ (ng/ml.h)	971 (248 – 2353)	76.0 (<6-1222.4)
$t_{1/2}$ (h)	6.3 (2.6 – 17.7)	9.8 (4.9 – 62.2) #
V_{ss} (L/kg)	1.1 (0.4 – 4.2)	NA
CL (ml/kg/min)	1.8 (0.7 – 6.7)	NA
MRT (h)	10.3 (4.0 – 25.6)	NA
C_{max} (ng/ml)	108 (48.8 – 217.0)	8.2 (<0.5-68.2)
T_{max} (h)	0.5 (0.5 – 4.0)	6 (1-12)
AUC ratio		0.09 (<0.001-1)

Data are expressed as median(range), # = data from 13 patients,
C_{max} = maximal concentration of drug in plasma, T_{max}= time to reach C_{max}, AUC_{ot} = area under the concentration-time curve from time zero to the last sampling time point, $AUC_{0-\infty}$ = area under the concentration-time curve from time zero to infinity, $t_{1/2}$ = elimination half-life, CL = total clearance and V_{ss} = volume of distribution at steady state, MRT = mean resident time, AUC ratio = 1-OH-midazolamAUC_{ot} / midazolamAUC_{ot}, NA = not available.

to indomethacin (n=11) had a significantly higher mean midazolam clearance as compared to infants who were not exposed to indomethacin (n=13) [0.17 (0.07-0.40) vs. 0.07 (0.04-0.24) ml/kg/min, p=0.003]. This effect of indomethacin on midazolam clearance was also found when only patients who were exposed to indomethacin, but did not have a patent ductus arteriosus at the time of the study (n=7) were compared to patients who were not exposed to indomethacin before [0.14 (0.07-0.40) vs. 0.07 (0.04-0.24) ml/kg/min, p=0.03]. Indomethacin treated infants also had a significantly higher volume of distribution, [1.7 (0.8-4.2) vs. 0.9 (0.4-1.6) L/kg, p=0.001], while mean half-life was not different between both groups [5.8 (3.9-17.7) vs 6.5 (2.6-14.2)h, p=0.93]. In addition, indomethacin-treated infants had a significantly higher postnatal age as compared to non-treated infants [median (range): 6.5 (3-11) days vs. 4.5 (3-9) days, p = 0.04]. Multiple regression was used to determine which factor was most predictive for midazolam plasma clearance. This analysis showed that midazolam clearance was significantly increased after indomethacin exposure (p=0.01) and when adjusted for this effect, there was no predictive value for postnatal age (p=0.43). We did not find an effect of any of the other clinical parameters [(i.e. feeding (enteral feeding n=6), ventilation (mechanically ventilated n=13), Apgar score, partus (spontaneous n=11), prenatal corticosteroid (n=20) or indomethacin administration (n=3), caffeine therapy (n = 15)] on midazolam pharmacokinetic parameters.

Additionally, there was no relationship detected between 1-OH-midazolam pharmacokinetic parameters (C_{max}, AUC, $t_{1/2}$) and postnatal, postconceptional or gestational age. No relationship was detected between any of the clinical parameters or concomitant drug therapy and 1-OH-midazolam pharmacokinetics. A significant

Figure 1 *Midazolam (A, n=24) and 1-OH-midazolam (B, n=13) concentration versus time curve after a single intravenous dose (0.1 mg/kg) to preterm infants.*



Each dot represents mean ± SEM concentration at each time point.

difference was not found with respect to comparison of demographic parameters between patients with or without detectable 1-OH-midazolam concentrations. In those infants where 1-OH-midazolam could be quantitated, the 1-OH-midazolam/midazolam AUC_{ot} ratio was determined as a surrogate marker for CYP3A activity. No association was observed between any of the demographic parameters and this ratio. Finally, there was not a statistically significant difference in this ratio between patients who were postnatally exposed to indomethacin and those who were not [0.08 (<0.001-0.3) vs 0.15 (<0.001-1), p = 0.90)].

Discussion

In preterm infants, midazolam clearance is lower than previously reported in older children and adults (11,12). Midazolam plasma clearance [1.8 (0.7-6.7) ml/kg/min] in our cohort of preterm neonates was comparable to values previously reported in newborn infants with gestational ages between 34 and 41 weeks (13), but was 1.5 to 5 times lower than reported in infants older than 3 months (3 - 9 ml/kg/min), children (5-13 ml/kg/min) and adults (6 - 11 ml/kg/min) (11,14-16). Accordingly, midazolam elimination half-life was longer in our patients [6.3 (2.6-17.7) h] than in older infants, children and adults (range: 1-2.5 h). This "impaired" midazolam elimination in preterm neonates as compared to older infants and children mirrors the known pattern for the ontogeny of CYP3A4 (7,8).

Interestingly, midazolam plasma clearance in our patients was somewhat higher [1.8 (0.7-6.7) ml/kg/min] than reported by Burtin et al. [1.2 ± 0.96 ml/kg/min] and Lee et al. [1.0 ± 0.2 ml/kg/min] (3,4). These previous investigations estimated clearance using population pharmacokinetics in preterm infants with gestational and postnatal ages similar to our patients, and who received midazolam as either a continuous infusion or as an intravenous bolus dose. This apparent difference between our mean midazolam clearance and those of earlier studies may be due to differences in patient population, co-medication received or simply, greater variability associated with pharmacokinetic parameter estimation from a population-based approach with sparse sampling. First, only about 50% of our infants were ventilated with a relatively low oxygen requirement (FiO_2 range 0.21 –0.29) while in the other studies (3,4) all patients were mechanically ventilated (oxygen demand not reported) suggesting that these patients were, on average, less stable than our patients. In adults, midazolam clearance appears to be reduced in critically ill patients possibly as a consequence of reduced CYP3A activity (17). Therefore, a difference in disease severity may have contributed to the lower midazolam clearance reported by Lee et al. (4) and Burtin et al. (3). Second, the increasing use of betamethasone over the last few years may also have contributed to the higher midazolam clearance reported in our patients compared to older studies. Twenty out of our 24 patients were antenatally exposed to betamethasone. If betamethasone is capable of inducing CYP3A activity as is dexamethasone (18), higher plasma midazolam clearance may have resulted from this particular drug interaction.

Whereas midazolam elimination in preterms shows age-related differences in relation to infants older than six months of age (11,19), we did not find a relationship between age (postconceptional, gestational or postnatal) and midazolam clearance or AUC ratio (as a surrogate marker for CYP3A4/5 activity) within our patient group. This

finding is in agreement with previous reports from preterm and term newborn infants with gestational ages ranging 24 from to 39 weeks (3,4). The lack of relationship between gestational or postnatal age and midazolam elimination mirrors the observation in vitro that CYP3A4 activity increases only marginally during the first two weeks of life (8). However, the lack of a relationship between age and midazolam clearance or AUC ratio in our study should be interpreted with caution given the relatively small sample size (n=24) of our study cohort and the narrow range of gestational (26 to 34 weeks) and postnatal ages (3 to 11 days) that characterized our subjects.

In our study the AUC ratio was used as a surrogate "marker" of CYP3A4/5 activity *in vivo*. Due to technical limitations (i.e. small sample volume), we were not able to measure the plasma 1-OH-midazolam-glucuronide concentrations (20). Therefore, the AUC ratio we calculated is not "corrected" for glucuronidation. Nonetheless, as the rate-limiting step in the formation of 1-OH-midazolam is catalyzed by CYP3A4, it was reasonable to assess the potential impact of development on enzyme activity using this AUC ratio.

Unexpectedly, postnatal indomethacin exposure, during or at any time before the study, was associated with a higher midazolam plasma clearance and a larger apparent volume of distribution. This may be an effect of altered pharmacokinetics as a result of resolution of a patent ductus arteriosus consequent to indomethacin treatment as has been reported for aminoglycosides, indomethacin and vancomycin (21). However, most of the patients received indomethacin more than 24 hours prior to midazolam administration and, based on clinical data, did not have a patent ductus arteriosus at the time of the study. As shown by van den Anker et al (22), indomethacin treatment of patent ductus arteriosus in the first days postnatally was associated with a larger apparent volume of distribution of ceftazidime up to two weeks of age. Hemodynamic consequences of the transition from fetal to neonatal circulation with resultant alterations in extracellular fluid dynamics and/or glomerular filtration may be sufficient to alter midazolam distribution and/or the clearance of 1-OH-midazolam as reflected by the increased elimination half-life in our patients.

Although 1-OH-midazolam concentrations have been measured in preterm infants (23, 24), the pharmacokinetics of 1-OH-midazolam in preterm infants have, to our knowledge, not been previously reported. The metabolite:drug AUC ratio appears to be lower in preterm infants than in older children and adults consequent to expected developmental reductions in CYP3A4/5 activity [0.09 (<0.001-1.0) vs. 0.13-0.26] (10,26). The increased median 1-OH-midazolam elimination half-life was longer in our neonates as compared to previous values from adults, where 1-OH-midazolam has a shorter half-life than the parent drug (10). This difference in 1-OH-midazolam elimination half-life may reflect lower renal clearance of 1-OH-midazolam and/or reduced glucuronidation in the neonate.

1-OH-midazolam concentrations and AUC ratio showed considerable interpatient variability (Table 2) and in five of our 24 patients, no 1-OH-midazolam could be detected. The intersubject variability in the AUC ratio is much larger in our cohort of preterm infants than the variability reported for midazolam and other CYP3A4/5 substrates in both pediatric and adult populations (10,25,26,27). This larger intersubject variability in newborns as compared to the intersubject variation in adults indicates that CYP3A4/5 activity in the newborn is certainly as variable as documented in adults, but probably

CYP3A4/5 activity in the newborn has a larger variability as compared to CYP3A4/5 activity in adults. Given the small age range of the preterm infants investigated in our study, it is highly unlikely that development per se is producing the aforementioned variability. Moreover, the lack of correlation between CYP3A4/5 activity and postconceptional age further supports that development does not explain the difference between variability in the newborn and adult in this study. Importantly, the larger variability in the newborn has potentially important implications for the treatment of newborns with CYP3A4/5 substrates.

Our inability to detect 1-OH-midazolam in a subset of patients may have been consequent to virtually absent constitutive CYP3A4/5 expression (ie. CYP3A7 predominance) with the production of metabolite concentrations below the limit of detection for the analytical method.

In conclusion, the elimination of midazolam in preterm infants between 26 and 34 weeks gestational age and less than two weeks of postnatal age is impaired relative to older infants, children and adults consequent to reduced CYP3A4/5 activity. Therefore, midazolam dosing regimens may need to be altered in young preterm neonates to prevent overdosing consequent to accumulation of midazolam and 1-OH-midazolam with repeated dosing. Part of the large variability in midazolam pharmacokinetics in this neonatal population could be explained by postnatal indomethacin exposure for closure of a patent ductus arteriosus and its effects on hemodynamic and/or renal function. Finally, as reflected by examination of the pharmacokinetic data for 1-OH-midazolam in the first two weeks of life, developmental dependence of CYP3A4/5 activity is either absent or alternatively, obscured by the marked interindividual variability in this enzyme.

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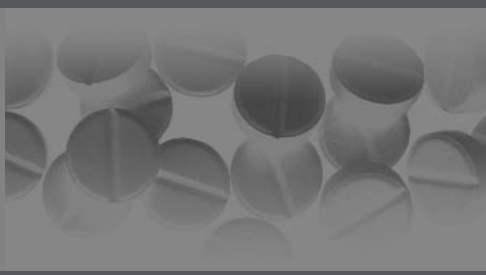
Pharmacokinetics and metabolism of oral midazolam in preterm infants

Chapter 5



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Submitted

Summary

Introduction Midazolam is a short-acting benzodiazepine that is finding expanded use in neonatal intensive care units. We studied the pharmacokinetics and metabolism of midazolam after a single oral dose in preterm infants.

Methods The pharmacokinetics of midazolam (M) and 1-OH-midazolam (1-OH-M) following a single 0.1 mg/kg oral dose of midazolam were determined in 15 preterm infants (gestational age: 26 to 31 weeks, postnatal age: 3 to 13 days). Blood was drawn prior to drug administration and at 0.5, 1, 2, 4, 6, 12 and 24 after oral administration. In 8 out of these 15 patients the pharmacokinetics of intravenous midazolam were also studied. Midazolam and 1-OH-midazolam concentrations were determined from plasma using GC-MS.

Results Apparent oral plasma clearance (CL/F), apparent volume of distribution (V_{ss}/F) and plasma half-life of midazolam were [median (range)]: 2.7 (0.67-15.5) ml/kg/min, 1.4 (0.3-12.1) L/kg and 7.6 (1.2-15.1) h, respectively. Midazolam C_{max} and T_{max} were [median (range)]: 64.4 (15.2-204) ng/ml and 2.0 (0.5-12) h, respectively. In 9 out of 15 preterm infants, 1-OH-midazolam concentrations could be detected. 1-OH-M C_{max}, T_{max} and 1-OH-M/M AUC ratio were [median(range)]: 10.0 (1.6-22.6) ng/ml, 4 (0.5-24) h and 0.03 (0.01-0.96), respectively. Median elimination half life could only be calculated in four patients and was 3.6 (0.81-10.3) h. Absolute bioavailability could be calculated in 8 patients and was [median (range)]: 0.49 (0.12-1.0), showing a large variability.

Discussion Consequent to immature intestinal and hepatic CYP3A4 activity, midazolam clearance and 1-OH-midazolam concentrations are markedly reduced in preterm infants as compared to previous reports from studies in older children and adults. The large variability of the bioavailability of midazolam (12-100%) most likely reflects ontogeny and variability in intestinal CYP3A4 activity.

Introduction

Midazolam, a short-acting benzodiazepine, is used for sedation in newborn infants, requiring prolonged mechanical ventilation and prior to invasive procedures (1). Despite the use of the drug in neonatal intensive care units, few pharmacokinetic data are available in preterm infants less than 34 weeks of gestation. Moreover, data describing midazolam disposition following oral administration in preterm infants are completely lacking. As well, there are no data on the absolute bioavailability of midazolam in preterm infants.

Midazolam undergoes extensive metabolism by members of the cytochrome P450 3A subfamily (e.g., CYP3A4 and CYP3A5) to a major hydroxylated metabolite (1-OH-midazolam) and several minor metabolites (2). CYP3A4 is primarily located in hepatocytes but also is found in the villus tip of enterocytes in the small intestine, the primary site of absorption for orally administered drugs. Therefore, following oral administration, midazolam is subject to hepatic and intestinal metabolism by CYP3A (3). In adults, 1-OH-midazolam concentrations are significantly higher following oral administration of midazolam compared to those seen after intravenous administration of the drug (4). In preterm infants, hepatic CYP3A4 activity is reduced, thereby resulting in prolonged plasma clearance of midazolam after intravenous administration (5). However, it is not known if intestinal CYP3A activity is also reduced as a consequence of age. Since hepatic and intestinal CYP3A are reportedly not co-regulated (6), a similar developmental pattern for intestinal CYP3A may not be assumed. Should the ontogeny of CYP3A4/5 in the small intestine mirror that observed for the liver, a reduction in the rate and extent of 1-OH-midazolam formation could be expected to occur.

Previous studies in adults (6) (7) (4) have used intravenous and oral midazolam to examine the relative contributions of hepatic and intestinal CYP3A4/5 to drug biotransformation. In this investigation, we examined the pharmacokinetics of oral midazolam in preterm infants (26 to 31 weeks of gestational age) who required the drug for pre-procedural sedation. In a cohort of these patients (n=8), midazolam disposition following intravenous administration was also characterized. The pharmacokinetic data from midazolam and its primary metabolite (i.e., 1-OH-midazolam) were used to assess developmental differences in CYP3A4/5 activity and also, to determine the oral bioavailability of midazolam in preterm neonates.

Methods

Patient population

The study was conducted in 15 preterm infants with gestational and postnatal ages ranging from 26 to 31 weeks and 3 to 13 days, respectively. The infants were recruited from the Neonatal Intensive Care Unit of the Sophia Children's Hospital. All children received midazolam prior to a stressful medical procedure (e.g. tracheal tube suction, elective nasopharyngeal intubation) and had a pre-existing indwelling arterial catheter previously placed for purposes of medical care not related to this pharmacokinetic study. In 8 of these patients the pharmacokinetics of intravenous midazolam were also studied, 72 hours before or after the patients received midazolam orally. Patients were excluded if they received morphine, dobutamine, dopamine or any drug known to affect CYP3A4

activity. In addition, patients were excluded if they had significant underlying hemodynamic, renal, hepatic or neurologic dysfunction. This research protocol was approved by the Human Ethical Committee of the Sophia Children's Hospital and the Network Steering Committee of the Pediatric Pharmacology Research Unit Network. Written, informed consent was obtained from parents or legal guardians prior to enrollment of subjects in the study.

Drug administration and sample collection

A single oral dose (0.1 mg/kg) of midazolam (Dormicum® injection, Roche Laboratories, The Netherlands) was given as a 0.5 ml glucose 5% solution via nasogastric tube, followed by 0.5 ml of glucose 5% to ensure complete drug delivery. Serial arterial blood samples (0.2 ml each) were obtained from an indwelling arterial catheter at baseline and at 0.5, 1, 2, 4, 6, 12 and 24 h after dosing. In eight of these patients, midazolam was also administered as a single 0.1 mg/kg dose in a 5% glucose solution (0.03 mg/ml) infused into a peripheral or central venous catheter by a syringe pump over 30 minutes through microbore tubing. Serial arterial blood samples were obtained in the same way as after oral dosing. Plasma was separated from whole blood by centrifugation (1000 X g for 10 minutes) and then stored at –80°C until analysis. The subjects were observed during the study drug administration for adverse reactions, with vital signs checked prior to dosing and at the time of blood samplings.

Analytical methods

Plasma samples were analyzed for midazolam and 1-OH-midazolam by gas chromatography with mass spectrometric detection (Hewlett Packard 6890, Agilent Technologies Inc, Palo Alto, CA). The column used was a J&W Scientific DB-17 EVDX [0.2 micron, 25 meters (J&W Scientific, Folsom, CA)]. Diazepam (Elkins Sinn, Cherry Hill, NJ), 5 µl of 500 ng/ml solution, was added to each sample as an internal standard and solid phase extraction was performed using a Varian Bond Elut Column (Varian Inc, Palo Alto, CA). The inter-day and intra-day coefficients of variation for the low standard (2ng/ml) were less than 10% midazolam and 1-OH midazolam, respectively. The lower limit of quantitation was 1 ng/ml for midazolam and 0.5 ng/ml for 1-OH-midazolam using 0.2 ml sample volume. All samples were analyzed in duplicate with the resultant mean concentration used in the pharmacokinetic analysis

Pharmacokinetic analysis

The maximal concentration of drug in plasma (C_{max}) and time to reach C_{max} (T_{max}) were determined by visual inspection of the plasma concentration vs. time curve for each subject. Initial polyexponential parameter estimates were generated from plasma concentrations vs. time data using a peeling algorithm for each subject. Final estimation of the apparent terminal elimination rate constant (λ_z) was accomplished by curve fitting using a non-linear, least-squares regression analysis with reciprocal (e.g., $1/Y^2$) weighting. Area under the concentration-time curve from time zero to the last sampling time point (AUC_{ot}) was calculated using the log-linear trapezoidal rule. Extrapolation of the AUC to infinity ($AUC_{o-\infty}$) was calculated by the summation of $AUC_{ot} + C_{pt}/ \lambda_z$, where C_{pt} represents the plasma concentration at the last sampling time (t) predicted from the fitted terminal elimination curve. The individual $t_{1/2}$ was calculated as $0.693/ \lambda_z$. The apparent

steady state volume of distribution (V_{ss}/F) and apparent oral plasma clearance (CL/F) were calculated using standard noncompartmental techniques. 1-OH-midazolam pharmacokinetic parameters (with the exception of V_{ss} and CL) were determined as described above for midazolam. The 1-OH-midazolam AUC_{0t}/1-OH-midazolam AUC_{0t} ratio (AUC ratio) was used as a "surrogate" marker of CYP3A activity. All pharmacokinetic analyses were performed using the Kinetica (version 2.0, Innaphase, Inc, Philadelphia, PA, USA) software package.

Statistical analysis

Results are expressed as mean ± standard deviation unless stated otherwise. Because most calculated pharmacokinetic parameters did not show a normal distribution, these are expressed as median (range). Comparison of groups of patients [which were defined according to the following dichotomous co-variates: partus (cesarean section/spontaneous), feeding (parenteral/enteral), prenatal indomethacin exposure (yes/no), mechanically ventilated (yes/no), prenatal betamethasone exposure (yes/no), postnatal indomethacin exposure (yes/no), mechanically ventilated (yes/no), caffeine therapy (yes/no) and detectable 1-OH-midazolam concentrations (yes/no)] with respect to calculated pharmacokinetic parameters was performed using the Mann-Whitney test. Association of continuous co-variates (e.g., postnatal age, gestational age, postconceptional age, Apgar score) and calculated pharmacokinetic parameters are given as Spearman's (r_s) correlation coefficients. Finally, absolute oral bioavailability (F) was calculated comparing the AUCs (i.e., $AUC_{0-\infty}$) of midazolam after intravenous and oral administration (i.e., $F = [AUC_{po} / AUC_{iv}]$) in the same patients corrected for both dose (i.e., 0.1 mg/kg) and apparent differences in λ_z between evaluation periods (i.e., PO vs. IV). These statistical analyses were obtained using the SPSS software (version 9.0.0, SPSS Inc, Chicago, Ill). The level of significance accepted for all statistical analysis was $\alpha = 0.05$.

Results

Clinical results

Fifteen preterm infants (7 female, 8 male) with a mean gestational age of 28 ± 1.6 weeks and a postnatal age between 3 and 13 days, participated in the study (Table 1). Median (range) FiO_2 was 0.22 (0.21-0.27) in patients who were mechanically ventilated (n=6) and 0.21 (0.21-0.33) in patients who received continuous positive airway pressure (CPAP) by nasopharyngeal tube (n=8). Fourteen patients were antenatally exposed to indomethacin (to prevent preterm labor) and/or betamethasone (to induce lung maturation). Six patients received both drugs, and 8 received only betamethasone. Nine patients were postnatally exposed to indomethacin. Of these patients, three had a patent ductus arteriosus, for which they received indomethacin during the study and 6 patients had received their last dose of indomethacin at least 24 hours before start of the study. Eleven patients received caffeine prior to or during the study for weaning of the ventilatory support or for treatment of neonatal apnea. Antibiotics, in most cases betalactams and aminoglycosides, were required before or during the study in all patients for suspected or proven infection. Additional drug therapy included

Table 1 *Demographic and clinical parameters of the study patients (n=15)*

Parameters		
GA (weeks)	28.0 ± 1.6	(26.0-30.7)
PNA (days)	6.1 ± 2.7	(3-13)
Birth weight (g)	1076 ± 240	(745-1630)
Study weight (g)	1070 ± 232	(825-1660)
Apgar 1 min	6.0 ± 2.0	(3-9)
Apgar 5 min	7.9 ± 1.3	(5-10)

Data are expressed as means ±SD (range) GA: gestational age, PNA: postnatal age

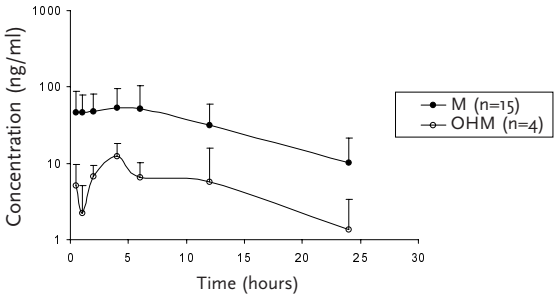
Table 2 *Oral midazolam calculated pharmacokinetic parameters in 15 preterm infants*

	Midazolam	**1-OH-midazolam**
Parameters		
AUC_{0t} (h.ng/ml)	613 (90-2286)	68.9 (<0.01-272.6)
$AUC_{0-\infty}$ (h.ng/ml)	642 (108-2465)	71.8 (1.6-305.0)#
$T_{1/2}$ (h)	7.6 (1.2-15.1)	3.6 (0.81-10.3)#
V_{ss}/F (L/kg)	1.4 (0.3-12.1)	NA
CL/F (L/kg/h)	0.16 (0.04-0.93)	NA
MRT (h)	12.0 (3.7-22.7)	9.7 (3.3-15.5)#
C_{max} (ng/ml)	64.4 (15.2-204.0)	10.3 (<0.01-22.1)
T_{max} (h)	2.0 (0.5-12.0)	4.0 (0.5-24.0)
AUC ratio		0.03 (<0.01-0.96)
F (%)	0.49 (0.12-1.0)	

Data are expressed as: median (range), # data of four patients

C_{max} = maximal concentration of drug in plasma, T_{max}= time to reach C_{max}, AUCot = area under the concentration-time curve from time zero to the last sampling time point, $AUC_{0-\infty}$ = area under the concentration-time curve from time zero to infinity, $t_{1/2}$ = elimination half-life, CL/F = total apparent clearance and V_{ss}/F = apparent volume of distribution at steady state, MRT = mean resident time, AUC ratio = 1-OH-midazolamAUC_{ot} / midazolamAUC_{ot}, F = oral bioavailability, NA = not available.

Figure 1 *Midazolam and 1-OH-midazolam mean concentration-time curves*



Concentration-time curve of midazolam and 1-OH-midazolam after 0.1 mg/kg oral midazolam: mean (SEM) concentrations of midazolam (n=15) and 1-OH-midazolam (n=4).

surfactant (n=9), morphine (n=4, >12 h before midazolam administration) and furosemide (n=1). No serious adverse events due to midazolam were reported throughout the course of the study.

Midazolam and 1-OH-midazolam pharmacokinetics

The mean plasma concentration-time curves for midazolam and 1-OH-midazolam are depicted in Figure 1. Apparent midazolam CL/F was [median (range)]: 2.7 (0.7-15.5) ml/kg/min, V_{ss}/F was: 1.4 (0.3-12.1) L/kg and $t_{1/2}$ was 7.6 (1.2-15.1) hours (Table 2). C_{max} was [median (range)]: 64.4 (15.2-204.0) ng/ml with a median T_{max} reached at 2 (0.5-12.0) h. In 9 out of 15 patients, 1-OH-midazolam could be quantitated over the post-dose sampling interval. Of these patients, median 1-OH-midazolam C_{max} was 10.3 (1.6-22.1) ng/ml with a median T_{max} reached at 4.0 (0.5-24) h. The median AUC ratio was low (0.03) with an almost 100-fold interindividual variation (range: 0.01-0.96). Sufficient concentration vs. time data were available in only four patients to reliably calculate the 1-OH-midazolam elimination half-life [mean (range)] which was 3.6 (0.81-10.3) h. In the 8 patients who received midazolam orally and intravenously the mean absolute oral bioavailability was 0.49 with a range of values (0.12-1.0) that also reflected considerable intersubject variability.

The effect of co-variates on midazolam and 1-OH-midazolam pharmacokinetics

No significant relationship was detected between age (postnatal, gestational or postconceptual age) and midazolam CL/F, V_{ss}/F or elimination half-life. We did not detect an effect of any of the clinical parameters or concomitant drug therapy [(i.e., feeding (enteral feeding n=3), ventilation (mechanically ventilated n=6), Apgar score, manner of delivery (spontaneous n=11), prenatal exposure to corticosteroids (n=14) or indomethacin (n=6), postnatal treatment with indomethacin (n=9), and caffeine therapy (n= 11)] on midazolam pharmacokinetic parameters.

Additionally, there was no relationship detected between age (postnatal, postconceptional or gestational) and 1-OH-midazolam pharmacokinetic parameters (C_{max}, AUC, $t_{1/2}$). Likewise, no relationship was detected between any of the clinical parameters or concomitant drug therapy and 1-OH-midazolam pharmacokinetics. A significant difference was not found with respect to comparison of age (i.e., both postnatal and postconceptional) between patients with or without detectable 1-OH-midazolam concentrations. In those infants where 1-OH-midazolam could be quantitated, the AUC ratio was used as a surrogate marker to assess total CYP3A activity. No association was observed between any of the demographic parameters and this ratio.

Discussion

In preterm infants, midazolam apparent oral clearance [2.7 (0.7-15.5) ml/kg/min] following oral administration was nearly 10-fold lower than previously reported in older children and adults (14.0-40.0 ml/kg/min) (8)). Accordingly, midazolam mean elimination half-life was longer in our patients (7.6 h) as compared to values reported in older children and adults (1.9-3.2 h) (6, 9, 10). This “impaired” midazolam elimination in preterm infants mirrors the recognized pattern for the ontogeny of CYP3A4 (11).

Moreover, in preterm infants, reduced midazolam clearance is also observed after intravenous administration consequent to low hepatic CYP3A activity shortly after birth (5). However, while intravenous midazolam clearance mainly reflects hepatic CYP3A activity (10), oral midazolam clearance is dependent on both intestinal and hepatic CYP3A activity (8). Therefore, the reduced midazolam clearance after oral administration to preterm infants suggests low hepatic ánd intestinal CYP3A4/5 activity directly after birth, which is supported by our observation that the median metabolite: drug AUC ratio is substantially lower [0.03 (0.01 - 0.96)] as compared to adults [0.43 ± 0.03 (mean ± SD)] (4).

In adults, 1-OH-midazolam plasma concentrations and the metabolite: drug AUC ratio were significantly higher following oral as compared to intravenous administration consequent to the role of intestinal 3A4 activity in midazolam biotransformation (4,14). Moreover, recent data indicate that the oral bioavailability of midazolam is almost entirely determined by CYP3A activity in the small intestine (7). Accordingly, low intestinal CYP3A4 activity would be expected to result in an increased oral bioavailability of midazolam, the converse being true for individuals with high intestinal CYP3A4 activity. This is further supported by the observation that oral administration of the CYP3A4 inhibitor clarithromycin resulted in a significant increase (from 31% to 75%) in the mean oral bioavailability of midazolam in healthy adult volunteers (17). Mexican adults with a systemic midazolam plasma clearance comparable to newborn infants (2.5 ± 0.4 vs. 2.0 ± 1.2 ml/kg/min) also demonstrated a relatively high oral absolute bioavailability [54 ± 6.1% (mean ± SEM)] of midazolam, confirming the aforementioned relationship between low CYP3A activity and increased absolute bioavailability of oral midazolam (15,16).

We were able to determine the absolute bioavailability of oral midazolam in approximately half of our study patients. Overall, the bioavailability of midazolam in these 8 patients averaged 49% which compares favorably with previous reports using a similar dose (per kilogram) given as an oral tablet or solution formulation in adult populations (24-38%) (4,10). Thus, it would appear that in preterm infants, both the rate and the extent of oral bioavailability may contribute in a substantial way to the substantial intersubject variability observed in the dose vs. plasma concentration relationship seen with oral administration of this drug. In preterm infants during the first two weeks of postnatal life, this most likely reflects intersubject variability in the constitutive expression of CYP3A4/5 in both the liver and the small intestine (18).

Whereas midazolam elimination shows a positive, linear association with age over the first years of life (11), we did not find a relationship between age (postconceptional, gestational or postnatal) and either the CL/F or AUC ratio within our study cohort. This finding is in agreement with previous reports from preterm and term newborn infants with gestational ages ranging from 24 to 39 weeks (5),13). This observation suggests that CYP3A4 activity increases only marginally during the first two weeks of postnatal life (12). However, the lack of a relationship between age and midazolam CL/F or AUC ratio in our study should be interpreted with caution given the relatively small sample size (n=15) of our study cohort in the face of apparent wide intersubject variability in the aforementioned parameters and the narrow range of gestational (26 to 31 weeks) and postnatal ages (3-13 days) that characterized our subjects.

As denoted previously, we used the AUC ratio of primary metabolite and parent

compound as a surrogate "marker" to assess CYP3A4/5 activity *in vivo*. Due to technical limitations (i.e., small sample volume), we were not able to measure the plasma 1-OH-midazolam-glucuronide concentrations. Therefore, the AUC ratio we reported is not "corrected" for glucuronidation (13). Nonetheless, given that the rate-limiting step in the formation of 1-OH-midazolam is catalyzed by CYP3A4, it was reasonable to evaluate the potential impact of development on enzyme activity using this AUC ratio.

Although 1-OH-midazolam concentrations have been measured in preterm infants (14), the pharmacokinetics of 1-OH-midazolam in preterm infants given an oral formulation of midazolam have, to our knowledge, not been previously reported. Despite considerable intersubject variability, the AUC ratio (Table 2) appears to be lower in preterm infants than values for this ratio previously reported in adults (4). The intuitive explanation for this finding would be reduced CYP3A4/5 activity in both liver and small intestine associated with immaturity of organ function. While midazolam is reportedly not a substrate for p-glycoprotein (15), it is unlikely that potential developmental differences associated with the functional competence of this transporter could have influenced the results of the AUC ratio. However, we can not rule out other "factors" (e.g., differences in gastrointestinal motility, loss of drug in stool) that could potentially impact either the rate or extent of midazolam absorption from the small intestine which, in turn, would influence the reliability of using the aforementioned AUC ratio as an indirect pharmacokinetic "surrogate" method.

The plasma concentrations of 1-OH-midazolam and the AUC ratio showed considerable interpatient variability (Table 2) and in 6 of our 15 patients, no 1-OH-midazolam could be detected. The intersubject variability in the AUC ratio is much larger in our cohort of preterm infants than the variability reported for midazolam and other CYP3A4/5 substrates after oral administration in adult populations (16). This variation may reflect organ-specific (i.e., intestine, liver) or CYP3A isoform-specific (i.e., CYP3A4, CYP3A5) differences in ontogeny. In addition to CYP3A4/5 activity, ontogenic differences in UGT activity may have contributed to the observed large intersubject variation in AUC ratios and 1-OH-midazolam plasma concentrations. Furthermore, although midazolam is almost completely metabolized in adults (10), renal excretion of unchanged midazolam may also contribute to the large intersubject variation in midazolam metabolism in preterm infants as is the case for caffeine, which is extensively metabolized in adults vs. approximately 90% renally excreted in preterm infants (22). Due to practical limitations (i.e., difficult and therefore, incomplete urine collections in preterm infants) we were not able to reliably quantitate midazolam and its metabolites in urine. Our inability to detect 1-OH-midazolam in a subset of patients may have been consequent to virtually absent constitutive CYP3A4/5 expression (i.e., CYP3A7 predominance) with the production of metabolite concentrations below the limit of detection for the analytical method. As well, the extreme intersubject variability in the AUC ratio may have been produced, in part, by non-enzymatic events (e.g. drug loss into stool, developmental differences in gastrointestinal motility capable of influencing the mean residence time of the drug at the absorptive surface area, intersubject differences in the size of the potential absorptive surface area in the small bowel) capable of influencing both the rate and extent of midazolam absorption.

The T_{max} for midazolam appears greater and more variable in preterm infants as compared to values previously reported in older children and adults [e.g., 2.0 (0.2-24)

h vs.: 0.90 ± 0.36 h and 0.37 ±0.05 h (mean ± SD), respectively](6, 17). Several factors, in addition to intestinal drug metabolism, may affect the rate of oral drug absorption in preterm infants (e.g., feeding, gastric pH, intestinal transit time) (18). While three out of our 15 patients received enteral feedings, we did not observe a difference in midazolam pharmacokinetics between patients who received oral feedings and those who did not. Our patient group may, however, have been too small to detect a truly significant difference in the pharmacokinetics of midazolam absorption associated with feeding *per se* or type of infant feeding. Finally, given that the median midazolam T_{max} is only reached after 2 hours and shows large interindividual differences, this route of administration is not suitable in preterm infants when a rapid sedative effect is needed for pre-procedural use.

In conclusion, the elimination of midazolam in preterm infants between 26 and 31 weeks gestational age and less than two weeks of postnatal age is impaired relative to older infants, children and adults consequent to reduced hepatic and intestinal CYP3A4/5 activity. Consequently, midazolam dosing regimens may need to be altered in young preterm neonates to prevent overdosing consequent to accumulation of midazolam and 1-OH-midazolam with repeated dosing. Based on absolute bioavailability of orally administered midazolam of approximate 49%, oral dosing in some infants may need to be increased to compensate for incomplete absorption as compared to the routine intravenous doses employed in clinical practice today. However, an apparent delayed rate of midazolam absorption in preterm neonates may limit the practical utility of the oral route when the drug is being given as a pre-procedural sedative agent. Finally, as reflected by examination of the pharmacokinetic data for 1-OH-midazolam in the first two weeks of life, developmental dependence of CYP3A4/5 activity is either absent or alternatively, obscured by the marked interindividual variability in this enzyme.

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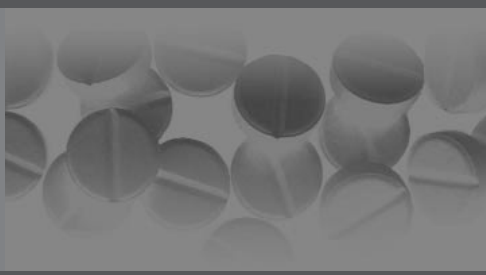
Population pharmacokinetics and metabolism of midazolam in pediatric intensive care patients

Chapter 6



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Submitted

Summary

Aim The aim of this study was to determine the pharmacokinetics and metabolism of midazolam in pediatric intensive care patients.

Methods The pharmacokinetics of midazolam and metabolites were determined in 21 pediatric intensive care patients with ages between 2 days and 17 years who received a continuous infusion of midazolam (0.05-0.4 mg/kg/h) for 3.8 hours to 25 days for conscious sedation. Blood samples were taken at different time points during and after midazolam infusion for determination of midazolam, 1-OH-midazolam and 1-OH-midazolam-glucuronide concentrations. A population analysis was conducted using a two-compartment pharmacokinetic model using the NPEM program. The final population model was used to generate individual Bayesian posterior pharmacokinetic parameter estimates.

Results Total body clearance, apparent volume distribution in terminal phase, and plasma elimination half-life were [mean ± SD, n=18]: 5.0 ± 3.9 ml/kg/min, 1.7 ± 1.1 L/kg and 5.5 ± 3.5 hr, respectively. The mean 1-OH-midazolam/midazolam ratio and (1-OH-midazolam + 1-OH-midazolam-glucuronide)/midazolam ratio were 0.14 ± 0.21 and 1.4 ± 1.1, respectively. Data from 3 patients with renal failure, hepatic failure and concomitant erythromycin/fentanyl therapy were excluded from the final pharmacokinetic analysis.

Conclusion We describe population and individual midazolam pharmacokinetic parameter estimates in pediatric intensive care patients using a population modeling approach. The derived population model can be useful for future dose optimization and Bayesian individualization. Lower midazolam elimination was observed in comparison to other studies in pediatric intensive care patients, probably due to differences in study design and patient differences such as age and disease state. Co-variates such as renal failure, hepatic failure and concomitant administration of CYP3A inhibitors are important predictors of altered midazolam and metabolite pharmacokinetics in pediatric intensive care patients.

Introduction

One of the most widely used sedative drugs in the pediatric intensive care is the short-acting benzodiazepine midazolam. Midazolam appears to reversibly potentiate the effects of γ-amino butyric acid (GABA), an inhibitory neurotransmitter of the central nervous system. Through this effect midazolam exerts its sedative, anxiolytic, anticonvulsive, muscle relaxant and amnesic effects, as has been illustrated in adults and children (1). Midazolam undergoes extensive metabolism by isoforms of the cytochrome P450 3A subfamily (e.g., CYP3A4 and CYP3A5) to a major hydroxylated metabolite (1-OH-midazolam) (2). 1-OH-midazolam is equipotent to midazolam and its concentrations are the resultant of formation by CYP3A and subsequent metabolism to 1-OH-midazolam-glucuronide by UDP-glucuronosyltransferases (UGTs). 1-OH-midazolam-glucuronide is subsequently renally excreted (3, 4). 1-OH-midazolam-glucuronide also appears to have sedative properties when concentrations are high, as has been observed in adult patients with renal failure (5).

CYP3A4/5 activity reaches adult levels between 3 and 12 months of postnatal age (6). Developmental differences in CYP3A activity may therefore alter the pharmacokinetics of midazolam in pediatric intensive care patients of different ages (7). Similarly, the UGTs exhibit developmental changes in activity. However, as the specific UGTs involved in the conjugation of 1-OH-midazolam are yet unknown, the impact of ontogeny on this reaction remains to be determined.

In addition, the pharmacokinetics of midazolam in critically ill patients can be altered due to single or multiple organ failure (8). The dysfunction of organs such as liver and kidney may affect the pharmacokinetics of midazolam in a different way for each patient (5) (9) (10). In adult intensive care patients, midazolam elimination is impaired in comparison to healthy adults (11) (12). In pediatric intensive care patients, however, no such difference in midazolam elimination between intensive care patients and healthy children has been found (13, 14) (15).

Although the pharmacokinetics of midazolam have been characterized in healthy children after a single bolus dose, few data are available on the pharmacokinetics of midazolam in pediatric intensive care patients (1, 11, 14, 16). Data on the disposition of 1-OH-midazolam and 1-OH-midazolam-glucuronide are scarce for both sick and healthy children in all age groups. Knowledge of the pharmacokinetics of midazolam and its metabolites may serve as a valuable tool for developing optimal infusion regimens for pediatric patients during intensive care. The aim of our investigation was, therefore, to determine the pharmacokinetics of midazolam and its metabolites in pediatric intensive care patients between birth and 18 years of age during continuous intravenous infusion of midazolam.

Methods

Patient recruitment

The children were recruited from the Pediatric Intensive Care Unit of the Sophia Children's Hospital, Rotterdam, The Netherlands between May 1999 and April 2000. The institutional review board approved this research protocol. Written, informed consent was obtained from patients and/or parents or legal guardians prior to enrollment in the study.

The pharmacokinetic data presented here are part of a prospective pharmacokinetic-pharmacodynamic investigation of intravenous midazolam infusion in pediatric intensive care patients used for conscious sedation.

Patients were eligible for study entry if they were between 0 and 18 years old, needed midazolam for conscious sedation and already had an indwelling arterial catheter placed for purposes of medical care. Patients were excluded if they (1) received concomitant neuromuscular blockade drugs, (2) were exposed to midazolam for longer than 12 hours prior to start of the investigation, (3) were exposed to midazolam prior to start of the investigation without exact information on midazolam dosing, (4) were exposed to recent (i.e. < 24 hours prior to dosing) or chronic treatment with medications known or suspected to alter the pharmacokinetics of midazolam. Since midazolam is a substrate for cytochrome P450 3A enzymes, potential patients were evaluated for exposure to drugs known to affect CYP3A activity (e.g. erythromycin, phenobarbital, dexamethasone, and cisapride) (17).

Study design

Midazolam (0.1 mg/kg in glucose 5%) was given as an intravenous bolus, after which an intravenous midazolam infusion was started at a rate of 0.1 mg/kg/h through microbore tubing into a peripheral vein or central catheter. Subsequently, the infusion rate was adjusted to keep the patient comfortable as estimated by the COMFORT score. The COMFORT score is a previously validated sedation scale for use in the pediatric intensive care patient and postoperative neonates (18) (19). If a patient was not adequately sedated, a bolus dose midazolam (0.1 mg/kg) was given, followed by an increase in infusion rate of 0.05 mg/kg/h. If a patient was too sedated, the infusion rate was decreased by 0.05 mg/kg/h. If sedation was no longer needed clinically, the infusion rate was decreased by 0.1 mg/kg/h every 12 hours.

Blood sampling and drug assay

A blank arterial blood sample was obtained before administration of the loading dose. During the infusion of midazolam, serial arterial blood samples were obtained at the following time points:

1 Prior to and 2 and 30 minutes after each midazolam loading dose
2 every morning at 8.00 am
3 prior to and 10, 30 minutes, 1, 2, 4, 6, 12 and 24 hours after discontinuation of the infusion.

Plasma was separated from whole blood by centrifugation (1000 X g for 10 minutes) and then stored at -80°C until analysis. Plasma samples were analyzed for midazolam and 1-OH-midazolam by validated high-pressure liquid chromatography (HP G13 series, Agilent, Amstelveen, The Netherlands) with diode array UV detection. (HP G1315A, Agilent, Amstelveen, The Netherlands) The column used was Novapak C18 (Waters, Ettenleur, The Netherlands). Diazepam (12.5 ng/100µl H2O, Bufa, Uitgeest, The Netherlands) was added to each sample as internal standard. Solid phase extraction was performed using alkalic extraction with DCM (Rathburn, Walkerburn, Switzerland)

The inter-day coefficients of variation at the low standard concentration (20 ng/ml) were less than 6.9% and 10.3% for midazolam and 1-OH-midazolam, respectively. The lower limit of quantitation was 20 ng/ml for both midazolam and 1-OH-midazolam using 0.5 ml plasma volume.

To measure 1-OH-midazolam-glucuronide in plasma, plasma samples were prepared with 50 µl β-glucuronidase (IBF Biotechnics, Villeneuve-la-Garenne, France, 100.000 Fishmann units/ml) and incubated at 40°C for 16 hours. Samples were processed and quantitated for total 1-OH-midazolam (conjugated plus unconjugated) in the same manner as described earlier. 1-OH-midazolam-glucuronide concentrations were then determined by using the following equation: [1 -OH-MG]= d[1-OH-M] x [M 1-OH-MG/M 1-OH-M], where: [1-OH-MG] = 1-OH-midazolam-glucuronide concentration, d[1-OH-M] = concentration difference of 1-OH-midazolam before and after hydrolysis, M 1-OH-MG = molecular weight of 1-OH-midazolam-glucuronide (517,9) and M 1-OH-M = molecular weight van 1-OH-midazolam (341,8).

Pharmacokinetic analysis

Population modeling

Midazolam concentration data were used to develop a population model by using the nonparametric expectation maximization algorithm developed by Schumitzky (NPEM program, USC*PACK clinical collection version 10.7, LAPK, Los Angeles, CA) employing all available data points per patient (20) (21).The model was parameterized in terms of volume of distribution of the central compartment V_1 (L/kg), elimination rate constant k_{el} (h^{-1}), and distribution constants k_{12} (h^{-1}) and k_{21} (h^{-1}). In order to weight each serum concentration correctly by the reciprocal of its variance an assay error pattern was used, fitted by the following equation: SD= 0.02 + 0.05C, where SD is the standard deviation of the assay and C represents the measured midazolam serum concentration (ng/ml). This error pattern was multiplied by gamma of 2.81, a factor determined by NPEM program to capture additional environmental noise, before calculating the population model. (22).

The modeling process consisted of two steps. The front part of the NPEM program was used to calculate individual parameter estimates for V_1, k_{el}, k_{12} and k_{21} in a two-compartment model with an iterative 2-stage Bayesian (IT2B) algorithm. The ranges for the parameter estimates were then used as input for the actual NPEM program resulting in the calculation of the entire probability density function (PDF). NPEM also provided mean and median population parameter estimates, SD's and the covariance and correlation matrices.

Population model and Bayesian feedback

The NPEM derived population model was entered into the MW\Pharm program (MW\Pharm, version 3.30, MediWare; Groningen, The Netherlands) and was used to generate the following individual Bayesian posterior pharmacokinetic parameter estimates: clearance (CL), volume of distribution of the central compartment and volume of distribution in terminal phase (V_1 and V_β, respectively), distribution half-life ($t_{1/2\alpha}$) and elimination half-life ($t_{1/2\beta}$). To test the internal consistency of the individual pharmacokinetic parameter estimates generated by the MW/Pharm program, we compared predicted and measured midazolam serum concentrations in individual patients by calculating bias and precision (23). The mean 1-OH-midazolam/midazolam ratio [OHM/M] and (1-OH-midazolam + 1-OH-midazolam-glucuronide)/midazolam ratio [(OHM+OHMG)/M] were calculated with and without correction for molecular weight of midazolam and metabolites.

Statistical analysis

Data are expressed as means ± SD unless stated otherwise. Mean drug and metabolite concentrations and metabolite: drug ratios were determined from the mean concentrations or ratios from each patient. Because the assumption of normality was not fulfilled for all pharmacokinetic parameter estimates, we calculated a Spearman's correlation coefficient to investigate a correlation between age and pharmacokinetic parameters. These statistical analyses were obtained using SPSS software (version 9.0.0, SPSS Inc., Chicago, Ill). The level of significance accepted for all statistical analysis was $\alpha = 0.05$.

Results

Patient clinical characteristics

21 patients were enrolled in this study ranging in age from 2 days to 17 years. The characteristics of the individual patients are listed in Table 1. All patients received concomitant drug therapy inherent in intensive care. Four patients required analgesia in the form of opioids (morphine, codeine, and fentanyl) during the midazolam infusion. Three additional patients received acetaminophen for fever reduction. Although the prescription of a drug known to affect CYP3A activity, was an exclusion criteria before entry in the study, two patients received such a drug after inclusion in the study. One patient (no 8) received a bolus of the CYP3A4 inducer dexamethasone (corticosteroid) on the second and third study day before a scheduled extubation (24). Another patient (no 11) received both the CYP3A4 inhibitor erythromycin (macrolide antibiotic) and the CYP3A4 substrate fentanyl during the whole study period (25-27). Other prescribed drugs were amphotericin B, amoxicillin, cefotaxime, cefuroxime, tobramycin, vancomycin, etomidate, furosemide, spironolactone and prostaglandin E1.

Midazolam dosing

In eleven patients study participation ended before midazolam was discontinued, for the following reasons: a) neuromuscular blockade (n=1), b) death (n=1), c) arterial line removal (n=4) and d) discharge from the intensive care unit (n=4). One patient (no 4) was withdrawn from the study by his guardian. In ten patients a (partial) washout curve could be established.

The median duration of infusion was 3.4 days (range 3.8 hours to 25 days) with a median infusion rate of 0.09 mg/kg/h (range 0.05 – 0.27 mg/kg/h). The median total dose of midazolam infused was 2.9 mg/kg (range 0.40 – 46.0 mg/kg). On average, patients received two (range 0 – 12) bolus injections of midazolam during the study period, and underwent 3 (range 1 – 10) infusion rate changes. In two patients the midazolam infusion was started without a loading dose. The reason for this protocol violation was that the attending physician considered possible hypotension associated with a midazolam bolus contraindicated given the patient's condition.

Midazolam pharmacokinetics

A two-compartment model was used to model the midazolam pharmacokinetics (28). We first modeled midazolam pharmacokinetics using data from all 21 patients. Three patients were excluded from the final population pharmacokinetic analysis, as their PK

Table 1 *Patient characteristics*

	Sex	Age	Weight (kg)	Origin	ICU admission reason
Patient					
1	Male	0.04 mths	3.5	Caucasian	Congenital heart disease
2	Male	0.12 mths	3.8	Caucasian	Congenital heart disease
3	Female	0.36 mths	3.6	Caucasian	Congenital heart disease
4	Male	0.48 mths	2.8	African	Postcardiac surgery
5	Female	0.60 mths	3.7	Caucasian	Congenital heart disease
6	Female	0.96 mths	4.3	Mediterranean	Respiratory insufficiency eci
7	Female	1.9 mths	4.8	Afro-Caribbean	Upper airway infection
8	Male	2.0 mths	3.6	Caucasian	Upper airway infection
9	Male	4.1 mths	7.5	Caucasian	Meningitis
10	Male	8.2 mths	20	Caucasian	Upper airway infection
11	Male	1.1 yrs	9.3	Asian	Pneumonia
12	Male	2.8 yrs	13	Asian	Empyema
13	Male	3.9 yrs	15	Afro-Caribbean	Acute laryngotracheobronchitis
14	Female	4.5 yrs	19	Caucasian	Pulmonary hypertension
15	Female	5.5 yrs	24	Caucasian	Staphylococcal scalded skin syndrome
16	Female	8.9 yrs	22	Middle-Eastern	Pulmonary hypertension
17	Male	9.1 yrs	25	Caucasian	Measles pneunomia
18	Female	13.1 yrs	40	Caucasian	Final stage ALL with multiple organ failure
19	Male	14.8 yrs	50	Caucasian	Ebstein-Barr virus infection
20	Female	15.1 yrs	52	Hispanic	Malignant hypertension
21	Male	17.0 yrs	60	Mediterranean	Postcardiac surgery

ALL = acute lymphatic leukemia, mths = months, yrs = years

parameters were very different in comparison to the other patients, as a result of renal failure, hepatic failure and erythromycin co-medication, respectively. Exclusion improved the final model (log-likelihood) and resulted in better estimates for the individual Bayesian pharmacokinetic parameter estimates, due to reduced inter-subject variability in the final population model.

The mean population pharmacokinetic parameter estimates provided by NPEM were: V_1 0.43 ± 0.20 L/kg; k_{el} 0.70 ± 0.40 /h; k_{12} 1.49 ± 0.84 /h and k_{21} 0.90 ± 0.48 /h. The mean individual Bayesian posterior pharmacokinetic parameter estimates were:

clearance (CL): 5.0 ± 3.9 ml/kg/min, volume of distribution (V_β): 1.7 ± 1.1 L/kg, distribution half-life ($t_{1/2\alpha}$): 0.24 ± 0.06 h and elimination half-life ($t_{1/2\beta}$): 5.5 ± 3.5 h. The median bias and precision of the observed vs. predicted midazolam concentrations, using the individual Bayesian pharmacokinetic parameter estimates was 12 ng/ml (3.8 %) and 47 ng/ml (14.6 %), respectively.

1-OH-midazolam and 1-OH-midazolam-glucuronide

Mean 1-OH-midazolam and 1-OH-midazolam-glucuronide concentrations were 60 ± 30 ng/ml and 556 ± 262 ng/ml, respectively. The mean 1-OH-midazolam/midazolam ratios were 0.21 ± 0.20 (not corrected for molecular weight, MW) and 0.14 ± 21 (corrected for MW). The mean (1-OH-midazolam +1-OH-midazolam-glucuronide)/midazolam ratios were 2.1 ± 1.5 (not corrected for MW) and 1.4 ± 1.1 (corrected for MW).

Effect of age on midazolam pharmacokinetics

No relationship could be detected between age and for body size corrected midazolam pharmacokinetic parameters or between age and for body size and dose corrected metabolite: drug ratios. We excluded patient 7, 16 and 19 from the analysis on the effect of age on midazolam disposition, as the pharmacokinetics of these patients were confounded by other co-variates than age (i.e. renal failure, hepatic failure and erythromycin co-administration).

Outliers

We also used the population model to estimate the individual Bayesian pharmacokinetic parameters for the three patients who were excluded from the final modeling. Patient 20 had renal insufficiency secondary to a non-functioning transplant kidney (creatinin 691 µmol/l). Elimination half-life was prolonged (40 hours), volume of distribution was large (16 L/kg), but midazolam clearance was within the population range (4.7 ml/kg/min). Her mean (1OHM+1OHMG)/M ratio was more than three times above the standard deviation of the population (i.e.: 14.8). Patient 18 had acute lymphatic leukemia and died within 24 hours after inclusion in the study of a secondary systemic aspergillosis infection. She had hepatic failure based on liver function tests (increased γGT, ASAT, ALAT, LD, reduced serum albumin concentration and impaired clotting function). Elimination half-life was extremely long (>100 hours), clearance very slow (<0.001 ml/kg/min), but volume of distribution (V) in the population range (1.4 L/kg). Her mean 1OHM/M and (1OHM+1OHMG)/M ratios were very low (0.06 and 0.09). Patient 11 received the CYP3A4 inhibitor erythromycin and CYP3A4 substrate fentanyl; consequently his midazolam elimination was also impaired [$t_{1/2\beta}$ 24.0 h, CL 2.2 ml/kg/min, OHM/M ratio 0.03 and (OHM +OHMG)/M ratio 0.40].

Discussion

Midazolam pharmacokinetics

This is the first report describing midazolam pharmacokinetics in pediatric intensive care patients using a population modeling approach. An additional advantage of the approach used is that, based on the derived model, we were able to estimate

pharmacokinetic parameters (CL, $t_{1/2\alpha}$, $t_{1/2\beta}$, V_1 and V_β) in individual patients with a high degree of precision using a Bayesian feedback algorithm. Moreover, using this approach, there will be no need for complete sets of post-infusion midazolam concentrations to estimate midazolam pharmacokinetic parameters in individual patients. Because three patients had a clinical condition associated with altered midazolam pharmacokinetics (renal failure, hepatic failure and concomitant erythromycin therapy), we excluded these patients from the final population pharmacokinetic analysis. A limitation of this study is that we did not validate our model prospectively in a separate group of patients, consequent to the limited sample size. Therefore, before using this model to predict midazolam pharmacokinetic parameters from other sparse data sets, prospective validation is needed

Midazolam undergoes extensive metabolism by members of the cytochrome 450 3A subfamily, i.e. CYP3A4, CYP3A5 and to a lesser extent by CYP3A7, to its main metabolite 1-OH-midazolam (2) (29). CYP3A4 activity is low at birth, with an increase thereafter to reach adult levels at one year of age (6). Therefore, midazolam clearance may alter as a function of age. Mean clearance rate in this study, with half of our patients less than 6 months of age, is lower than reported in patients older than two years of age (9.6 ± 3.5 ml/kg/min and 13.0 (range 2.3-66.7) ml/kg/min (14, 15). In contrast, mean midazolam clearance rate is higher in this study than reported in neonatal intensive care patients [median 1.8 (range 0.6-2.7) ml/kg/min] (3) (14, 30). Midazolam clearance in our study was comparable to data from another study in pediatric intensive care patients between 26 days and 5 years of age, when studied during the first 24-48 hours of midazolam infusion (5.8 ± 3.8 ml/kg/min vs. 5.0 ± 3.9 ml/kg/min) (16). Hence, our data in addition to data from other studies in pediatric patients, support an age-related increase in midazolam clearance, consequent to an age-related surge in CYP3A4/5 activity (7). Since our patient population covers the whole pediatric age range, we also studied the relationship between age and midazolam elimination in our patient sample. However, mean midazolam clearance was not statistically different between patients younger and older than 6 months of age (3.8 ± 1.9 ml/kg/min vs. 6.2 ± 5.0 ml/kg/min, p=0.39). Moreover, none of the other pharmacokinetic parameters showed a relationship with age.

Other differences in study design may also contribute to the disparate results on midazolam disposition. First, we used a population modeling approach to calculate pharmacokinetic parameters, while others calculated midazolam clearance by dividing infusion rate by 'steady-state' plasma concentration. Our data show that midazolam half-life appears to be longer than in healthy children (5.5h vs. 1.2h) (13). Since time of steady-state was defined as 6 or 24 hours after an infusion rate change, steady-state may not have been reached in patients in those studies (14, 16). This may very well have resulted in an overestimation of midazolam clearance values. In this study, clearance rates estimated using steady-state (>24 hours) do not differ significantly from clearance rates estimated using population pharmacokinetics (data not shown). Second, differences in disease state may also contribute to reported differences in midazolam pharmacokinetic parameters. Intrinsic hepatic disease or cytokines associated with systemic infection have been shown to alter CYP3A activity (10) (8). Moreover, disease-related changes in body composition or hepatic blood flow may alter midazolam pharmacokinetics of intensive care patients (9) (11). (10).

Midazolam elimination half-life (5.5 ± 3.5h) is longer than reported for healthy children between 3 and 10 years of age (1.2 ± 0.3 h) (13). These data are in agreement with adult data, where midazolam elimination is also impaired in critically ill patients as compared to healthy adults (11) (12). Hence, midazolam can not be considered a drug with a short elimination half-life in pediatric intensive care patients. Without a loading dose, steady-state of a midazolam infusion will only be reached after approximately 20 hours. Therefore, midazolam sedation should be initiated with a bolus followed by a continuous infusion in pediatric intensive care patients.

1-OH-midazolam and 1-OH-midazolam-glucuronide

The mean 1OHM/M ratio (0.21 ± 0.20) in our patients is in agreement with the OHM/M ratio in pediatric patients who received midazolam after cardiac surgery [mean 0.25 (SEM ± 0.03)] (31). In contrast, the OHM/M ratio was higher in our patients than in newborn infants less than two weeks of age during continuous infusion of midazolam (0.06 ± 0.05) (3). Since the OHM/M ratio may be a surrogate measure of CYP3A activity, these findings also imply rapid developmental changes in CYP3A4/5 activity between birth and the first weeks of life.

The ratio between the plasma concentrations of 1-OH-midazolam + 1-OH-midazolam-glucuronide and midazolam is governed by the metabolic rate of midazolam and 1-OH-midazolam and the renal excretion rate of 1-OH-midazolam-glucuronide, which is in turn related to the glomerular filtration rate of the patient (9). Therefore, the OHM/M and (OHM+OHMG)/M ratios will provide insight in the contribution of the different metabolic pathways. The following observations suggest that the metabolism of 1-OH-midazolam to 1-OH-midazolam-glucuronide by UGT isoforms is not importantly impaired in children between 2 days and 17 years of age compared to adults. First, the mean (OHM+OHMG)/M ratio in our patients (2.1 ± 1.5) is higher than reported in adult intensive care patients [median 0.96 (range 0.1-23.2)] (9). Second, the OHM/M ratio is almost 7 times lower than the (OHM+OHMG)/M ratio. Finally, the (OHM+OHMG)/M ratio did not differ between children younger or older than 6 months of age (Mann-Whitney p=0.754). However, reduced renal excretion of 1-OH-midazolam-glucuronide consequent to immature renal function in the first weeks after birth, may obscure lower UGT activity in this period (32).

Outliers

Three patients had clinical characteristics, which are associated with altered midazolam pharmacokinetics. Pharmacokinetic analysis of data from these patients did indeed show altered midazolam pharmacokinetics.

First, consequent to renal failure the elimination half-life of patient 20 was extremely long, volume of distribution was extremely large, but midazolam clearance was in the normal range of our patient group. As discussed by Reves et al. (33), the free fraction of midazolam is significantly increased in renal-failure patients. Consequently, after correction for free-fraction no significant difference in midazolam pharmacokinetics will remain (33). Also consequent to renal failure, her mean 1-OH-midazolam-glucuronide concentration was high (1100 ng/ml) as well as her (OHM+OHMG)M ratio. Since this patient received midazolam for only 12 hours, the metabolite did not accumulate to a great extent (5). However, if renal-failure patients receive higher doses

of midazolam or an infusion for a longer period of time, prolonged sedation (> 3 days after cessation of the infusion) may occur due to accumulation of the 1-OH-midazolam-glucuronide in the absence of detectable concentrations of midazolam or 1-OH-midazolam (5).

In patient 18, who was critically ill with hepatic failure, midazolam elimination was significantly impaired due to impaired midazolam metabolism (low metabolite : drug ratios). This impairment in midazolam metabolism was most likely due to liver failure, as has been shown in critically ill adult patients (8). In addition, inflammatory mediators in serum may also have impaired midazolam metabolism consequent to reduced CYP3A activity (10).

The concomitant administration of the CYP3A4 inhibitors erythromycin and fentanyl in patient 11 also reduced midazolam elimination as a result of inhibition of midazolam metabolism (14, 34).

In summary, using a population pharmacokinetic approach we were able to estimate pharmacokinetic parameters for individual patients. The derived population pharmacokinetic model can be useful for future dose optimization and Bayesian individualization in pediatric intensive care patients. Lower midazolam elimination was observed in comparison to other studies in pediatric intensive care patients, probably due to differences in study design and patient differences such as age and disease state. Renal insufficiency, hepatic failure and concomitant administration of CYP3A inhibitors are important predictors of altered midazolam and metabolite pharmacokinetics in pediatric intensive care unit patients for which alternative dosing strategies have to be developed.

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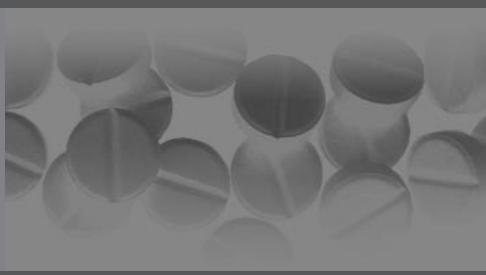
CYP3A4-V polymorphism detection by PCR-RFLP and its allelic frequency among 199 Dutch Caucasians

Chapter 7



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Summary

Background Interindividual variation in drug metabolism may complicate the treatment of patients in modern medicine. Detection of genetic polymorphisms in cytochrome P450 enzymes may contribute to an improvement of effective pharmacotherapy. Recently, a genetic polymorphism was identified in the nifedipine-responsive element in the promoter region of the abundantly expressed cytochrome P450 3A4 enzyme (CYP3A4).

Methods We developed a simple and rapid assay for the specific detection of the CYP3A4-V polymorphism. The assay uses a polymerase chain reaction (PCR) with specifically designed oligonucleotide primers, followed by restriction fragment length polymorphism (RFLP) detection.

Results Applying this assay on genomic DNA obtained from 199 healthy Dutch Caucasian volunteers, we identified 21 heterozygotes and no homozygotes for this polymorphism. This corresponds to an allelic frequency of 5.3% for the CYP3A4-V allele in the group studied.

Conclusion A PCR-RFLP procedure is described which can be used for screening purposes for the *CYP3A4-V* allele. Using this assay, we found that the allelic frequency in Dutch Caucasians is significantly lower than the allelic frequency reported by Rebbeck et al. (J Natl Cancer Inst 1998; 9:1225-1229) in U.S. Caucasians.

Introduction

Interindividual variation in drug metabolism is a complicating factor in pharmacotherapy. Enzymes of the cytochrome P450 system are involved in the metabolism of several endogenous substrates and a broad range of foreign compounds, such as drugs, environmental pollutants and carcinogens (1). The cytochrome P450 enzyme family consists of several subfamilies, with CYP3A4 being the most abundant P450 enzyme in human liver (2). CYP3A4 is involved in the metabolism of >50% of all drugs used in humans (3, 4). Interindividual differences in CYP3A4 expression may account for the observed interindividual differences in pharmacokinetics of drugs metabolized by this enzyme (4-6). Variations in CYP3A4 expression may be caused by factors inhibiting or stimulating transcription and/or translation (e.g. concomitant drug administration), and by genetic polymorphisms.

In a recent study, an A (-290) G substitution was described in the 5'-regulatory region of the *CYP3A4* gene (7, 8). This allele was termed *CYP3A4-V*, but was recently also referred to as *CYP3A4*1B* (9). The allelic frequency, determined by using conformation sensitive gel electrophoresis, was estimated to be 9.6% among U.S. Caucasians (7). In 94 healthy unrelated male volunteers, 3.2% were found to be homozygous and 12.8% heterozygous for the CYP3A4-V allele (7). In a recent publication, the allelic frequency was shown to display major interethnic differences, ranging from 0.0% among Chinese Americans to 54.6% among black Americans (10, 11). The *CYP3A4-V* allele has recently been associated with higher clinical stage and grade of prostate cancer (11-13).

In this study, a simple PCR-RFLP procedure was developed that can be used in routine screening procedures to detect the *CYP3A4-V* polymorphism. The assay was used on 199 DNA samples obtained from Dutch Caucasian volunteers to determine the allelic frequency of the *CYP3A4-V* allele in this group.

Materials and Methods

EDTA- whole blood was collected from 199 healthy Caucasian volunteers after informed consent. The Medical Ethical Committee of the University Hospital Rotterdam approved the study. From 300 µL of blood, genomic DNA was isolated using the GenomicPrep Blood DNA Isolation Kit (Amersham Pharmacia Biotech, Buckinghamshire, UK). DNA yields were estimated by measuring the absorbance at 260 nm (A_{260}). For the polymerase chain reaction (PCR), approximately 50 ng of genomic DNA was used in a total PCR volume of 50 µL. The PCR-mixture contained 1X buffer (10 mmol/L Tris-HCl pH 8.3, 1.5 mmol/L $MgCl_2$, 50 mmol/L KCl and 0.01 g/L (w/v) gelatin) (Perkin-Elmer, Norwalk, CT, USA), 0.2 mmol/L of each dNTP (Boehringer Mannheim, Germany), 1.25 U AmpliTaq Gold (Perkin-Elmer), 40 pmol forward primer 5'-GGA CAG CCA TAG AGA CAA CTG CA-3' (position -315 to -291 (8)) and 40 pmol reverse primer 5'-CTT TCC TGC CCT GCA CAG-3' (position +22 to +5 (8)). The underlined nucleotides are mismatches with the *CYP3A4* sequence, creating a *Pst*I restriction site in the *CYP3A4-V* PCR product. The 3' end of the forward primer lies in the nifedipine-responsive element, a sequence that is characteristic for CYP3A4 and is absent in the homologous CYP3A7 sequence, ensuring specific amplification of CYP3A4 (8). PCR conditions were as follows: 7 min 94 °C, 35 cycles of [1 min 94 °C, 1 min 55 °C, 1 min 72 °C] and finally 7 min 72 °C. A 334-bp fragment was amplified using the CYP3A4-V primers described

(Fig 1. Lane 0). Fifteen µL of PCR product was digested for 1 1/2 hours at 37 °C by adding 10 U PstI (Boehringer Mannheim). The digested product was analyzed on a 3% agarose/Tris-borate-EDTA gel with ethidium bromide staining. The DNA sequences of the nifedipine-responsive element were verified by direct sequencing. Genomic DNA was amplified by PCR: 7 min 94 °C, 35 cycles of [30 sec 94 °C, 30 sec 55 °C, 30 sec 72 °C], followed by 7 min 72 °C using forward primer 5'-AAC AGG GGT GGA AAC ACA AT-3' and reverse primer 5'-CTT TCC TGC CCT GCA CAG-3' (7). Direct sequencing was performed on an automated ABI 310 capillary sequencer (Perkin-Elmer) using the Big Dye Terminator Cycle Sequencing Kit (Perkin-Elmer).

Results

PCR-RFLP for CYP3A4-V

A 334 bp fragment was amplified; using the *CYP3A4-V* primers described (Fig. 1B, lane 0). Digestion of the PCR product with PstI results in 220, 81 and 33 bp fragments for the wild-type sequence (Fig. 1B, lane wt/wt), or 199, 81, 33 and 21 bp fragments for the CYP3A4-V allele (Fig. 1B, lane V/V). Two internal PstI sites in this fragment serve as an internal control for digestion (Fig. 1A). In case of heterozygosity, both the 220 bp and the 199 bp fragments are present (Fig. 1B, lane wt/V). The DNA sequences of the nifedipine-responsive element of these controls were verified by sequencing.

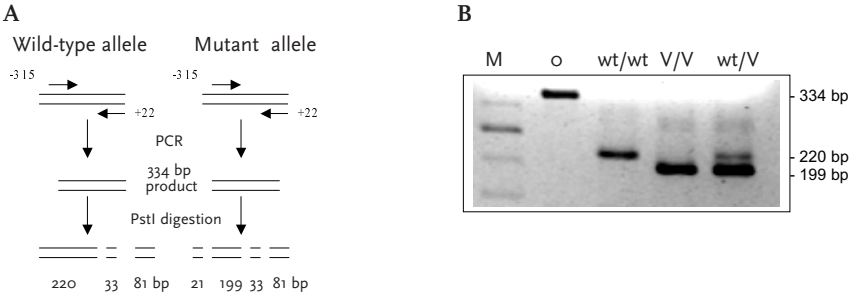
Allelic frequency in a group of 199 Dutch Caucasians

Using the described PCR-RFLP, we screened 199 unselected and unrelated healthy Caucasians for the CYP3A4-V allele using the wt/wt, wt/V and V/V samples described above as controls. We identified 21 CYP3A4-V alleles, resulting in an allelic frequency of 5.3%. In total, 21 individuals were found to be heterozygous for this allele, giving a

Figure 1

Panel A *Schematic outline PCR-RFLP procedure for the CYP3A4-V allele. The starting positions of the primers are indicated (8).*

Panel B *Undigested PCR product (0), and PstI-digested PCR product for wild-type (wt/wt), homozygote (V/V) and heterozygote DNA samples (wt/V) for CYP3A4-V. The 81, 33 and 21 bp bands are not visible. Analysis on a 3% agarose/TBE gel; picture is printed negative. M= basepair marker (50 bp ladder).*



heterozygote frequency of 10.6%. No individuals were found that were homozygous for this polymorphism, indicating that the homozygote frequency is likely to be under 2% in this population. DNA sequencing of all heterozygous individuals confirmed the A(-290)G substitution in all cases; no additional mutations were detected in the nifedipine-responsive element. As a control, 10 wild-type samples were also sequenced, confirming that the wild-type DNA sequence was present in the nifedipine-responsive element in all cases.

Discussion

The initial publication describing the *CYP3A4-V* polymorphism made use of PCR of the -571 to +22 bp region of the *CYP3A4* allele in which the PCR product was analyzed using conformation-sensitive gel electrophoresis (7). In order to simplify the procedure for large scale screening purposes, and to make screening specific for the described *CYP3A4-V* mutation, we developed a PCR in which a simple digestion with PstI reveals the presence of this polymorphism. The forward primer has two mismatches, creating a PstI restriction site when the *CYP3A4-V* allele is amplified. Due to the fact that two additional PstI sites are present in the PCR-product, an internal control of digestion is automatically included. The 3' end of the forward primer lies in the nifedipine-responsive element, a sequence which is characteristic for *CYP3A4* and which is absent in the homologous *CYP3A7* sequence, ensuring specific amplification of *CYP3A4* (8). DNA sequencing of 31 samples confirmed the reliability of the assay. Interestingly, in the heterozygous samples, the 220 bp wild-type derived band was usually less intense when compared to the 199 bp *CYP3A4-V* derived band. We assume that the first base to be added by the Taq-polymerase, being a G for the *CYP3A4-V* sequence and an A for the wild-type sequence, increases the stability of the forward primer-genomic DNA complex better for the *CYP3A4-V* allele when compared to the wild-type sequence. We tried to minimize this effect by having a forward primer synthesized missing the 3' A base, making the stability of the primer-DNA complex less dependent on the first base to be added by the Taq-polymerase. PCR-RFLP with this alternative forward primer in combination with the described reverse primer, yielded 220 bp and 199 bp bands of equal intensity (results not shown). However, the overall efficiency of the PCR reaction was dramatically decreased, probably because the two mismatches with the *CYP3A4* sequence are now in too close proximity of the 3' end of the primer, decreasing efficiency of the PCR. Application of this alternative primer therefore needed 45 cycles of PCR and 25 µL of PCR product for digestion and subsequent detection on gel.

Recently, another PCR-RFLP procedure was described for detection of the *CYP3A4-V* allele, which is based on creating a MboII restriction site (15). This procedure makes use of nested PCR, which is more laborious and has an increased risk of sample contamination. Moreover, the *CYP3A4-V* allele PCR products cannot be digested, which means that this assay lacks an important internal control on digestion efficiency.

Applying the assay on 199 samples obtained from Dutch Caucasian volunteers identified 21 heterozygotes for the *CYP3A4-V* allele, resulting in an allelic frequency of 5.3%. This is significantly different ($p<0.05$, Fisher's Exact Test) from the reported allelic frequency of 9.6% (18 *CYP3A4-V* alleles in 94 individuals) by Rebbeck et al. for U.S. Caucasians (7). In that specific study on 94 healthy volunteers, 3.2% appeared to be

homozygous for this mutation. We did not find any *CYP3A4-V* homozygotes among the 199 individuals studied. The allele and genotype frequencies were in Hardy-Weinberg equilibrium (P=0.342); the absence of homozygotes in our study population of 199 individuals is consistent with a Hardy-Weinberg distribution. In Swedish Caucasians, 3 *CYP3A4-V* alleles were recently found when studying 39 individuals, giving an allelic frequency of 3.8% (16), whereas Sata et al. (9) reported an allelic frequency of 4.2% in 59 white subjects. These data are more in agreement with our results.

CYP3A4, the most abundant form of the cytochrome P450 enzyme family present in human liver, is involved in the metabolism of a large number of drugs (3, 4, 14). Recently, a genetic polymorphism in the 5' regulatory region was discovered, in which an A(-290)G substitution changes the nifedipine-responsive element (7). This mutation potentially alters the transcription efficiency and thus the overall enzymatic activity of CYP3A4. Initially, the effect of this mutation on transcription was believed to be a decrease, based upon the clinical presentation of prostate cancer (7) and drug-induced leukemia (12). Later experiments, in which protein expression and enzymatic activity using liver samples were compared, suggested that the *CYP3A4-V* mutation had no effect on transcription (15). This was supported by experiments on the 6ß-hydroxylation of testosterone in three microsomal liver samples from individuals heterozygous for the *CYP3A4-V* allele (16), although this conclusion was questioned later by others (17). Experiments, in which the promoter region of CYP3A4 was fused to the luciferase reporter gene, followed by expression of these constructs in HepG2 and MCF7 cells, showed that the CYP3A4-V allele actually gives, rise in an increase in *CYP3A4* transcription compared to the *CYP3A4* wild-type allele (18).

In conclusion, we have described and validated a fast and simple PCR analysis, which can be applied for specific screening for the *CYP3A4-V* allele. This assay will greatly facilitate studies on the effect of this polymorphism in endogenous processes, environmental susceptibility to cancer and individual ability to metabolize drugs.

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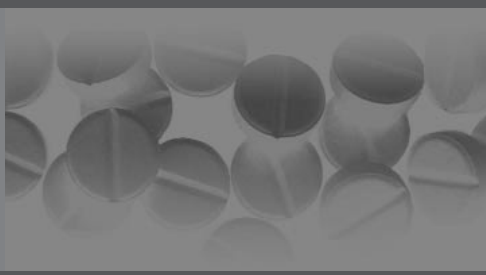
The CYP3A4*3 allele: is it really rare?

Chapter 8



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Clin Chem 2001; 47 (6): 1104-1106

Summary

Introduction Cytochrome P450 3A4 (CYP3A4) is the major cytochrome involved in drug metabolism. Genetic polymorphisms in the *CYP3A4* allele, affecting enzymatic activity, may contribute to the observed interindividual variation in drug metabolism. Two genetic polymorphisms have been described: *CYP3A4*1B* and *CYP3A4*2*. A third variant allele, *CYP3A4*3*, was found only in one individual and was therefore referred to as a rare allele.

Methods We developed a PCR restriction fragment length polymorphism (RFLP) assay for detection of the *CYP3A4*3* allele. A total of 762 individuals was screened for this variant allele.

Results Applying this assay on genomic DNA obtained from 499 Caucasians, we identified 13 heterozygote and no homozygote individuals for *CYP3A4*3*. When 263 patient samples were screened, 3 additional heterozygote individuals were identified.

Conclusion We conclude that *CYP3A4*3* is not a rare allele, but can be found in 1.1 % of the population studied. Therefore, the *CYP3A4*3* allele may represent a genuine polymorphism. A PCR-RFLP procedure is described which can be used to screen for the *CYP3A4*3* variant allele.

Introduction

Enzymes of the cytochrome P450 system are involved in the metabolism of a broad range of foreign compounds, such as drugs, environmental pollutants and carcinogens (1). The most abundant enzyme in the human liver is cytochrome P450 3A4 (CYP3A4) (2). This enzyme is involved in the metabolism of over 50% of all drugs used in humans (3, 4) and the interindividual differences in the pharmacokinetics of these drugs are thought to be related to variations in CYP3A4 activity (4-6). These variations may be caused by age and disease related differences, by drugs inducing or repressing transcription/translation, or from genetic polymorphisms. Although the *CYP3A4* gene was initially thought not to be polymorphic, recent reports described three genetic variants of this gene: *CYP3A4*1B*, *CYP3A4*2* and *CYP3A4*3* (7, 8). The allelic frequency for the *CYP3A4*1B* allele, which contains an A(-290)G substitution in the promoter region of *CYP3A4*, ranges from 0.0% in Chinese and Japanese Americans to over 54% in African Americans (8, 9). White Americans and European Caucasians were reported to have an allelic frequency of approximately 4-5% (8-11). The *CYP3A4*2* allele, which encodes a Ser222Pro change, has an allelic frequency of 2.7% in the white (Finnish) population (8). Because variant alleles that are found in more than 1% of the population are defined as genetic polymorphisms (12), both the *CYP3A4*1B* and the *CYP3A4*2* allele are considered to be genetic polymorphisms of *CYP3A4*. In addition, a variant allele found in the DNA of a single Chinese subject contained a T1437C substitution (8). Because this allele, encoding a Met445Thr change, was not found in any other of the 91 subjects investigated in that study, it was referred to as a rare allele.

Methods

In this study, we developed a PCR-restriction fragment length polymorphism (RFLP) procedure for the detection of the *CYP3A4*3* allele. We used this assay to determine the allelic frequency of *CYP3A4*3*. EDTA-whole blood was obtained from 499 healthy Dutch Caucasian volunteers and from 66 pediatric patients involved in a midazolam pharmacokinetic study after informed consent. The study was approved by the Medical Ethical Committee of the University Hospital Rotterdam. We isolated genomic DNA from 300 µL of blood, using the GenomicPrep Blood DNA Isolation Kit (Amersham Pharmacia Biotech). DNA yields were estimated by measuring the absorbance at 260 nm (A_{260}). A total of approximately 50 ng of genomic DNA was used in a PCR volume of 50 µL. The PCR mixture contained 1X buffer (10 mmol/L Tris-HCl pH 8.3, 1.5 mmol/L $MgCl_2$, 50 mmol/L KCl and 0.001% (w/v) gelatin (Perkin-Elmer)), 0.2 mmol/L each dNTP (Roche), 1.25 U of AmpliTaq Gold (Perkin-Elmer), and 40 pmol of each of forward primer [5'-TGG ACC CAG AAA CTG CAT ATG C-3' (nucleotide 23,255-23,276; GenBank sequence AF209389)] and reverse primer [5'-GAT CAC AGA TGG GCC TAA TTG-3' (nucleotide 23,483-23,503; GenBank sequence AF209389)]. The nucleotides underlined are mismatches with the *CYP3A4* sequence, creating a *Nsi*I restriction site in the wild-type *CYP3A4* PCR product. When the *CYP3A4*3* allele is amplified, this *Nsi*I site is disrupted. PCR conditions were as follows: 7 min at 94 °C; 35 cycles of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C; and finally 7 min at 72 °C. The 249 bp PCR product of 5 DNA samples were sequenced, confirming that indeed only the *CYP3A4* gene was amplified. The PCR product (15 µL) was digested with 10 U *Nsi*I (Roche) for

2 hours at 37 °C, and analyzed on a 3% MP agarose/Tris-borate-EDTA gel with ethidium bromide staining. Samples that produced a heterozygote signal were re-analyzed by PCR-RFLP. Subsequently, heterozygosity for *CYP3A4*3* was confirmed by sequencing of the PCR product with the reverse primer on an automated ABI 310 capillary sequencer (Perkin-Elmer) using the Big Dye Terminator Cycle Sequencing Kit (Perkin-Elmer). To check reproducibility, the *CYP3A4*3* assay was performed 10 times at a wild-type and 10 times at a heterozygote sample, which resulted each time in the correct genotype.

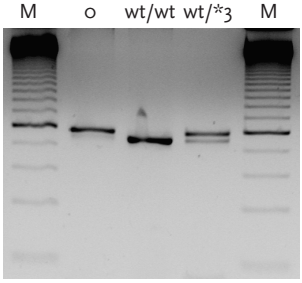
Results

A 249 bp fragment was amplified, using the primers described (Fig 1b, lane 0). Digestion of the PCR product with *Nsi*I resulted in 226 and 23 bp fragments for the wild-type sequence (Fig. 1, lane wt/wt) and 249, 226 and 23 bp for heterozygote sequences (Fig. 1, lane wt/*3). The 23-bp fragments are not visible on gel. To check reproducibility, the *CYP3A4*3* assay was performed 10 times at a wild-type and 10 times at a heterozygote sample, which resulted each time in the correct genotype.

The *CYP3A4*3* PCR-RFLP assay was applied to 499 genomic DNA samples obtained from Dutch Caucasian volunteers and 66 samples from pediatric patients who participated in a midazolam pharmacokinetic study. This resulted for the 499 volunteers in 488 cases in digestion of the 249 bp PCR product into 226 and 23 bp (not visible) fragments, as expected for wild-type samples and in 11 cases (2.2%) in the heterozygote signal. No homozygotes were detected. The allelic frequency of *CYP3A4*3* in these Caucasians is therefore 1.1%. In the 66 pediatric patients, one heterozygous individual for CYP3A4*3 was detected. These allele and genotype frequencies are in Hardy Weinberg equilibrium (p=0.80). In the heterozygote samples, direct sequencing showed a mixed T/C peak corresponding to position 1437, indicating that the nucleotide change was indeed T1437C in all cases.

Because only one pediatric patient was heterozygous for CYP3A4*3, it was not possible to determine the effect of this mutation on midazolam pharmacokinetics in pediatric patients.

*Figure 1 PCR-RFLP procedure for the CYP3A4*3 allele. Undigested PCR product (0), and NsiI-digested PCR fragments of 226 and 23 bp for a wild-type (wt/wt) and 249, 226 and 23 bp for a heterozygote DNA sample (wt/*3). The 23 bp fragment is not visible. Analysis on a 3% agarose/TBE gel; picture is printed negative. M= basepair marker (50 bp ladder).*



Discussion

Variant *CYP3A4* alleles in the population may contribute to interindividual variability in CYP3A4 activity and detecting genetic polymorphisms may help to predict an individual's ability to respond to certain drugs. The *CYP3A4*3* allele, which has a T1473C change leading to a Met445Thr substitution in exon 12, was found only in one Chinese subject from Shanghai and could not be detected in 91 other individuals (8). The investigators concluded that *CYP3A4*3* is a rare allele, which may result in low priority in performing functional studies on this allele. Our data indicate that the *CYP3A4*3* allele is not restricted to a single individual, but has an allelic frequency of 1.1% in Caucasians. This implies that the variant *CYP3A4*3* allele is not a rare allele, but instead represents a genetic polymorphism which can be found in a substantial part of the population. The identification of the *CYP3A4*3* variant allele as a genetic polymorphism adds up to the two other described genetic polymorphisms for CYP3A4. The *CYP3A4*1B* allele potentially alters the transcription efficiency and thus the overall enzymatic activity of CYP3A4: although initial reports suggested decreased activity *in vivo* (7, 13, 14), increased activity *in vitro* (15, 16) and no effect (10, 14, 17). For the variant allele *CYP3A4*2*, a decreased enzymatic activity was observed for nifedipine, but not testosterone (8). For *CYP3A4*3*, the location of the amino acid which is changed in the CYP3A4 protein is near the cysteine that is involved in the active site of the enzyme (8). This might induce structural differences leading to alteration in enzymatic activity. However, expression studies need to be performed to prove this. Taking into account the allelic frequencies of the genetic polymorphisms in *CYP3A4* (10% heterozygotes for *CYP3A4*1B*, 2.7% heterozygotes for *CYP3A4*2* and 2.2% heterozygotes for *CYP3A4*3*), this implies that approximately 10-15% of the (Caucasian) population may carry a genetic polymorphism in this allele. Since genetic polymorphisms may exhibit strong variation in occurrence among different ethnic groups, other populations need to be investigated to determine the allelic frequency of *CYP3A4*3*.

In conclusion, we have described and validated a PCR-RFLP analysis for the *CYP3A4*3* allele. The frequency of this variant allele in the Caucasian population of 1.1% indicates that it might be important when predicting CYP3A4 activity based on genotype. Future research should be directed upon elucidating the effect of this polymorphism on the CYP3A4 enzymatic activity and to establish whether we are dealing with only a genetic, or also a functional polymorphism.

Acknowledgements

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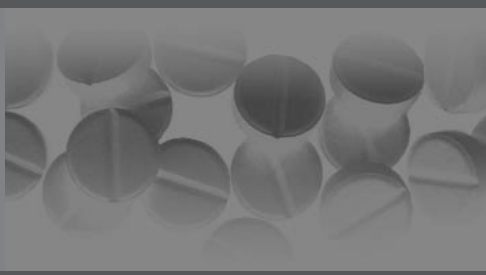
Midazolam pharmacokinetics in preterm infants: lack of an association with the CYP3A4*1B genetic polymorphism

Chapter 9



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Submitted

Summary

Introduction The aim of this study was to investigate whether the CYP3A4*1B polymorphism is associated with altered midazolam clearance in preterm infants.

Methods The association between CYP3A4*1B genotype and midazolam pharmacokinetics was examined in preterm neonates (gestational age 26-34 weeks, postnatal age 3-13 days) who received a single intravenous (n=24) and/or oral (n=15) midazolam dose (0.1 mg/kg). All patients were genotyped for CYP3A4*1B with PCR-RFLP. Midazolam and 1-OH-midazolam concentrations were quantitated from plasma by GC-MS. Pharmacokinetic parameters were determined using standard noncompartmental techniques.

Results Median clearance of midazolam after intravenous midazolam did not differ significantly between patients with and without the CYP3A4*1B mutation [0.18 (range 0.06-0.40) vs 0.10 (range 0.04-0.26) L/kg/h, p=0.22]. Interestingly, median apparent oral clearance of midazolam was approximately 4-fold higher in the 3 preterm infants with the CYP3A4*1B mutation [0.43 (range 0.18-0.93 L/hr/kg)] as compared to 12 infants without the CYP3A4*1B mutation [0.12 (range 0.04-0.6) L/hr/kg, p=0.048]. However, multivariate analysis showed that after correction for indomethacin exposure, no difference in midazolam apparent oral clearance between infants with and without the CYP3A4*1B mutation could be detected.

Discussion The CYP3A4*1B mutation does not appear to alter midazolam disposition after intravenous and oral administration in preterm infants.

Introduction

Cytochrome P450 3A4 (CYP3A4) is abundantly expressed in human liver and intestine where it catalyzes the biotransformation of over 50 currently prescribed drugs in addition to several endogenous compounds and environmental pollutants. CYP3A4 expression shows large interindividual variability (1, 2). Co-variates known to affect CYP3A4 activity include age, medication, disease-state, nutrition, race and potentially, genotypic expression (3-6).

Recently, a CYP3A4 variant allele has been reported, CYP3A4*1B (7). This variant allele contains an A to G mutation in the nifedipine-specific element (NFSE) located –287bp from the transcription start site. The exact role of this NFSE element in the regulation of the CYP3A4 gene expression is currently unknown (8). Interestingly, the CYP3A4*1B mutation appears to be associated with a worse clinical presentation of prostate cancer and a lower risk for treatment-related leukemia in children (7, 9). In addition, Rebbeck and co-workers have shown that the CYP3A4*1B gene promoter variant may be associated with higher expression and activity of CYP3A4 *in vitro* as compared to the CYP3A4 wild-type promoter (10, 11). These observations could not, however, be confirmed by other investigators (12). A pharmacokinetic study in adults using the CYP3A4 probe midazolam showed a statistically significant decreased clearance of midazolam only after intravenous administration in individuals with the *1B mutation. In contrast, no effect of the CYP3A4*1B allele was detected using the erythromycin breath test, nifedipine clearance or oral midazolam clearance. (13, 14). The reduction of intravenous midazolam clearance in subjects with the *1B mutation was very modest and therefore was felt to be of minimal clinical importance with respect to its impact on CYP3A4 activity.

Genetically determined events that control the activity (i.e., affinity and capacity) of enzyme(s) responsible for the metabolism of several drugs appear to vary as a consequence of development (15). However, it is unknown whether specific polymorphisms in the CYP3A4 gene may be associated with the acquisition of functional activity (4). If the NFSE is associated with the expression of CYP3A4 activity after birth, genetic polymorphisms in this element may explain part of the large interindividual variation found in the pharmacokinetics of CYP3A4 substrates in newborn infants (16).

The aim of this study was to investigate if the plasma clearance of the CYP3A4 probe midazolam in newborn infants after intravenous or oral administration was associated with the CYP3A4*1B mutation.

Materials and Methods

Patient population

This study was conducted using specimens from 30 preterm infants (gestational age: 26 to 33 weeks, postnatal age: 3 to 13 days) who participated in a pharmacokinetic study of midazolam. Infants were recruited from the Neonatal Intensive Care Unit of the Sophia Children's Hospital from June 1997 through May 2000. All children received midazolam prior to a stressful procedure (e.g. tracheal tube suction, elective nasopharyngeal intubation) and had an indwelling arterial catheter placed upon medical indication not

related to this study. No patients received drugs known to alter the activity of CYP3A4 and were excluded from participation if they received morphine, dobutamine or dopamine. Furthermore, patients were excluded from study participation if they had significant underlying renal, hepatic or neurological dysfunction.

This research protocol was approved by the Medical Ethical Committee of the Sophia Children's Hospital. Written, informed consent was obtained from parents or legal guardians of all infants prior to their enrollment in the study.

Drug administration and sample collection

Midazolam pharmacokinetics were determined after intravenous (IV, n=24) or after oral (PO, n=15) administration. Nine patients received both formulations in random order with a washout period of at least 72-hours between study periods. Midazolam (Dormicum® injection, Roche Laboratories, The Netherlands) was administered as a single intravenous (IV) dose, 0.1 mg/kg, diluted to a final concentration of 0.03 mg/ml in a 5% glucose solution. Midazolam (IV) was infused over 30 minutes through microbore tubing at a constant rate into a peripheral vein or into a central catheter. Midazolam (PO) at a concentration of 0.5 mg/ml was diluted with glucose 5% to a final volume of 0.5 ml and given as a single 0.1 mg/kg dose through the gastric tube, followed by 0.5 ml of glucose 5% to ensure that no midazolam remained in the tube. Arterial blood samples (0.2 ml each) were collected for the quantitation of midazolam and 1-OH-midazolam concentrations from plasma and were stored at –80°C until they were analyzed using a validated GC-MS method. Blood samples were obtained immediately prior to the infusion (IV) or bolus (PO) administration (i.e., at time = 0) and at 0.5, 1, 2, 4, 6, 12 and 24 h after dosing. The subjects were observed for the whole study period for adverse reactions, with vital signs checked prior to infusion and at the time of blood sampling.

Sampling for pharmacogenetic analyses

For all patients who had completed the companion pharmacokinetic study, a buccal brush was used to obtain epithelial cells for DNA isolation and subsequent analysis. From patients who enrolled in the pharmacokinetic study after development of CYP3A genotyping, we obtained 0.3 ml whole blood samples (EDTA anticoagulant) via an arterial catheter for DNA isolation and CYP3A*1B genotyping.

Drug assay methods

Plasma samples were analyzed for midazolam and 1-OH-midazolam by gas chromatography with mass spectrometric detection (Hewlett Packard 6890, Agilent Technologies Inc, Palo Alto, CA). The column used was a J&W Scientific DB-17 EVDX [0.2 micron, 25 meters (J&W Scientific, Folsom, CA)]. Diazepam (Elkins Sinn, Cherry Hill, NJ), 5µl of 500 ng/ml solution, was added to each sample as an internal standard and solid phase extraction was performed using a Varian Bond Elut Column (Varian Inc, Palo Alto, CA). The inter-day and intra-day coefficient of variation for the low standard (2 ng/ml) was consistently less than 10% for both midazolam and 1-OH midazolam. The lower limit of quantitation was 1 ng/ml for midazolam and 0.5 ng/ml for 1-OH-midazolam using 0.2 ml sample volume. All samples were analyzed in duplicate with the resultant mean concentration used in the pharmacokinetic analysis.

Genotyping

To detect individuals who carried the CYP3A4*1B mutation, genomic DNA was isolated from blood using the GenomicPrep Blood DNA Isolation Kit (Amersham Pharmacia Biotech, Buckinghamshire, UK). In a subset of patients DNA was isolated from buccal epithelium, using the MasterAmp Buccal Swab DNA Extraction Kit (BIOzym). DNA yields were estimated by measuring OD_{260}. Evidence for the CYP3A4*1B mutation was examined from each DNA sample using the PCR-RFLP method developed by van Schaik et al. (17).

Pharmacokinetic analysis

Pharmacokinetic parameters were determined for midazolam following both intravenous and oral administration using standard non-compartmental methods. The area under the curve for both midazolam and 1-OH-midazolam were determined by the log-linear trapezoidal rule (AUC_{0t}) with extrapolation to infinity ($AUC_{0\infty}$) using the apparent terminal elimination rate constant (λz) determined via non-linear curve fit. The 1-OH-midazolam AUC_{0t} /midazolam AUC_{0t} ratio (i.e. metabolite:parent drug) was used as a "surrogate" measure of CYP3A4 activity. Only subjects with measurable concentrations of 1-OH-midazolam were used to construct this metabolic ratio. All pharmacokinetic analyses were conducted using models resident in the Kinetica® (version 2.0, Innaphase, Inc., Philadelphia, PA, USA) software package.

Statistical analysis

Data between subject groups with and without the CYP3A4*1B mutation were compared using the Mann-Whitney test. Multivariate linear regression analysis was performed to determine the predictive value of other co-variates, in addition to presence/absence of the CYP3A4*1B allele, on midazolam pharmacokinetics. For the multivariate analysis we used the logarithm of midazolam $AUC_{0-\infty}$ and clearance to normalize the distribution of these parameters. These statistical analyses were conducted using the SPSS software package (version 9.0, Chicago, Illinois, USA). Data are expressed as mean ± SD unless stated to the contrary. A significance limit of $\alpha = 0.05$ was accepted for all statistical analyses.

Results

Data from 30 patients were included in the analysis. Midazolam was administered intravenously in 15 patients and orally in six. In an additional 9 patients midazolam was administered both intravenously and orally. Patient demographic characteristics are summarized in Table 1. Out of the 30 patients studied, two were homozygous for the CYP3A4*1B mutation (one Black, one Hispanic) and four were heterozygous (three of Caucasian and one of unknown decent) (Table 2).

Median apparent oral clearance of midazolam was greater in the 3 preterm infants with the CYP3A4*1B mutation as compared to the 12 infants without the mutation [0.43 (range 0.18-0.93) vs. 0.12 (range 0.04-0.6) L/kg/h, p=0.048]. In addition, the apparent volume of distribution after oral administration was also larger in the patients with the mutation as compared to those without [3.7 (range 1.9 – 12.1) vs. 1.3 (range 0.3-9.2) L/kg, p=0.048]. However, elimination half-life nor metabolite:drug AUC ratio differed

Table 1 *Demographic data of the patients*

	Intravenous midazolam		Oral midazolam	
	N=24		N=15	
Parameters				
GA (weeks)	29.1 ± 2.3	(26.3-33.6)	28.0 ± 1.6	(26.0-30.7)
PNA (days)	5.8 ± 2.6	(3-11)	6.1 ± 2.7	(3-13)
Birth weight (g)	1092 ± 233	(745-1630)	1076 ± 240	(745-1630)
Study weight (g)	1105 ± 230	(770-1645)	1070 ± 232	(825-1660)
Apgar 1 min	6.1 ± 2.0	(2-9)	6.0 ± 2.0	(3-9)
Apgar 5 min	8.0 ± 1.2	(5-10)	7.9 ± 1.3	(5-10)

Data are expressed as means ± SD (range), GA = gestational age, PNA = postnatal age.

Table 2 *Ethnic distribution of the CYP3A4*1B mutation*

	Genotype			
	A/A	A/G	G/G	Total
Race				
Caucasian	21 (15*,10#)	3 (3,1)		24 (18,11)
Mediterranean	2 (2,2)			2 (2,2)
Black			1 (1,1)	1 (1,1)
Hispanic			1 (1,1)	1 (1,1)
Mixed-unknown	1 (1,0)	1 (1,0)		2 (2,0)
Total	24 (18,12)	4 (4,1)	2 (2,2)	30 (24,15)

** = number of patients who received intravenous midazolam, # = number patients who received oral midazolam.*

*AA = wild-type, A/G = heterozygous for CYP3A4*1B, GG = homozygous for CYP3A4*1B.*

significantly between patients with and without the *1B mutation. Multivariate linear regression analysis was performed to test if, in addition to presence or absence of the CYP3A4*1B allele, any of the following co-variates was associated with altered midazolam $AUC_{0-\infty}$ or plasma clearance: gestational age, postnatal age, postconceptional age, birth weight, study weight, delivery mode, feeding, ventilation, prenatal exposure to betamethasone and/or indomethacin, postnatal exposure to indomethacin, caffeine or antibiotics. When the apparent oral midazolam clearance (i.e., CL/F; ml/min/kg) was

corrected for the effect of postnatal indomethacin treatment, the *1B mutation was no longer of significant predictive value for midazolam clearance. None of the other co-variates tested impacted the predictive value of the CYP3A4*1B mutation on the pharmacokinetics of oral midazolam.

No relationship with the CYP3A4*1B mutation was apparent with respect to midazolam clearance or $AUC_{0-\infty}$ after intravenous midazolam administration. When a similar multi-variate analysis was performed for midazolam clearance after intravenous administration, no single association between midazolam clearance and CYP3A4*1B mutation was detected.

Discussion

Inheritance of the CYP3A4*1B mutation does not appear to alter midazolam disposition in preterm infants. The fact that there was no genotype/phenotype relationship with respect to the apparent oral clearance of midazolam in these infants is in agreement with data derived from healthy adults (19). However, the apparent absence of a genotype-phenotype correlation associated with the *1B mutation in our population of preterm infants who received intravenous midazolam is not in agreement with previous data from healthy adults that demonstrated a small but statistically significant reduction in midazolam plasma clearance (19). Examination of these data suggests, however, that this reduction in midazolam elimination is modest and given the normal, wide interindividual variability in CYP3A4/5 activity as reflected by midazolam clearance (19), may be of limited clinical significance.

In preterm infants other co-variates such as age and concurrent medication have also been identified as causes of variability in midazolam clearance (16,20). Even after correction for several co-variates, we could not detect an effect of the CYP3A4*1B mutation on midazolam clearance. Interestingly, all patients with the CYP3A4*1B allele had been exposed postnatally to indomethacin, while only half of the patients without the mutation had received indomethacin for closure of a patent ductus arteriosus. Therefore postnatal exposure to indomethacin was seen as a confounding treatment and as such, was considered during the final analysis. A limitation of our study, in addition to the uneven distribution of co-variates across the patient groups was the small sample size of our cohort. An effect or lack of effect of one or more CYP3A4*1B alleles may have been obscured due to a lack of statistical power. The normal intersubject variability in CYP3A activity and the effect of ontogeny on CYP3A activity necessitates a large sample of newborns to be included in such a pharmacogenetic study in order to enable discrimination of genotype-phenotype associations. A developmentally related regulated surge in CYP3A activity during the first months of life as suggested by the data of Lacroix et al. (17), may completely obscure the role that specific mutations of the CYP3A4 gene might have on enzyme activity. As reflected by previous data on N-acetyl tranferase 2 (NAT2) (15,23), developmental regulation of a gene product may have profound effect on the expression of both enzyme activity and phenotype.

Despite the reported allelic variants for CYP3A4, their significance with respect to the clearance of pharmacologic substrates for this enzyme remains inconclusive. In contrast, mutations in the CYP3A5 gene that appear to convey polymorphic activity of the enzyme were recently reported (18). One of the well described limitations of using pharmacologic "probe" compounds to assess the activity of CYP3A4 resides with the

potential for overlapping substrate specificities between CYP3A4 and CYP3A5. In the case of midazolam, both CYP3A4 and CYP3A5 are involved in its metabolism, with a differential capability of these isoforms on the formation of midazolam metabolites (19). The potential relevance of these findings to our data resides with the fact that we did not specifically assess CYP3A5 genotype or phenotype in our subjects but did examine the relative extent of midazolam conversion to 1-OH-midazolam as a potential "surrogate" marker for CYP3A4/5 activity. Thus, it is possible that the constitutive level of CYP3A5 activity in our subjects may have obscured our ability to discriminate a significant role of the *1B mutation as a determinant of midazolam disposition in preterm neonates.

Finally, data from Ozdemir et al. (6) suggest that approximately 60% of the individual variability in adult hepatic and intestinal CYP3A activity appears to be under genetic control. This is exemplified by the known polymorphisms for both CYP3A4 and CYP3A5, the latter being of functional significance with respect to the biotransformation of CYP3A5 substrates (18). Therefore, a continuous effort in elucidating the causes of the large variability in the pharmacokinetics of CYP3A substrates is necessary that takes into account the rapid discovery of new and potentially important genetic polymorphisms for the isoforms of CYP3A (8,24).

In conclusion, the results from our pilot study conducted in preterm neonates suggests that the CYP3A4*1B mutation does not appear to alter midazolam disposition after intravenous and oral administration of the drug. Before these results can be generalized and applied to the pediatric population, it will be necessary for much larger studies to be conducted in infants and children so that the impact of polymorphisms in both the CYP3A4 and CYP3A5 genes as a determinant of midazolam disposition in patients can be clearly elucidated.

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Pharmacodynamics of intravenous and oral midazolam in preterm infants

Chapter 10

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Submitted

Summary

Introduction The aim of this study was to evaluate the pharmacodynamics and safety of oral and intravenous midazolam in preterm infants.

Methods Twenty-four preterm infants (gestational age: 26 to 34 weeks, postnatal age: 3 to 13 days) received a 30-minute intravenous infusion (n=20) and/or oral (n=11) bolus dose of midazolam (0.1mg/kg) in a random order. Pharmacodynamic measurements consisted of a COMFORT® score (a previously validated sedation scale for pediatric patients) at baseline and at 0.5, 1, 2, 4 and 6 hours postdose. Midazolam and 1-OH-midazolam concentrations were measured and vital signs were recorded at all pharmacodynamic measurement time points.

Results Overall, mean COMFORT scores decreased (=more sedated) significantly within 30 minutes after intravenous (p=0.002) and within one hour after oral midazolam administration (p=0.003). However, in 45% of patients the COMFORT scores decreased little or not at all after midazolam. The sedative response to midazolam did not differ after intravenous or oral midazolam administration. Blood pressure decreased significantly after intravenous (approximately 11%) but not after oral midazolam administration. No serious adverse events were reported.

Discussion In summary, midazolam administered as a 30-minute intravenous infusion or oral bolus dose appears to be effective and safe in a majority of preterm infants. However, a considerable proportion of neonates do not appear to respond to midazolam. The lack of response may be due to the fact that patients truly experienced therapeutic failure and/or consequent to the inability of the COMFORT score to adequately reflect sedation uniformly in sick preterm infants.

Introduction

In contrast to earlier beliefs, it is now acknowledged that preterm infants are not only able to experience stress in response to invasive and nursing procedures, but that their response to these stressful stimuli may also compromise their clinical condition (1). Therefore, the necessity for effective sedation in preterm infants is obvious. However, little is known regarding the benefits and/or risks of sedatives in preterm infants (2). One of the drugs used for sedation in preterm infants is the benzodiazepine midazolam (3). Midazolam appears to reversibly potentiate the effects of γ-amino butyric acid (GABA), an inhibitory neurotransmitter of the central nervous system. Through this effect midazolam exerts its sedative, anxiolytic, anticonvulsive, muscle relaxant and amnesic effects, as has been illustrated in adults and older children (4).

In a placebo-controlled trial designed to study the sedative effect of midazolam, continuous infusion of the drug induced effective sedation in preterm infants on mechanical ventilation. (5). In another placebo-controlled pilot study, designed to study the effect of sedation on neurological outcome in preterm infants, sedation scores (COMFORT scores) were significantly lower (more sedated) in patients who received continuous midazolam infusion as compared to those who received placebo (6) (7). Midazolam has also been used for premedication prior to intubation in newborn infants (8). However, little is known regarding either the extent or duration of the sedative effect from a single dose of oral or intravenous midazolam in preterm infants.

Adverse events of midazolam have occasionally been described in preterm infants including hypotension and brief periods of myoclonic activity (3). While hypotension has been observed after intravenous bolus injection of midazolam(2), administration of the drug as a 30-minute infusion or oral bolus dose conceivably may prevent hypotension associated with intravenous bolus dosing.

To evaluate the efficacy and safety of a single dose of midazolam given as a 30-minute infusion or as an oral bolus to preterm infants, we conducted a controlled clinical pharmacodynamic study that examined the plasma concentration vs. effect relationship.

Methods

Patient recruitment

This was a randomized, prospective pharmacodynamic investigation of intravenous and oral midazolam in preterm infants given for preprocedural sedation (e.g. endotracheal suction, elective nasopharyngeal intubation). The children were recruited from the Neonatal Intensive Care Unit of the Sophia Children's Hospital, Rotterdam. The institutional review board approved this research protocol. Written, informed consent was obtained from parents or legal guardians prior to subject enrollment.

Patients were included if they had a gestational age between 26 and 34 weeks, needed midazolam for preprocedural sedation and had an indwelling arterial catheter, previously placed for purposes associated with medical care. Patients were excluded if: (1) they received concomitant or recent (i.e. 12 hours prior to dosing) morphine, dobutamine or dopamine; (2) were exposed to any sedating agents within a 12-hour period prior to study drug administration or (3) had significant underlying hemodynamic, renal, hepatic or neurological dysfunction.

Concomitant, recent (i.e. 24 hours prior to dosing) or chronic treatment with medications known or suspected to alter the pharmacokinetics of midazolam was prohibited. Since midazolam is a substrate for cytochrome P450 3A enzymes, potential study objects were specifically assessed for exposure to drugs known to induce, inhibit or serve as substrates for CYP3A4 (e.g. erythromycin, phenobarbital, rifampin, dexamethasone, fentanyl, cisapride) (9).

The concomitant use of sedative/anxiolytic drugs that could potentially affect the pharmacodynamics of midazolam was also prohibited during the 6-hour postdose period.

Study design and treatment groups

Patients were randomly assigned to initially receive midazolam as a 30-minute intravenous infusion or an oral bolus, followed in cross-over fashion by midazolam administration via the alternate route. To ensure complete washout of the first dose of midazolam, the minimum interval between doses was 72 hours. A nurse not involved in the investigation or clinical care of the study subject installed the treatment according to a computer generated randomization list. Both the patient's nurse and the investigator (S.N.W.) were blinded to the drug formulation given.

Dosing

Midazolam (Dormicum® injection, Roche Laboratories, The Netherlands) was administered as a single 0.1 mg/kg dose in a 5% glucose solution (0.03 mg/ml) infused by syringe pump over 30 min through microbore tubing into a peripheral vein or into a central catheter. Oral (PO) midazolam was administered via the gastric tube using a syringe at a concentration of 0.5 mg/ml diluted with glucose 5% to a final volume of 0.5 ml. Next, the gastric tube was flushed with 0.5 ml glucose 5% to ensure complete drug delivery.

Plasma sampling and drug concentration analysis

Serial arterial blood samples (0.2 ml) were obtained at baseline and at 0.5, 1, 2, 4, and 6 hours from the time of dosing. Plasma was separated from whole blood by centrifugation (1000 X g for 10 minutes) and then stored at –80°C until analysis. Plasma samples were analyzed for midazolam and 1-OH-midazolam by gas chromatography with mass spectrometric detection (Hewlett Packard 6890, Agilent Technologies Inc, Palo Alto, CA). The column used was a J&W Scientific DB-17 EVDX [0.2 micron, 25 meters (J&W Scientific, Folsom, CA)]. Diazepam (Elkins Sinn, Cherry Hill, NJ), 5 µl of 500 ng/ml solution, was added to each sample as internal standard and solid phase extraction was performed using a Varian Bond Elut Column (Varian Inc, Palo Alto, CA). The inter-day and intra-day coefficients of variation at the low standard concentration (2ng/ml) were less than 10% for both midazolam and 1-OH midazolam. The lower limit of quantitation was 1 ng/ml for midazolam and 0.5 ng/ml for 1-OH-midazolam using a 0.2 ml sample volume.

Pharmacodynamic measurements

Sedation level was quantitated prior to dosing and at 0.5, 1, 2, 4 and 6 hours after midazolam administration. Sedation levels were determined with the COMFORT score, a previously validated sedation scale that rates eight behavioral or physiologic dimensions of distress (*Table 1.*) (10). Each dimension is scored on a subscale from 1 to 5. Each observation consisted of a 2-min period of intensive evaluation of the patient. After each observation,

Table 1 *(adapted with permission from van Dijk et al. (11)*

Scale item	Score
Alertness	
Deeply asleep	1
Lightly asleep	2
Drowsy	3
Fully awake and alert	4
Hyperalert	5
Calmness	
Calm	1
Slightly anxious	2
Anxious	3
Very anxious	4
Panic	5
Respiratory response	
No coughing and no spontaneous respiration	1
Spontaneous respiration with little or no response to ventilation	2
Occasional cough or resistance to ventilator	3
Actively breathes against ventilator or coughs regularly	4
Fights ventilator; coughing or choking	5
Crying	
Quiet breathing, no crying	1
Sobbing or gasping	2
Moaning	3
Crying	4
Screaming	5
Physical movement	
No movement	1
Occasional, slight movement	2
Frequent, slight movements	3
Vigorous movement limited to extremities	4
Vigorous movements including torso and head	5
Muscle tone	
Muscles totally relaxed; no muscle tone	1
Reduced muscle tone	2
Normal muscle tone	3
Increased muscle tone and flexion of fingers and toes	4
Extreme muscle rigidity and flexion of fingers and toes	5
Facial tension	1
Facial muscles totally relaxed	2
Facial muscle tone normal; no facial muscle tension evident	3
Tension evident in some facial muscles	4
Tension evident throughout facial muscles	5
Facial muscles contorted and grimacing	
Blood pressure	1
Blood pressure below baseline	2
Blood pressure consistent at baseline	3
Infrequent elevations of 15% or more above baseline (1-3 during 2 minutes observation)	4
Frequent elevations of 15% or more above baseline (> 3 during 2 minutes observation)	5
Sustained elevations of 15% or more	
Heart rate	
Heart rate below baseline	1
Heart rate consistent at baseline	2
Infrequent elevations of 15% or more above baseline (1-3 during 2 minutes observation)	3
Frequent elevations of 15% or more above baseline (> 3 during 2 minutes observation)	4
Sustained elevations of 15% or more	5

the COMFORT scores were totaled by a single trained observer (minimal 8, maximal 40). The COMFORT score at 0.5 h after midazolam administration was determined immediately prior to the stressful procedure, which was carried out at 32 minutes after midazolam dosing. We assumed that the effect of the procedure on sedation levels did not carry over to the next pharmacodynamic observation point (i.e., 1 hour after dosing).

Safety

Observation of each patient for adverse events was performed at each of the pharmacodynamic assessment points. An adverse event was defined as a clinically significant change from the baseline (pretreatment) condition for any of the following: pulse rate, systolic and diastolic blood pressure by manometry and oxygen saturation of hemoglobin by pulse oximetry. Respiratory depression was defined as $SpO_2 < 85\%$ for greater than 10 consecutive seconds.

Adverse event severity was categorized as: (1) mild, (2) moderate or (3) severe. The relationship of an adverse event to midazolam dosing was adjudicated by the investigator as 'not related', 'remotely related', 'possibly related' or 'probably related'. Serious adverse events were defined as those that were lethal or life threatening, and were immediately reported to the institutional review board.

Data analysis

Sedation

COMFORT scores were categorized as *oversedated* (8-16), *awake/calm* (17-26) or *agitated* (>26) as previously validated in pediatric intensive care patients (10). Sedation scores from patients who had a baseline and at least one posttreatment assessment were included in the analysis. Patients were considered unevaluable if a sedation score was not obtained at a specified observation point. The proportion of patients in each sedation category and for each midazolam formulation group was determined at all pharmacodynamic measurement time points.

The change in COMFORT score from baseline was determined at all individual time points. The effect of formulation at each pharmacodynamic time-point on change in COMFORT score from baseline was also determined. Next, changes in COMFORT score were used to calculate area under the effect curve (AUEC) from baseline to 6 hours postdose, after which the overall effect of formulation was determined

We defined patients with a COMFORT AUEC of –5 or greater as "non-responders" given that the mean decrease in COMFORT score was less than 1 per time point. Patients with a COMFORT AUEC lower than –5 were classified as "responders". Patients whose COMFORT AUEC was higher than -5, but who had a decrease in COMFORT score of more than 2 points at one time point, were also defined as responders.

Pharmacokinetic-pharmacodynamic relationship

The relationship between concentrations of midazolam and/or 1-OH-midazolam and COMFORT scores or changes in COMFORT score from baseline (i.e. all paired values at baseline and 5 postdose intervals) were determined. The analysis was performed with overall data and data grouped per pharmacodynamic time-point.

Hemodynamic parameters

Changes in hemodynamic parameters (heart rate, systolic and diastolic blood pressure) from baseline were also determined and used to calculate respective area under the effect curves (AUEC) from baseline to 6 hours postdose. As with the COMFORT scores, formulation and post-dose time-point related differences were determined.

Statistical methods

The effect of formulation or time-point on the pharmacodynamic parameters (COMFORT, change in COMFORT, heart rate, diastolic and systolic blood pressure) was determined using a mixed model ANOVA allowing for inter- and intra-patient differences [SAS PROC MIXED software (version 6.12, SAS institute, Inc, Cary, N.C)]. The relationship between COMFORT scores or changes in COMFORT score and midazolam, 1-OH-midazolam, and the sum of 1-OH-midazolam plus midazolam plasma concentrations was also determined using mixed model ANOVA.

Data are expressed as means ± SEM. However, if a given parameter was not normally distributed medians (range) are used. AUECs and AUC were calculated by the linear trapezoidal rule. All other statistical analyses were obtained using SPSS software (version 9.0.0, SPSS Inc., Chicago, Ill). The level of significance accepted for all statistical analysis was p=0.05.

Results

Patients

24 preterm infants were enrolled of whom seven patients received both intravenous and oral midazolam, 13 patients received only intravenous midazolam and four only oral. Demographic characteristics for all patients are summarized in Table 2.

All patients received medications prior to study enrollment, as well as during the study period, including antibiotics (100 %), surfactant (65% in the IV midazolam group, 46% in the PO midazolam group), caffeine (60% in the IV midazolam group, 73% in the PO midazolam group), indomethacin (45% in the IV midazolam group, 55% in the PO midazolam group), morphine at least 12 hours before study drug administration (43% in the IV midazolam group, 27% in the PO midazolam group) and furosemide (5% of patients in the IV midazolam group).

Sedation

Before study drug administration, 5% of patients in the IV group was agitated, 60 % was awake/calm and 35% was oversedated, according to the COMFORT score. In the oral group, none of the patients was agitated, 82% was awake/calm and 18% was oversedated. The mean COMFORT score before midazolam administration was higher in the oral group than in the intravenous group, but this difference was not statistically significant (20.5 ± 3.8 SD vs. 18.8 ± 3.6 SD, p=0.28). After drug administration the proportion of patients that was oversedated was 76 % at one hour after start of the intravenous infusion and 64 % at two hours after oral dosing (*Figure 1*).

Figure 2 illustrates a significant reduction in COMFORT score starting at the end of the 30-minute infusion of midazolam. The nadir was reached one hour after start

of the infusion [-3.7 ± 1.1 (p = 0.002)]. After oral administration, the COMFORT score also decreased significantly and reached a nadir one hour after dosing [-3.7 ± 1.2 (p=0.003)]. However, in individual patients, the time-point at which the maximum change in COMFORT score was observed varied widely between 0.5 and 6 hours postdose. When the COMFORT score was integrated over time ($AUEC_{COMFORT}$), the total sedative effect of midazolam was not different between the two treatment groups (p=0.74).

Table II *Demographic and physical characteristics of patients who participated in the investigation*

	Intravenous midazolam	**Oral midazolam**
Parameters		
Patients (n)	20	11
Sex (M/F)	7/13	8/7
Gestational age (weeks)	29.0 ± 2.2# (26.3 - 33.6)*	27.6 ± 1.3 (26 - 30.7)
Postnatal age (days)	6.0 ± 2.7 (3 - 11)	6.5 ± 3.1 (3 - 13)
Birth weight (g)	1081 ± 245 (745 - 1630)	1037 ± 241 (745 - 1630)
Study weight (g)	1094 ± 245 (770 - 1600)	1040 ± 239 (825 - 1660)
Mechanically ventilated (n)	12	5
Nasal CPAP (n)	7	5

Figure 1 *This bar-graph depicts the proportion of preterm infants who are sedated after a single midazolam dose. The X-axis represents the time before (0) and after (start of) midazolam dosing in hours. The Y-axis represents the proportion of patients sedated (<COMFORT score <17). The black bars represent the patients who received a 30-minute midazolam intravenous infusion (0.1 mg/kg). The white bar represents the patients who received an oral midazolam bolus dose (0.1 mg/kg)*

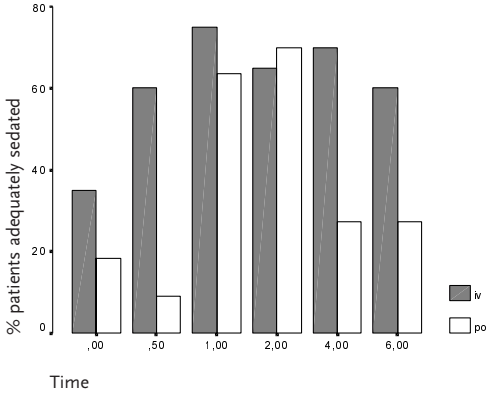
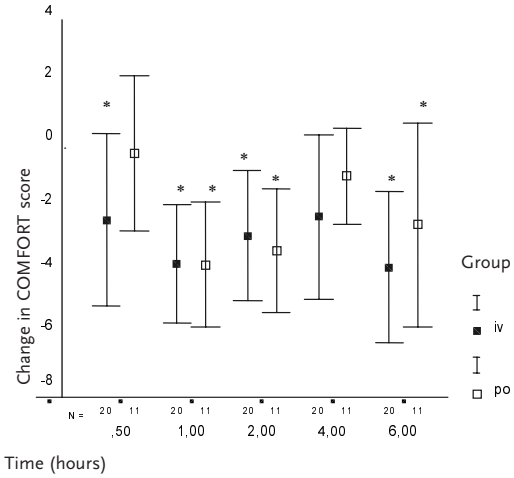
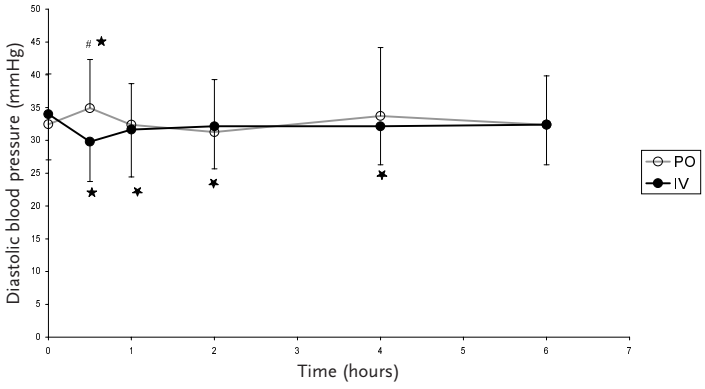


Figure 2 *This graph depicts the change in COMFORT score after 0.1 mg/kg midazolam in preterm infants according to the intravenous (iv) or oral (po) route. The x-axis represents the time after (start of) midazolam dosing in hours. The y-axis represents the mean (± 2 SEM) change in COMFORT score from baseline.*



* = *p<0.05 before vs. after midazolam administration*

Figure 3 *This plot depicts diastolic blood pressure after 0.1 mg/kg of midazolam in preterm infants. The x-axis represents the time before (0) and after (start of) midazolam dosing in hours. The y-axis represents the diastolic blood pressure (mmHg). The dots represent the mean (± 2 SEM) diastolic blood pressure at the various time-points. ∗ p<0.05 before vs after midazolam administration. # p=0.05 intravenous vs oral midazolam administration.*



Nine out of the 20 patients who received intravenous midazolam and five out of the eleven who received oral midazolam were non-responders. First, we determined if differences in drug concentration might account for the difference in response. However, midazolam, 1-OH-midazolam and midazolam plus 1-OH-midazolam concentrations were not statistically different between responders and non-responders. Being a responder or non-responder was also not consistent patients who received midazolam twice by either route. We also did not find an effect of sequence or period of drug administration, postnatal age or mechanical ventilation (present/absent) on the response to midazolam.

Pharmacokinetic-pharmacodynamic relationships

Plasma drug sampling and associated sedation assessment sufficient for examination of possible pharmacokinetic relationships was performed in all 24 patients (216 pairs). No relationship was found between COMFORT scores or change in COMFORT score from baseline and midazolam (p=0.18), 1-OH-midazolam (p=0.50), or midazolam plus 1-OH-midazolam concentrations (p=0.25).

Safety

Overall, during the 6-hour postdose interval, diastolic blood pressure decreased significantly after intravenous (baseline 34.0 ± 7.0 mmHg, $AUEC_{DPB}$= -12.6 ± 4.0 mmHg*h, p=0.003), but not after oral midazolam administration (baseline 32.5 ± 7.7 mmHg, $AUEC_{DBP}$= -2.5 ± 3.7 mmHg*h, p=0. 52, $AUEC_{DBP}$ IV vs. PO p= 0.04), *(Figure 3)*. The maximum decrease in diastolic blood pressure was observed directly after the 30-minute midazolam infusion (- 3.8 ± 0.7 mmHg, p=0.0001) and returned to baseline at 6 hours after start of the infusion (-1.6 ± 0.9, p= 0.08). Although diastolic blood pressure decreased significantly, no hemodynamic instability requiring infusion of crystalloid or colloid or administration of vaso-active drugs, occurred. After oral midazolam administration, diastolic blood pressure never decreased below baseline. In contrast, diastolic blood pressure significantly increased from baseline (2.4 ± 0.9 mmHg, p=0.01) at 30 minutes after oral midazolam administration.

The changes in systolic blood pressure were less pronounced than the changes in diastolic blood pressure. Overall, systolic blood pressure did not decrease significantly after intravenous (baseline 52.8 ± 10.4 mmHg, $AUEC_{SBP}$ = -9.1 ± 7.1 mmHg*h, p=0.21) or after oral midazolam administration (baseline 51.2 ± 9.3 mmHg, $AUEC_{SBP}$ = 3.4 ± 9.4 mmHg*h, p=0.72, $AUEC_{SBP}$ IV vs. PO, p=0.31). After intravenous midazolam, the systolic blood pressure only decreased significantly 30 minutes following midazolam infusion (-4.7 ± 1.4 mmHg), but returned to baseline within one hour (-2.2 ± 1.4 mmHg, p=0.13). After oral midazolam administration, systolic blood pressure did not decrease below baseline at any time point within the 6-hour postdose interval.

Overall, heart rate did not change significantly after intravenous (baseline 150 ± 9.9 bpm, $AUEC_{HR}$ -0.4 ± 1.5 bpm*h, p=0.80) or after oral midazolam administration (144 ± 9.2 bpm, $AUEC_{HR}$ 3.6 ± 0.1 bpm*h, p=0.77) during the 6-hour postdose interval.

The following other adverse events were observed after intravenous and oral midazolam administration: myoclonus (n=1 for intravenous and oral, respectively), apnea (n=2 for IV with O_2sat 61%, n=1 for PO with O_2sat 80%) and bradycardiac episodes

(n=1). Both apneas and bradycardia were defined as 'possibly related' to the study drug, since these symptoms were already present before drug administration, but an effect of midazolam could not be ruled out. Myoclonus was defined as ' probably related' to study drug. All adverse events were categorized as 'mild' and resolved spontaneously without treatment or residua.

Discussion

Sedation

The COMFORT scores decreased significantly (more sedated) in preterm infants after both intravenous and oral midazolam administration. The mean peak sedative effect of midazolam was seen one hour after dosing. As expected, the mean onset of action after intravenous midazolam administration was observed earlier than that of oral midazolam (at 30 minutes after start of the infusion vs. 1 hour after dosing). The onset of action, time of peak effect and duration of effect showed, however, large variation between individual patients.

We found that before midazolam administration, approximately one third of our patients were already oversedated according to the ranges provided by the COMFORT score. A similar high proportion of sedated patients (as defined by a different sedation score) was reported by other investigators before start of a continuous midazolam infusion in preterm infants (5). Moreover, other investigators reported that the mean COMFORT score before start of a continuous midazolam infusion in preterm infants was even lower than observed in our study cohort [18.8 ± 3.6 vs. 15.9 ± 3.8] (6). The COMFORT score has been validated for the assessment of sedation in pediatric intensive care patients and in neonates postoperatively (10, 11). Despite the fact that the COMFORT score has not been validated for use in preterm infants it was used in this study as a quantitative measurement to examine the sedative effect of midazolam and to discriminate between sedated and non-sedated preterm infants. It is important to note that premature infants have a decreased level of alertness and limited muscle development, which suggests a less dramatic visible response to stress (12, 13). Consequently, these developmental differences may confound the use of sedation scores that have been validated in older infants and children and thus, somewhat limit the extrapolation of findings (i.e., the comparison to other older populations) pertaining to the assessment of drug effect. In an attempt to minimize these particular effects, we investigated the change of the COMFORT score in the individual patient as a measure of the sedative effect of midazolam.

In our subjects, the COMFORT score decreased significantly from baseline after a single midazolam dose. This observation is in agreement with data from older preterm infants (gestational age 34 to 40 weeks) where a change in sedation level was observed 10 to 20 minutes after an IV midazolam bolus (0.2 mg/kg) and appeared to last more than 2 hours (3). Moreover, in a placebo-controlled study on the effect of continuous sedation on neurological outcome, the mean COMFORT score was lower during midazolam infusion than during placebo (p=0.04) (6, 7). In contrast, these investigators did not find a significant decrease in COMFORT score 24 hours after start of the continuous midazolam infusion in preterm infants (6). In comparing preterm infants

to older children and adults, the time to peak effect (30 minutes vs. 2-3 minutes) and duration of effect (2-6 hours vs. 0.5-1 hour) for intravenous midazolam appear to be longer and more variable (14) (15).

In contrast to the rather uniform response reflected by the mean COMFORT scores in our patients, only 55% of the preterm infants demonstrated a sedative response after midazolam administration; a proportion which is lower than observed with the use of the absolute COMFORT score of < 17 (i.e., 65% and 76% after oral and intravenous midazolam, respectively). Several reasons may explain these apparent discrepant findings. First, as discussed earlier, the COMFORT score has not been validated in preterm infants and may lack sufficient sensitivity to accurately detect changes in distress in preterm infants. Second, physiologic instability associated with prematurity and its attendant disease states, may leave the preterm infant too weak to respond and consequently, blunt observations. We therefore propose that the lack of response to midazolam in many of our subjects may have been due to physical instability of these particular infants. Another potential limitation of our study resides with the fact that we did not determine severity of illness as measured, for example, by the Clinical Risk Index for Babies (6) but rather, relied upon the need for mechanical ventilation as a surrogate measure for illness which revealed that the proportion of ventilated patients was not higher in the non-responder as compared to the responder group [p=0.7 (IV) and p=1.0 (PO), Fisher Exact test). Finally, another potential reason for the lack of sedative response to midazolam in some preterm neonates may reside in developmental differences in benzodiazepine receptor density and functional activity. Benzodiazepine-binding sites are expressed early in life, with the overall benzodiazepine receptor densities increasing three- to fourfold postnatally in cortices and cerebellum (16). However, the exact ontogeny of the different benzodiazepine receptor subtypes during human development has not been elucidated.

Pharmacodynamic-pharmacokinetic relationship

The lack of an apparent relationship between midazolam plasma concentrations and change in sedation level in our subjects corroborates the results of other investigators who determined the relationship between midazolam plasma concentrations and sedation levels at 24 and 48 hours after the start of a continuous infusion of the drug in preterm infants (5). In older children and adults, however, a relationship between midazolam concentrations and level of sedation is apparent (14, 17). The apparent absence of a definable pharmacokinetic-pharmacodynamic relationship in preterm infants as compared to older children and adults supports our assertion that the lack of response to midazolam in a substantial portion of preterm infants is due to developmental differences in drug action and/or the relative inability of sick infants to demonstrate distress at levels sufficient to be detected with the operative constraints of the COMFORT score.

Safety

Midazolam was administered as a 30-minute infusion or as an oral bolus dose in an attempt to minimize the increased risk of hypotension in preterm infants given intravenous bolus doses of the drug (18). As a consequence, we expected midazolam peak plasma concentrations to be lower and thus, to obviate or attenuate any adverse

hemodynamic effects of midazolam. Our data support that this particular safety objective of the investigation was attained. Following intravenous infusion of midazolam, plasma concentrations were higher after intravenous as compared to oral administration [median (range): 108 (48-217) vs. 64.4 (15.2-204) ng/ml]. Only after intravenous midazolam administration did blood pressure decrease slightly, albeit significantly. A previous study (24) suggested that hypotension may reduce cerebral blood flow velocity; a finding that has been disputed with regard to its significance (25). While we did not specifically evaluate cerebral blood flow in or study, evaluation of our subjects suggested that the reduction in blood pressure following a 30 minute intravenous infusion of midazolam was not clinically significant as it was transient and did not require pharmacologic or fluid treatment. Thus, a 30 minute intravenous infusion of a single 0.1 mg/kg dose of midazolam in preterm neonates appears to be well tolerated.

The frequency and type of adverse events observed in our patients after midazolam administration are comparable to those described in a previous investigation (2). We qualified apneas and bradycardia as possibly related to midazolam administration because these symptoms are inherent in prematurity and they were also observed with the same frequency and severity in the 24-hour pre-study period. Myoclonus was observed in one patient in each formulation group after midazolam administration. In newborn infants, myoclonus following midazolam administration has been reported by several investigators. (19). However as shown previously (26), EEG recording during myoclonus episodes in 6 newborn infants following midazolam administration did not show any epileptic activity.

In summary, midazolam (0.1 mg/kg) as a 30-minute intravenous infusion or a single oral bolus dose appears to be well tolerated in preterm infants but produces effective sedation in only a small majority of patients. This developmental difference (as compared to older infants and children) appears to have a pharmacodynamic basis as opposed to a pharmacokinetic basis. Further evaluation of midazolam pharmacodynamics in the neonatal period is warranted as is the validation of scoring "systems" that are both sensitive and specific enough to better quantitate drug effects. Finally, it is unclear whether dose escalation (i.e., doses > 0.1 mg/kg) might produce sedation in those preterm infants who have apparent therapeutic failure with the 0.1 mg/kg dose. Caution in this regard would be prudent given the potential for higher midazolam doses to produce potentially significant adverse effects (e.g., hypotension, desaturation) that may not be well tolerated in a small, sick preterm infant.

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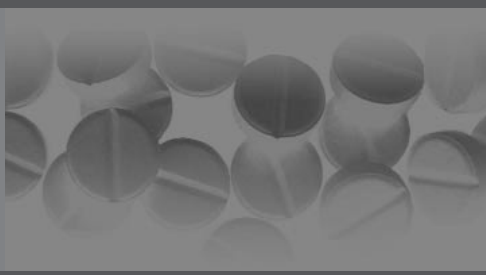
Pharmacodynamics of midazolam in pediatric intensive care patients

Chapter 11



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Summary

Aim To aim of the study was to determine the pharmacodynamics of midazolam in pediatric intensive care patients using the COMFORT scale as validated sedation scale.

Methods The pharmacodynamics of midazolam and its metabolites were determined in 21 pediatric intensive care patients with ages between 2 days and 17 years who received a continuous infusion of midazolam (0.05-0.4 mg/kg/h) for 3.8 hours to 25 days for conscious sedation. The rate of midazolam infusion was titrated according to sedation level, using the COMFORT scale as a validated tool for the assessment of sedation. Blood samples were taken at different time points during and after midazolam infusion for determination of midazolam, 1-OH-midazolam and 1-OH-midazolam-glucuronide with HPLC-UV assay.

Results In twenty out of the 21 patients the rate of midazolam infusion could be effectively titrated to the desired level of sedation. However, the COMFORT scale ranges, as previously validated to reflect an adequate level of sedation, could not be applied to all patients as in specific disease states a deeper level of sedation was clinically indicated. A relationship between pharmacokinetic and pharmacodynamic assessment using the COMFORT scale could not be detected

Discussion Desired levels of sedation could be reached with midazolam in almost all pediatric intensive care patients. Based on our findings that there is no relationship between pharmacokinetic parameters and pharmacodynamic outcome, we recommend that midazolam dosing should be titrated according to the desired clinical effect with the use of the COMFORT scale.

Introduction

Most pediatric intensive care patients are in pain, fearful and anxious. This impairs cooperation with treatment and negatively influences clinical outcome (1) (2). Sedation is, therefore, often required in children in the intensive care unit.

One of the most widely used drugs in the pediatric intensive care is the short-acting benzodiazepine midazolam. The efficacy of continuous midazolam infusion for sedation in pediatric intensive care patients has been shown by several investigators (3) (4) (5) (6). These studies demonstrated that midazolam resulted in effective sedation in the majority of patients studied. Unfortunately, all these studies show limitations with respect to the overall usefulness of their results for midazolam dosing in pediatric intensive care patients. The two main limitations of these studies are the concomitant administration of other sedative-analgesic drugs and the use of non-validated sedation scores, varying from the subjective assessment of sedation by the patient's nurse to a 5-item sedation scale. (4) (5) (6)

The COMFORT scale is specifically validated to assess sedation in pediatric intensive care patients and postoperative neonates (1) (7) (8). This scale has been successfully used to investigate the pharmacodynamics of other drugs for continuous sedation or analgesia in pediatric intensive care patients (9). Hence, the COMFORT scale appears to be a useful surrogate measure for the efficacy of midazolam infusion in pediatric intensive care patients.

The aim of this study is to investigate the pharmacodynamics of midazolam and its metabolites in pediatric intensive care patients, using the COMFORT scale as a validated tool for measurement of sedation.

Methods

Patient recruitment

Patients were recruited from the Pediatric Intensive Care Unit of the Sophia Children's Hospital, Rotterdam, The Netherlands. The institutional review board approved this research protocol. Written, informed consent was obtained from patients and/or parents or legal guardians prior to enrollment in the study.

Patients were eligible for study entry if they were between 0 and 18 years old, needed midazolam for conscious sedation and already had an indwelling arterial catheter placed for purposes of medical care. Patients were excluded if they (1) received concomitant neuromuscular blockade drugs, (2) were exposed to midazolam for longer than 12 hours prior to start of the investigation, (3) were exposed to midazolam prior to start of the investigation without exact information on midazolam dosing, (4) were exposed to recent (i.e. < 24 hours prior to dosing) or chronic treatment with medications known or suspected to alter the pharmacokinetics of midazolam. Since midazolam is a substrate for cytochrome P450 3A enzymes, potential patients were evaluated for exposure to drugs known to affect CYP3A activity (e.g. erythromycin, phenobarbital, dexamethasone, and cisapride) (10). Severity of illness during the first 24 hours of ICU stay was assessed with the Pediatric Risk Mortality score (PRISM) (11).

Sedation assessment

The COMFORT scale was used as primary measure of effectiveness of midazolam (Table 1). The COMFORT scale rates eight behavioral or physiologic dimensions of distress (1). Each dimension is scored on a subscale from 1 to 5 by a trained observer. Six individuals were trained as observer and were allowed to score children for the study when interrater reliability was acceptable as described by Van Dijk et al. (7). For the non-ventilated patients, an item on crying replaced the item 'respiratory response'(7). Each observation consisted of a 2-min period of intensive observation of the patient. After each observation, the trained observer calculated the COMFORT score (minimal score 8, maximal score 40). COMFORT scores of < 17 were considered reflective of oversedation, scores between 17 and 26 as effective/optimal sedation and scores of >26 as distressed, undersedated and in need of further intervention (1).

Study design

We used a midazolam dosing schedule to obtain optimal sedation as assessed by the COMFORT scale. If the patient's COMFORT score was <17 or >26 during the midazolam infusion the dose was either reduced or increased, respectively. The midazolam infusion was continued until the attending physician decided that additional sedation was no longer needed (e.g. when extubation was anticipated that same day). The time of end of study was determined by completion of wash-out sampling, removal of the arterial line, discharge from the ICU, a midazolam infusion rate > 1.0 mg/kg/h, neuromuscular blockade, death or by request of the patients parent/guardian

Midazolam dosing and pharmacodynamic assessment

Drug dosing was started with midazolam, 0.1 mg/kg in glucose 5%, (Dormicum®, Roche, Mijdrecht, The Netherlands) as an intravenous bolus followed immediately by an intravenous midazolam infusion of 0.1 mg/kg/h into a peripheral vein or central catheter. COMFORT scores were determined before, 2 and 30 minutes after start of the infusion.

If midazolam infusion was already started before study entry, the midazolam infusion was continued and a baseline COMFORT score was determined. Next, for all patients, COMFORT scores were determined every 8 hours (at 8 am, 4 pm and 12 pm) and, in addition, when the nurse considered the patient to be not optimally sedated at any other time. The infusion rate was adjusted according to the COMFORT score as follows: if a patient was in distress (COMFORT>26), a bolus dose midazolam (0.1 mg/kg) was given, followed by an immediate increase in infusion rate of 0.05 mg/kg/h. If a patient was considered oversedated (COMFORT<17), the infusion rate was decreased by 0.05 mg/kg/h. Additional COMFORT scores were determined, 2 and 30 minutes after an increase in midazolam infusion rate and 60 minutes after a decrease in midazolam infusion rate. Deviations from the protocol were allowed at the discretion of the attending physician as based on clinical needs.

If sedation was no longer clinically needed, the infusion rate was decreased by 0.1 mg/kg/h every 12 hours. Immediately prior to and 10, 30 minutes, 1, 2, 4, 6, 12 and 24 hours after discontinuation of the infusion, COMFORT scores were determined.

Table 1 *(adapted with permission from van Dijk et al. (7)*

Scale item	Score
Alertness	
Deeply asleep	1
Lightly asleep	2
Drowsy	3
Fully awake and alert	4
Hyperalert	5
Calmness	
Calm	1
Slightly anxious	2
Anxious	3
Very anxious	4
Panic	5
Respiratory response	
No coughing and no spontaneous respiration	1
Spontaneous respiration with little or no response to ventilation	2
Occasional cough or resistance to ventilator	3
Actively breathes against ventilator or coughs regularly	4
Fights ventilator; coughing or choking	5
Crying	
Quiet breathing, no crying	1
Sobbing or gasping	2
Moaning	3
Crying	4
Screaming	5
Physical movement	
No movement	1
Occasional, slight movement	2
Frequent, slight movements	3
Vigorous movement limited to extremities	4
Vigorous movements including torso and head	5
Muscle tone	
Muscles totally relaxed; no muscle tone	1
Reduced muscle tone	2
Normal muscle tone	3
Increased muscle tone and flexion of fingers and toes	4
Extreme muscle rigidity and flexion of fingers and toes	5
Facial tension	1
Facial muscles totally relaxed	2
Facial muscle tone normal; no facial muscle tension evident	3
Tension evident in some facial muscles	4
Tension evident throughout facial muscles	5
Facial muscles contorted and grimacing	
Blood pressure	1
Blood pressure below baseline	2
Blood pressure consistent at baseline	3
Infrequent elevations of 15% or more above baseline (1-3 during 2 minutes observation)	4
Frequent elevations of 15% or more above baseline (> 3 during 2 minutes observation)	5
Sustained elevations of 15% or more	
Heart rate	
Heart rate below baseline	1
Heart rate consistent at baseline	2
Infrequent elevations of 15% or more above baseline (1-3 during 2 minutes observation)	3
Frequent elevations of 15% or more above baseline (> 3 during 2 minutes observation)	4
Sustained elevations of 15% or more	5

'Crying' is used instead of 'respiratory response' if a patient is not mechanically ventilated

Blood sampling and drug assay

Blood samples were taken for later analysis of midazolam and 1-OH-midazolam and 1-OH-midazolam-glucuronide concentrations. Blood samples were taken simultaneously with the COMFORT scores: i.e.:

1 before, 2 and 30 minutes after each midazolam loading dose
2 before and 60 minutes after each decrease in midazolam infusion rate
3 every morning at 8.00 AM, but not at 4 PM and 12 PM
4 prior to and 10, 30 minutes, 1, 2, 4, 6, 12 and 24 hours after discontinuation of the infusion.

Plasma was separated from whole blood by centrifugation (1000 X g for 10 minutes) and then stored at -80°C until analysis. Plasma samples were analyzed for midazolam and 1-OH-midazolam by validated high-pressure liquid chromatography (HP G13 series, Agilent, Amstelveen, The Netherlands) with diode array UV detection. (HP G1315A, Agilent, Amstelveen, The Netherlands) The column used was Novapak C18 (Waters, Ettenleur, The Netherlands). Diazepam (12.5 ng/100µl H_2O, Bufa, Uitgeest, The Netherlands) was added to each sample as internal standard and solid phase extraction was performed using alkalic extraction (pH=9) with dichloromethane (Rathburn, Walkerburn, Switzerland)

The inter-day coefficients of variation at the low standard concentration (20 ng/ml) were less than 6.9% and 10.3% for midazolam and 1-OH-midazolam, respectively. The lower limit of quantitation was 20 ng/ml for both midazolam and 1-OH-midazolam using 0.5 ml plasma volume (12).

Table 2 *Patient characteristics*

	Sex	Age	Weight (kg)	Origin	ICU admission reason	PRISM (11)
Patient						
1	Male	0.04 mths	3.5	Caucasian	Congenital heart disease	14
2	Male	0.12 mths	3.8	Caucasian	Congenital heart disease	23
3	Female	0.36 mths	3.6	Caucasian	Congenital heart disease	9
4	Male	0.48 mths	2.8	African	Postcardiac surgery	22
5	Female	0.60 mths	3.7	Caucasian	Congenital heart disease	17
6	Female	0.96 mths	4.3	Mediterranean	Respiratory insufficiency eci	30
7	Female	1.9 mths	4.8	Afro-Caribbean	Upper airway infection	14
8	Male	2.0 mths	3.6	Caucasian	Upper airway infection	20
9	Male	4.1 mths	7.5	Caucasian	Meningitis	19
10	Male	8.2 mths	20	Caucasian	Upper airway infection	6
11	Male	1.1 yrs	9.3	Asian	Pneumonia	19
12	Male	2.8 yrs	13	Asian	Empyema	7
13	Male	3.9 yrs	15	Afro-Caribbean	Acute laryngotracheobronchitis	14
14	Female	4.5 yrs	19	Caucasian	Pulmonary hypertension	24
15	Female	5.5 yrs	24	Caucasian	Staphylococcal scalded skin syndrome	11
16	Female	8.9 yrs	22	Middle-Eastern	Pulmonary hypertension	2
17	Male	9.1 yrs	25	Caucasian	Measles pneunomia	9
18	Female	13.1 yrs	40	Caucasian	Final stage ALL with multiple organ failure	20
19	Male	14.8 yrs	50	Caucasian	Ebstein-Barr virus infection	5
20	Female	15.1 yrs	52	Hispanic	Malignant hypertension	12
21	Male	17.0 yrs	60	Mediterranean	Postcardiac surgery	4

ALL = acute lymphatic leukemia, PRISM = pediatric risk of mortality score, mths = months, yrs = years

To measure 1-OH-midazolam-glucuronide in plasma, 50 µl β-glucuronidase was added to plasma samples (IBF Biotechnics, Villeneuve-la-Garenne, France, 100.000 Fishmann units/ml) and incubated at 40°C for 16 hours. Samples were processed and quantitated for total 1-OH-midazolam (conjugated plus unconjugated) in the same manner as described before. 1-OH-midazolam-glucuronide concentrations were then determined by using the following equation: [1 -OH-MG] = d[1-OH-M] x [M 1-OH-MG/M 1-OH-M], where: [1-OH-MG] = 1-OH-midazolam-glucuronide concentrations, d[1-OH-M] = concentration difference of 1-OH-midazolam before and after hydrolysis, M 1-OH-MG = molecular weight of 1-OH-midazolam-glucuronide (517,9) and M 1-OH-M = molecular weight of 1-OH-midazolam (341,8).

Statistical analysis

COMFORT scores of patients who had a baseline score and a minimum of one 'post-infusion rate change' score were included in the analysis relating dose-adjustments to sedation level. The proportion of patients at each sedation level group was determined before and two and 30 minutes after the initial midazolam dose, during steady state, before and two and 30 minutes after each midazolam dose increment, before and 60 minutes after midazolam dose reduction and during the wash-out period. The paired student t-test was used to compare COMFORT scores, drug and metabolite concentrations before and after start of the infusion. The effect of time-points on the parameters COMFORT score, midazolam, 1-OH-midazolam and 1-OH-midazolam-glucuronide concentrations were determined using a mixed model ANOVA allowing for inter- and intra-patient differences. The relationship between COMFORT score, sedation level category (i.e. oversedated, sedated or distressed) and drug or metabolite concentrations was also determined using a mixed model ANOVA. Data are expressed as mean ± SD unless stated else. These statistical analyses were obtained using SPSS software (version 9.0.0, SPSS Inc., Chicago, Ill) and SAS software [PROC MIXED (version 6.12 SAS institute, Inc, Cary, N.C)]. α =0.05 was considered the limit of significance.

Results

Patient clinical characteristics

21 patients in the age range of 2 days to 17 years were enrolled in the study. The characteristics of the individual patients are listed in Table 2. As reflected by the PRISM score, disease severity among our patients varied considerable. All patients received concomitant drug therapy inherent to intensive care treatment. Seven patients required analgesia (morphine, codeine, fentanyl and acetaminophen) during the midazolam infusion. Data from these patients are included in the analysis unless stated otherwise. Although the prescription of a drug known to affect CYP3A activity, was an exclusion criteria before entry in the study, two patients received CYP3A substrates after inclusion in the study. One patient (no 8) received a bolus of the CYP3A4 inducer dexamethasone (corticosteroid) on the second and third study day before a planned extubation (12). One patient (no 11) received both the CYP3A4 inhibitor erythromycin (macrolide antibiotic) and the CYP3A4 substrate/inhibitor fentanyl (analgesic) during the whole study period (13-15).

In eleven patients study participation ended before the washout period for the following reasons: a) neuromuscular blockade (n=1), b) death (n=1), c) removal of the arterial line (n=4) and d) discharge from the intensive care unit (n=4). One patient (no 4) was withdrawn from the study by his guardian. From one patient no COMFORT score immediately before cessation of the infusion was determined. Hence, in nine patients a (partial) washout curve could be established.

Midazolam dosing

The median duration of midazolam infusion was 3.4 days (range 3.8 hours to 25 days) with a median infusion rate of 0.09 mg/kg/h (range 0.05 – 0.4 mg/kg/h). The median total dose of midazolam infused was 2.9 mg/kg (range 0.40 – 46.0 mg/kg). On average, patients needed two (range 0 – 5) midazolam dose increments and one (range 0 – 3) infusion rate reductions.

In all cases of a COMFORT score >26 (n=14), the midazolam dosing was increased according to the protocol. Two times, midazolam dosing was increased, while the COMFORT score was less than 26, because a deeper level of sedation was clinically needed. All downward adjustments of the infusion rate were in keeping with a COMFORT score of <17. At 30 occasions (of which 9 and 5 times in two patients) of a COMFORT <17, the midazolam infusion was not decreased, because the clinicians judged a deeper level of sedation necessary.

Pharmacokinetic-pharmacodynamic relationship

Plasma drug sampling and associated sedation assessment sufficient to permit an evaluation for possible pharmacokinetic-pharmacodynamic relationships were performed in all 21 patients (242 data pairs) and in patients without analgesic co-administration (n=14, 200 observations). The relationship between sedation level category was compared with corresponding drug and metabolite concentrations (Table 3). No significant relationship between sedation level category and drug or metabolite concentrations was found. Midazolam concentrations associated with a COMFORT score between 17 and 26 varied from 100 ng/ml (25th percentile value) and 450 ng/ml (75th percentile value) in patients without analgesic co-medication.

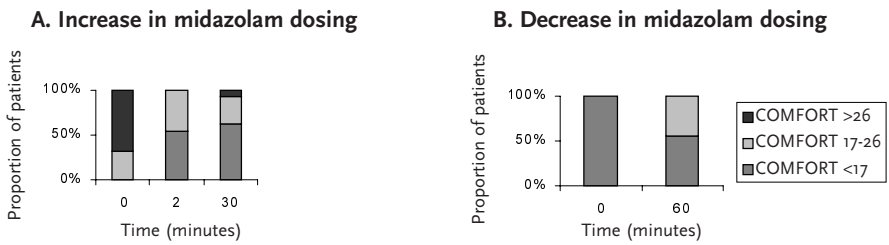
Sedation level

Only five patients did not receive any midazolam twelve hours prior to study enrollment. The COMFORT scores prior to midazolam dosing were <26 in all patients. Two and 30 minutes after midazolam dosing (bolus and start infusion), two and five patients became oversedated (COMFORT <17), respectively. Their mean COMFORT score prior to any study drug administration was 19.8 ± 4.0, which decreased significantly to 16.2 ± 3.0 two minutes and to 12.2 ± 2.7 (p=0.012) 30 minutes thereafter.

The effect of midazolam dosage adjustments during the infusion period on the proportion of patients in each sedation category is shown in Figure 1. The mean COMFORT scores were 26.6 ± 1.3 prior to midazolam dosage increment and decreased significantly to 17.7 ± 1.4 (p<0.01), 2 minutes, and to 15.9 ± 1.3 (p<0.01), thirty minutes thereafter. The mean COMFORT score increased significantly from 12.9 ± 1.1 to 17.0 ± 1.1 (p=0.01) one hour after decrease of the midazolam infusion rate with 0.05 mg/kg/h.

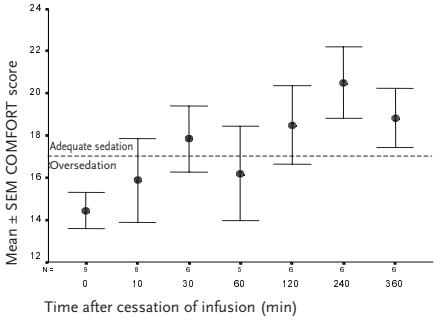
From only six patients COMFORT score were available up to six hours after

Figure 1 *Effect of midazolam dosage adjustment on sedation level in pediatric intensive care patients*



a. midazolam bolus dose (0.1 mg/kg) followed immediately by an infusion rate increase with 0.05 mg/kg/h (16 times, n=6)
b. midazolam infusion rate decrease with 0.05 mg/kg/h (24 times, n=13). The X-axis represents time (minutes) before (0) and after midazolam dosage adjustment. The Y-axis represents proportion of patients in each sedation level category.

Figure 2 *Effect of midazolam infusion cessation on COMFORT score in pediatric intensive care patients.*



The X-axis represents time (minutes) after cessation of the midazolam infusion. The Y-axis represents the mean (± SEM) COMFORT score.

Table 3 *Relationship between sedation level category and midazolam or metabolite concentrations in pediatric intensive care patients.*

	COMFORT scale ranges			
	<17	**17-26**	**>26**	**p-value**
Midazolam	**Concentrations**			
All patients	553 ± 164	577 ± 169	572 ± 210	0,96
Patients without co-medication#	370 ± 56	311 ± 57	258 ± 78	0,08
1-OH-Midazolam				
All patients	49 ± 11	49±11	47±14	0,97
Patients without co-medication	37 ± 8	35 ± 9	27 ± 11	0,40
1-OH-Midazolam-glucuronide				
All patients	593 ± 105	622 ± 110	549 ± 154	0,83
Patients without co-medication	649 ± 140	624 ± 142	587 ± 192	0,90

All drug concentrations are expressed in ng/ml. COMFORT scale <17: oversedated, 17-26 effectively sedated, >26 undersedated, #=other sedative-analgesic drugs.

cessation of the infusion (Figure 2). The mean COMFORT score of these patients increased from 13.9 ± 5.6 before to 20.8 ± 4.2 (p=0.03 Wilcoxon's test, n=6) four hours after cessation of the infusion.

Outliers.

Two patients needed very high concentrations of midazolam to achieve an adequate level of sedation. Patient 18 was terminally ill due to an aspergillus infection secondary to ALL and died within 24 hours after inclusion in the study. She was very restless (i.e. tried to remove her central venous and arterial lines and her tube several times) and needed several midazolam infusion rate increments to a maximum infusion rate of 0.4 mg/kg/h. In contrast to our expectations, her midazolam concentrations were disproportional high (up to 8200 ng/ml), probably due to reduced midazolam metabolism secondary to hepatic failure. Patient 11, a patient with Down's syndrome, experienced recurrent episodes of breakthrough agitation during concomitant midazolam and fentanyl infusions, despite high midazolam concentrations (mean 1590 ng/ml).

Discussion

We determined the pharmacodynamics of midazolam in a heterogeneous group of intensive care patients with different clinical diagnoses. In most patients, we were able to establish a level of sedation that would allow the children to remain sedated during mechanical ventilation or spontaneous breathing, as well as during nursing activities. Moreover, in most patients, there was no need for additional drugs as opioids to achieve effective sedation. The ranges of midazolam infusion rates associated with effective sedation in our study (0.05-0.4 mg/kg/h) were in agreement with data from a study in 24 pediatric intensive care patients who were between 26 days and 5 years old (0.05-0.5 mg/kg/h) (5). Midazolam infusion rates associated with effective sedation were lower in two other studies in pediatric intensive care patients (0.02-0.4 mg/kg/h) (3, 16). However in both studies frequent temporary midazolam infusion rate increments or single doses of other sedative-analgesic drugs were needed. The results of studies in pediatric cardiac surgery patients are not well comparable with our results because midazolam infusion was used in conjunction with the administration of morphine (6) (17) (18). Hence, the observed sedative effect is the result of the synergistic action of both drugs, as discussed by Somma et al. (19).

Since mean COMFORT scores change considerably after a midazolam dosage adjustment a concentration-effect relationship appears to be present in individual patients. However, no significant concentration-effect relationship could be detected when data from all patients are taken together. This lack of a significant relationship between midazolam or metabolite concentrations and sedation is in agreement with other studies in pediatric and neonatal intensive care patients (5, 17, 20). Interestingly, Hartwig et al. (5) found a significant concentration-effect relationship during the first 24 to 48h of midazolam infusion, but no longer after 80-120 h of infusion duration. In healthy adult volunteers and pediatric patients (21, 22), a significant relationship between midazolam concentrations and sedation is apparent after a single bolus dose midazolam. In pediatric intensive care patients, a concentration-effect relationship may be obscured by several

factors. First, mental status changes during sleep. Most persons alternate from awake to sedated to asleep during the day. Clearly, the level of sedation is not entirely a function of plasma concentrations. Second, in many studies, a possible concentration-effect relationship may be complicated by the concomitant administration of other sedative-analgesic drugs. In this study, the concentration-effect relationship seemed to improve after exclusion of the patients with co-medication (Table 3). Third, development of tolerance to midazolam after prolonged infusion may also obscure a concentration-effect relationship (2) (23). Due to the small number of patients in this study who received midazolam for more than 72 hours (n=6) we were, however, not able to investigate such an effect. Finally, the absence of adequate sedation despite high concentrations as observed in patient 11 may be associated with Down's syndrome (18). Children with Down's syndrome respond less predictable to sedative drugs and often need more than one drug to achieve adequate sedation (24). Concluding the lack of an overall concentration-effect relationship precludes the use of standardized dosing schedules to reach a similar sedative effect in all pediatric intensive care patients.

The use of standard COMFORT scale ranges (i.e. <17, >26) for all patients and consequent changes in midazolam dosing needs careful consideration. In our experience, these ranges are not valid for all patients with different needs of sedation. In more than half of the occasions with a COMFORT score of less than 17, the attending physician felt dose reduction not appropriate in view of the patient's clinical condition. Clinical conditions which warranted a deeper sedation level, to prevent breakthrough agitation, were e.g. pulmonary hypertension or systemic malignant hypertension.

Our results demonstrate that episodes of distress can be treated effectively and rapidly by a midazolam bolus dose in conjunction with an increase in infusion rate using the COMFORT scale as a measure of sedation (Figure 1a). If an immediate sedative effect is needed, a bolus dose is needed in addition to an increase in infusion rate, given the pharmacokinetics of midazolam. With the reported midazolam elimination half-lives between 1 to 4 hours (and possibly longer) in pediatric intensive care patients, a new steady-state midazolam concentration will only be reached after 3.5 to 16h (18) (17, 25) (26). Although frequently employed (16), the administration of other drugs to overcome breakthrough agitation, will complicate the titration of sedation afterwards by the effect of two drugs instead of one. Moreover, especially in newborn infants, the concomitant use of midazolam and opioids has been associated with hypotension (27).

Our results also show that a decrease in midazolam dosage or cessation of the midazolam infusion results in a relatively slow and variable increase in COMFORT score (less sedated), consequent to the wide interindividual variability in midazolam clearance (18) (17, 25) (26, 28). These data are in agreement with other studies in pediatric intensive care patients were a clinical state allowing extubation was achieved between two and four hours after cessation of the midazolam infusion (6, 18).

We also determined the concentrations of 1-OH-midazolam and 1-OH-midazolam-glucuronide in addition to midazolam, because these metabolites are also clinically active. The reasons for the lack of a pharmacokinetic-pharmacodynamic relationship for the metabolites by themselves, or when the sum of midazolam and metabolites was taken, are probably similar as discussed for midazolam. High 1-OH-midazolam-glucuronide concentrations may lead to prolonged sedation, when present in high concentrations as may occur patients with renal failure. Only one patient (no 20) in our patient sample

had serious renal failure. Although her 1-OH-midazolam-glucuronide/midazolam ratio was very high (data not shown), she did not become oversedated, probably because she received midazolam only for approximately 12 hours.

In conclusion, midazolam dosing can be successfully titrated using the COMFORT score to achieve adequate sedation in pediatric intensive care patients. The lack of a pharmacokinetic-pharmacodynamic relationship in pediatric intensive care patients forces midazolam dosing to be titrated according to its desired effect. Using the pharmacodynamic data generated in the current study, the following dosing schedule can be suggested. First, although the COMFORT score adequately reflects sedation in pediatric intensive care patients, individual COMFORT score ranges, based on each patient's clinical condition, should be defined reflecting adequate sedation. Second, this dosing scheme incorporates a single 0.1 mg/kg loading dose of midazolam to achieve immediate, effective sedation while simultaneously beginning a continuous infusion at a rate of 0.1 mg/kg/h. Further dosage increment, at least 30 minutes after a prior dosage increment consists of a single bolus dose (0.05-0.1 mg/kg) with a simultaneous increasing of infusion rate with 0.05 mg/kg/h. Downward dosage adjustment consists of a decrease of midazolam infusion rate with 0.05 mg/kg/h. Downward dosage adjustment at night or early morning should be done with care, as the COMFORT score may reflect sleep instead of sedation.

Acknowledgement

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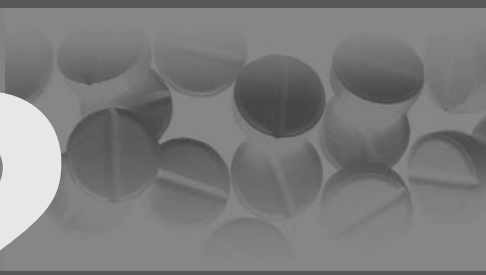
Summary, discussion and future directions

Chapter 12



Maturation of organ systems and somatic growth during fetal life and throughout childhood exerts a profound effect on drug disposition. The maturation of drug-metabolizing enzymes is the predominant factor accounting for age-associated changes in nonrenal drug clearance.

The various studies described in this thesis were performed to study the impact of age on midazolam metabolism, as surrogate measure to assess the impact of development upon both CYP3A and UGT activity, and to examine the most well characterized polymorphism of the CYP3A4 gene with respect to its potential to influence CYP3A activity; a determinant of midazolam pharmacokinetics and pharmacodynamics.

Summary

Introduction

Chapter 1 describes the aims of the studies conducted as part of this thesis.

Chapter 2 reviews the current literature on the ontogeny of the cytochrome P450 3A subfamily of drug-metabolizing enzymes. The available data indicate that profound, often clinically important, changes occur in the activity of CYP3A isoforms during all stages of development. However, several critical information gaps exist with regard to the overall impact of ontogeny on CYP3A activity. The exact developmental pattern of CYP3A expression and its impact on the pharmacokinetics of CYP3A substrates has not been fully characterized during the first 3 to 5 years of life. The contribution of intestinal and renal CYP3A to the disposition of CYP3A substrates in children has not been well studied and thus, it is not known if development *per se* influences the functional expression of CYP3A activity in these organs. Finally, the genetic and possibly, neurohumoral, factors that govern the upregulation of CYP3A after birth are also still largely unknown.

Chapter 3 reviews the current literature on the developmental aspects of the UDP-glucuronosyltransferases (UGT) superfamily of drug-metabolizing enzymes. At least 10 different UGT isoforms have been identified, which all appear to display a different developmental pattern. Despite the presence of pharmacokinetic studies of UGT substrates in pediatric patients (e.g., lorazepam, morphine, acetaminophen), isoform specificity of enzyme action and its implications with respect to the disposition of pharmacologic substrates is generally not known. It is concluded that current knowledge of UGT ontogeny is insufficient and future investigations must consider the impact of development on isoform specific biotransformation of therapeutic drugs and xenobiotics.

Pharmacokinetics

Chapter 4 describes the pharmacokinetics and metabolism of the CYP3A substrate midazolam, given as an intravenous bolus dose to 24 preterm infants, who were between 26 and 33 weeks of gestational age and between 3 and 11 days of postnatal age. Consequent to immature hepatic CYP3A4 activity, midazolam clearance and 1-OH-midazolam concentrations were markedly reduced in preterm infants as compared to previous reports from studies in older children and adults. Midazolam apparent volume of distribution and total plasma clearance were increased in those infants who

had postnatal indomethacin exposure as compared to those who did not. This finding suggests that indomethacin treatment of a patent ductus arteriosus produces a drug-treatment effect capable of altering the disposition of midazolam, an extensively metabolized drug, in premature neonates.

The pharmacokinetics, absolute oral bioavailability and metabolism of midazolam given as an oral bolus dose to 15 preterm infants are presented in Chapter 5. The oral bioavailability of midazolam is increased while the midazolam oral plasma clearance and 1-OH-midazolam concentrations are markedly reduced as compared to previous reports in older children and adults. These latter findings correspond to reduced functional activity of both intestinal and hepatic CYP3A4/5; developmental findings that characterize prematurity. These findings may also have important consequences for the oral availability of other CYP3A4 substrates as higher oral bioavailability may lead to higher plasma concentrations of CYP3A4 substrates which in turn, could increase the risk of toxicity in newborn infants should the dose of a drug not be modified *a priori* for developmental differences in the rate and extent of drug absorption.

Chapter 6 describes the population pharmacokinetics and biotransformation of midazolam administered as a continuous infusion in 21 pediatric intensive care patients who were between 2 days and 17 years of age. Midazolam plasma clearance in our subjects appeared to be lower as compared to data from other studies in pediatric intensive care patients; a potential consequence of reduced CYP3A4 activity associated with the lower mean age of patients in our study cohort. These studies demonstrated production and persistence of the glucuronide conjugate of 1-OH midazolam, the primary oxidative metabolite of midazolam catalyzed by CYP3A, in all infants. This finding suggests that the yet uncharacterized UGT isoforms responsible for the phase II metabolism of 1-OH midazolam are present at two days of postnatal age in preterm neonates. This study also identified the potential for important disease (e.g., renal and/or hepatic compromise) and treatment (e.g., concomitant administration of CYP3A4 inhibitors) co-variates to serve as predictors of altered midazolam pharmacokinetics and metabolism in pediatric intensive care patients. The derived population pharmacokinetic model may have clinical applicability for individualization and resultant optimization of midazolam therapy in children.

Pharmacogenetics

Chapter 7 describes a new detection method for the CYP3A4*1B allele and its allelic frequency among 199 Dutch Caucasians. A simple and specific PCR-restriction fragment length polymorphism (RFLP) was developed. Allelic frequency for this mutation was found to be 5.3%. All identified individuals were heterozygous for this allele, giving a heterozygote frequency of 10.6%. It was concluded that this assay could greatly facilitate studies on the effect of this polymorphism in endogenous processes, environmental susceptibility to cancer and individual ability to metabolize drugs that are substrates for CYP3A4.

A new PCR-RFLP method for the detection of the CYP3A4*3 allele is described in Chapter 8. The frequency of this allele in 499 Dutch Caucasians and 66 pediatric patients was determined. Thirteen of 499 and 1 of 66 individuals were found to be heterozygous

for the allele, which renders an allelic frequency of approximately 1%. The results indicated that the CYP3A4*3 may be a true genetic polymorphism and thus, has the potential to influence the activity of this enzyme reflected by altered pharmacokinetics of CYP3A4 substrates. Future research was suggested to elucidate the effect of this polymorphism on CYP3A4 activity.

Chapter 9 describes an exploratory, pilot investigation that examined the possible association of the CYP3A4*1B allele with the pharmacokinetics of midazolam in 30 preterm infants. The data from this study demonstrated that the CYP3A4*1B allele was not apparently associated with changes in intravenous or oral midazolam clearance in preterm infants in the first 14 days of postnatal life. Recognizing the implicit limitations associated with sample size in this pilot pharmacogenetic study, it was concluded that further studies are needed to elucidate the potential impact of CYP3A*1B and other allelic variants of CYP3A4 and CYP3A5 on its activity in man.

Pharmacodynamics

Chapter 10 describes the pharmacodynamics of midazolam given as a 30-minute intravenous infusion or oral bolus dose to 24 preterm infants. The results suggest that midazolam is effective in a majority of infants as reflected by reductions in COMFORT scores within 30 minutes after intravenous and one hour after oral midazolam administration. This evidence of a desired pharmacologic effect (i.e., midazolam was used to induce pre-procedural sedation) was observed without a clinically important change in hemodynamic or other safety parameters. However, a considerable proportion of patients did not appear to respond to midazolam. It was suggested that this lack of response may be due to the fact that patients truly experienced therapeutic failure consequent to developmental immaturity of drug-receptor interaction and/or was associated with the inability of the COMFORT score to adequately reflect sedation uniformly in preterm infants where the pharmacodynamic response may have been blunted by their physical instability.

Chapter 11 describes the pharmacodynamics of continuous midazolam infusion in 21 pediatric intensive care patients. The data indicate that midazolam dosing can be successfully titrated to achieve adequate sedation in pediatric intensive care patients. However, the need for different levels of sedation in different patient groups reduces the possibility of a single COMFORT score range to be used as indicator for adequate sedation. It is, therefore, suggested that individual COMFORT score ranges that are demonstrated to produce adequate sedation should be defined for each patient who receives midazolam by continuous intravenous infusion.

Limitations

Pharmacokinetics

The initial goal of this research project was to determine the ontogeny of CYP3A4 activity *in vivo*, in order to tailor drug therapy with CYP3A4 substrates according to age-related changes in CYP3A4 activity. When studying the impact of co-variates such as age on

drug metabolism *in vivo*, the need for a validated, safe and non-invasive pharmacologic probe sufficient to accurately profile the impact of development on enzyme activity is evident. At the time the studies described in this thesis were started (1997), midazolam clearance and the ^{14}C-erythromycin breath test (EBMT) were the best validated probes to assess CYP3A activity *in vivo* (1). The latter probe has disadvantages, which render it less optimal for use in infants and young children. Since the EBMT uses radioactively labeled erythromycin, this method is unethical for use in newborn infants. We therefore decided to focus on midazolam plasma clearance as surrogate measure of CYP3A activity. Initially, we planned to investigate the possibility of an urinary metabolite:drug ratio as an alternative for plasma clearance, the latter requiring frequent blood sampling for accurate determination. However, during the first year of the studies, it became clear that CYP3A (i.e., CYP3A5) is also present in the kidney and is capable of biotransforming midazolam intrarenally. This, in turn, would be expected to affect the urinary metabolite:drug ratio and thereby, reduce the validity of this ratio as measure of hepatic (and intestinal) CYP3A activity (2). Consequently, we used midazolam plasma clearance as an *in vivo* pharmacologic probe to assess CYP3A activity. In order to reduce the number of samples taken for AUC calculations, we also investigated the possibility of a minimal sampling method (3). It was, however, not possible to derive such a minimal sampling schedule for use in preterm infants. The consequent need for repeated blood sampling restricted the recruitment of patients to those who already had an indwelling arterial catheter placed prior to the study for clinical purposes.

This restriction explains the gap in gestational ages studied (i.e. newborn infants between 33 and 42 weeks of gestational age). Preterm infants with higher gestational ages were not frequently admitted to our neonatal intensive care unit or were too unstable to receive midazolam, the latter being associated with hypotension in hemodynamically unstable patients (4).

Other important limitations of our studies are represented by the relatively small numbers of patients included and the need for an indwelling vascular catheter necessary for repeated blood sampling to accomplish the pharmacokinetic objectives of the study. This may explain why we did not find an age-related difference in midazolam disposition within our study populations in contrast to the differences observed (e.g., comparison of midazolam plasma clearance) between the neonatal and pediatric intensive care patients. However, other factors may also explain the lack of apparent developmental differences in midazolam disposition within the neonatal and pediatric patients groups.

First, midazolam is not only metabolized by CYP3A4 but also, by CYP3A5 and to an unknown extent by CYP3A7, the fetal CYP3A isoform (5). The contribution of CYP3A43 to the metabolism of midazolam is yet unknown (6). CYP3A4 expression is transcriptionally activated during the first week of life and is accompanied by a simultaneous decrease in the level of CYP3A7 expression (7). Additionally, CYP3A4 activity is low directly after birth and attains 30 to 40 percent of adult activity at 1 month of postnatal age. The ontogenic pattern of CYP3A5 and CYP3A43 remains to be elucidated. However, during the transition from fetal to neonatal life, total CYP3A content appears to be relatively stable (7). Hence, it is possible that the contribution of other CYP3A isoforms to the metabolism of midazolam may have obscured a postnatal increase in CYP3A4 activity early in life.

Second, these studies were performed in ill patients housed in intensive care units. In these patients, the disposition of midazolam is more likely to be influenced by both exogenous factors (e.g. drugs capable of altering CYP3A activity or the disposition of midazolam by other pathways of metabolism [glucuronidation] or elimination [renal compromise]) and endogenous factors (e.g. intrinsic hepatic disease or the effect of cytokines associated with systemic infection on CYP3A activity (8)). It was not possible to control for these co-variates consequent to the small number of patients included in the studies and also, their inherent medical conditions.

In contrast to cytochrome P450, knowledge concerning the impact of development on UGT activity is far from complete. The lack of specific probe drugs capable of assessing the activity of individual UGT isoforms and knowledge concerning isoform specificity for the biotransformation of important UGT substrates have contributed to this lack of knowledge. For example, our preliminary data suggest that glucuronidation of the CYP3A4/5 catalyzed primary metabolite of midazolam, 1-OH midazolam, occurs at levels comparable to those reported in adults at between 2 days and 17 years of postnatal life. This finding is in marked contrast to the observation that morphine glucuronidation is impaired at birth and increases to adult levels between 6 and 30 months of age (9). This discrepancy may be explained by the involvement of different UGT isoforms in the glucuronidation of midazolam and morphine. It is however unknown which UGT isoform(s) is/are involved in the formation of 1-OH-midazolam-glucuronide from 1-OH-midazolam. Therefore, our findings regarding midazolam can not be generalized with respect to their potential therapeutic implication to other UGT substrates used to treat infants and children.

Pharmacogenetics

Individual differences in drug metabolism during childhood are only partially explained by the developmental changes involving drug-metabolizing enzymes. It is now recognized that genetic variation directs the expression of many drug-metabolizing enzymes and therefore also contributes to the interindividual differences in drug metabolism (10). At the time this research project started no genetic polymorphisms associated with altered CYP3A activity were known. Recently, many mutations in the CYP3A4, 3A5 and 3A7 genes have been described (11). We determined the allelic frequency of two CYP3A4 mutations in Dutch Caucasians and showed that based upon the frequency of both alleles studied, they may be considered as true genetic polymorphisms in this population. However, evidence is emerging that large interethnic differences exist in the frequency of CYP3A genetic polymorphisms (12). In the Netherlands, an increasing part of the populations is not Caucasian but rather, from Mediterranean, African and Middle-Eastern descent. Given that we did not study these distinct ethnic groups or a population that was the product of genetic admixture between Dutch Caucasians and the other populations, our data can not be extrapolated to the whole Western European population.

Next, we conducted a pilot study investigating a possible association between the CYP3A4*1B mutation and midazolam plasma clearance in preterm infants. The lack of such an association as observed in our patient cohort does, however, not rule out the existence of a genotype-phenotype relationship for CYP3A4*1B in preterm infants. The

number of patients in our study was small and the numbers of patients with and without the mutation was not balanced. However, *in vivo* data from adults suggest that the CYP3A4*1B mutation is not associated with clinically important differences in pharmacokinetics of CYP3A4 substrates (13) and thus, corroborates our experimental findings. Finally, due to the low allelic frequency of the CYP3A4*3 mutation, we were not able to investigate a possible genotype-phenotype relationship for this mutation consequent to the limited population of infants who received midazolam in our investigations.

Pharmacodynamics

Our pharmacodynamic studies of midazolam illustrate the problems associated with the assessment of sedation in nonverbal children. To date, no validated sedation score for preterm infants is available, which leaves the distinction between therapeutic failure or 'sedation score' failure uncertain. Moreover, we encountered limitations of the use of the COMFORT score in pediatric intensive care patients. The COMFORT score that should be determined in situations when the patient is at rest does not adequately predict agitation associated with therapeutic nursing or medical procedures, or other "handling" (e.g., parental agitation) of young infants. Also, we experienced that the COMFORT score ranges defined to reflect adequate sedation in pediatric intensive care patients do not always reflect adequate sedation in all patient groups. For example, we found that deeper sedation appears to be needed in patients with pulmonary hypertension or malignant systemic hypertension. Finally, the heterogeneity (e.g., differences in disease state, organ function, concomitant medications, etc.) which characterizes the pediatric intensive care unit population may have confounded our ability to detect a pharmacokinetic-pharmacodynamic association for midazolam in neonatal and pediatric intensive care patients.

Future directions

Pharmacokinetics

Clearly, several information gaps exist with regard to the overall impact of ontogeny on CYP3A and UGT activity. *In vitro* and *in vivo* studies should together provide greater insights regarding the ontogeny of CYP3A and UGT activity and its impact on the pharmacokinetics of important substrates. First, *in vitro* studies should be conducted to elucidate isoform-specific developmental patterns for both the CYP3A and UGT, not only for hepatic tissue but also for intestinal and renal tissue. This is especially true given that co-regulation of hepatic and intestinal CYP3A4 is not apparent while the activity of the enzyme in both tissues demonstrates a clear developmental pattern(7) (14). Second, *in vitro* metabolism and isoform-specificity of CYP3A and UGT substrates frequently used in children (e.g. midazolam, cisapride, fentanyl, cyclosporin, morphine, ibuprofen) should be determined using relevant tissues (e.g., microsomes derived from liver, intestine, kidney) obtained not from adults but rather, from neonates, infants and children. In these experiments, relevant (i.e., therapeutic) drug concentrations and concentrations of enzyme should be used. Third, the pharmacokinetics and *in vivo* metabolism of these important CYP3A and UGT substrates should be completely

characterized from birth through the first 3 to 5 years of life. Also, other drugs frequently used in children should be investigated in order to clarify a possible 'compensatory' role of CYP3A and UGT activity, when the activity of other drug-metabolizing enzymes is developmentally impaired. Such studies are required to examine the developmental "breakpoints" of enzyme activity so that population extremes of sufficient magnitude to alter the dose vs. concentration vs. effect relationship for selected drugs and their active metabolites can be identified.

The information derived from these studies may aid the physician in designing truly age-appropriate dosing regimens for CYP3A and UGT substrates, which should improve both the safety and efficacy of drug treatment.

Pharmacogenetics

Although many genetic polymorphisms of the CYP3A and UGT genes have been reported, the clinical importance of these polymorphisms with respect to the pharmacokinetics of frequently used drugs in children remains to be elucidated.

Recently, genetic polymorphisms underlying the large interindividual variation in CYP3A5 expression have been found (12). The investigators suggest that CYP3A5 may be the most important genetic contributor to interindividual and interethnic differences in CYP3A activity *in vivo*. Since interindividual differences in CYP3A activity, as reflected by midazolam clearance, appear to be even larger in preterm infants than in adults, this may reflect the interplay between genetic and developmental factors determining CYP3A5 activity. Therefore the investigation of the developmental expression of CYP3A5 and the effect of genetic polymorphisms on this expression should receive increased attention.

Also, recent reports suggest a role for nuclear receptors as PPAR, PXR and CAR in the regulation of drug metabolizing enzyme activity (15). The role of these and other factors in the developmental regulation of CYP3A and possibly, UGT activity should also be elucidated.

Pharmacodynamics

Validated sedation scores for use in preterm infants are lacking. Although the COMFORT score is validated to assess sedation in pediatric intensive care patients and postoperative neonates (16), it is not specifically validated for use in preterm infants. A possible lack of response to stress, as often observed in preterm infants, may be due to an inability to respond resulting from physical instability consequent to prematurity related disease. Validation of the COMFORT score or other sedation scores for preterm infants should improve the quality of pharmacodynamic studies on sedative drugs in this patient population. Also, although the COMFORT score and its ranges associated with adequate sedation are validated for pediatric intensive care patients, it does not take into account circadian differences in sedation level, distress caused by nursing procedures and different needs of sedation for different groups of patients. In future studies of the pharmacodynamics of sedative drugs, these aspects should also be taken into account. As well, more work must be done to evaluate the impact of ontogeny and genetic polymorphisms on receptor expression as a possible basis for true developmental differences in pharmacodynamics.

In conclusion, this thesis illustrates that age is an important determinant of pharmacokinetics and pharmacodynamics of midazolam in preterm infants and children. Moreover, physiological changes consequent to development and disease such as patent ductus arteriosus, hepatic failure and renal failure all contribute to the variability in disposition and effect of midazolam in children. The dependence of many drugs on metabolic elimination underscores the need to obtain specific data in pediatric patients when developmental differences in drug metabolism serve as a primary determinant of drug disposition and/or action.

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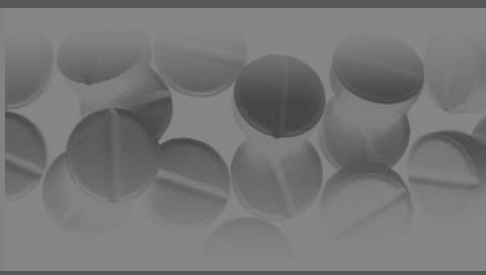
Samenvatting



Samenvatting

Rijping van verschillende organen en veranderingen in lichaamssamenstelling gedurende de neonatale periode en de kinderleeftijd hebben een duidelijk effect op de farmacokinetiek van geneesmiddelen. De uitscheiding van geneesmiddelen door het lichaam (klaring) geschiedt hoofdzakelijk via afbraak in de lever (metabolisme door enzymen) en excretie door de nieren (renale klaring). De ontwikkeling van de activiteit van de enzymen die geneesmiddelen metaboliseren is de belangrijkste factor voor leeftijdsgerelateerde veranderingen in de klaring van geneesmiddelen die niet renaal geklaard worden. Belangrijke enzymfamilies zijn CYP3A en UGT. De CYP3A subfamilie is betrokken bij het metabolisme van meer dan 50% van alle geneesmiddelen. De UGT familie is betrokken bij de omzetting van tientallen belangrijke geneesmiddelen (bv. paracetamol en morfine). Echter over de precieze ontwikkeling van deze enzymactiviteit is niet veel bekend, alsmede over welk effect deze ontwikkeling heeft op de farmacokinetiek van geneesmiddelen bij kinderen op verschillende leeftijden. Dit wordt beschreven in het inleidende gedeelte van dit proefschrift. In het tweede deel van dit proefschrift wordt gekeken naar het effect van leeftijd op de ontwikkeling van CYP3A and UGT activiteit door op verschillende leeftijden de farmacokinetiek te bepalen van midazolam. Midazolam kan als 'modelmedicijn' gebruikt worden voor de enzymactiviteit van CYP3A en UGT. Naast leeftijdsgerelateerde veranderingen in farmacokinetiek, blijken ook verschillen in erfelijke aanleg een rol te spelen bij geneesmiddelen die gemetaboliseerd worden. In het derde deel van dit proefschrift hebben we gekeken naar het vóórkomen van twee mutaties (afwijkingen) in het gen van CYP3A4 en naar het effect van één deze mutaties op de farmacokinetiek van midazolam bij premature pasgeborenen.
Tenslotte is niet alleen de farmacokinetiek onderhevig aan leeftijdsgerelateerde veranderingen, ook de farmacodynamiek (werking van het geneesmiddel) is onderhevig aan leeftijdsgerelateerde veranderingen in bijvoorbeeld geneesmiddel-receptor binding. In het laatste deel van dit proefschrift hebben we gekeken naar het effect van midazolam bij zowel premature pasgeborenen als bij oudere kinderen (3 dagen tot 17 jaar) die opgenomen waren op een intensive care.

Inleiding

Hoofdstuk 1 beschrijft de doelen van de studies die zijn uitgevoerd zoals beschreven in dit proefschrift.

In hoofdstuk 2 wordt een literatuuroverzicht gegeven met betrekking tot de ontwikkeling van de enzymen van de cytochroom P450 3A subfamilie bij kinderen. De beschikbare data laten zien dat er belangrijke, vaak klinisch relevante veranderingen in de CYP3A activiteit plaatsvinden tijdens alle ontwikkelingsstadia. Echter, het precieze patroon van de ontwikkeling van CYP3A expressie en het belang daarvan voor de farmacokinetiek van CYP3A substraten is voor de eerste 3 tot 4 levensjaren niet volledig gekarakteriseerd. De bijdrage van intestinaal en renaal CYP3A aan de farmacokinetiek van CYP3A substraten is evenmin goed in kaart gebracht op de kinderleeftijd en het is niet bekend of ontwikkeling op zich de functionele expressie van CYP3A in deze organen beïnvloedt. Tenslotte zijn de genetische en mogelijk neurohumorale factoren, die de activering van CYP3A expressie en activiteit reguleren na de geboorte, eveneens grotendeels niet bekend.

In hoofdstuk 3 wordt de recente literatuur besproken met betrekking tot de ontwikkeling van de UDP-glucuronosyltransferase (UGT) superfamilie. Tenminste 10 verschillende UGT enzymen (isoformen) zijn tot nu toe geïdentificeerd, die zich ieder verschillend lijken te ontwikkelen. Ondanks studies naar de farmacokinetiek van UGT geneesmiddelen bij kinderen (lorazepam, morfine, paracetamol) zijn enzym specificiteit en de betekenis daarvan voor de farmacokinetiek, over het algemeen nog niet bekend. Geconcludeerd wordt dat de huidige kennis over het effect van ontwikkeling op de activiteit van de UGTs onvoldoende is en dat onderzoek in de toekomst zich moet richten op het effect van ontwikkeling van de invididuele UGTs op de farmacokinetiek van veel gebruikte UGT geneesmiddelen.

Farmacokinetiek

Hoofdstuk 4 beschrijft de farmacokinetiek en het metabolisme van het CYP3A modelmedicijn midazolam, toegediend als een intraveneuze bolus aan 24 premature neonaten die minder dan 12 dagen oud waren. Passend bij een nog niet volledig ontwikkelde hepatische CYP3A4/5 activiteit waren de klaring van midazolam en de concentraties van de belangrijkste metaboliet 1-OH-midazolam sterk afgenomen bij deze neonaten in vergelijking tot oudere kinderen en volwassenen. Het verdelingsvolume en de klaring van midazolam waren verhoogd in die neonaten die na de geboorte indomethacine kregen voor de behandeling van een open ductus botalli. Deze bevinding suggereert dat indomethacine een (nog onbekend) effect tot gevolg heeft, gerelateerd aan de open ductus Botalli of aan het geneesmiddel, dat in staat is de farmacokinetiek van midazolam bij premature pasgeborenen te veranderen.

De farmacokinetiek, absolute orale beschikbaarheid en metabolisme van midazolam na orale bolus aan 15 premature neonaten wordt beschreven in hoofdstuk 5. De resultaten laten zien dat de orale beschikbaarheid en de klaring van midazolam hoger zijn, terwijl de 1-OH-midazolam concentraties lager zijn dan bij oudere kinderen en volwassenen. Dit is in overeenstemming met de verminderde functionele activiteit van zowel intestinaal als hepatisch CYP3A4/5; bevindingen die passen bij prematuriteit. Mogelijk hebben deze resultaten belangrijke consequenties voor de orale beschikbaarheid van andere CYP3A4/5 geneesmiddelen. Een hogere orale beschikbaarheid kan namelijk leiden tot hogere plasma concentraties van deze geneesmiddelen. Hierdoor wordt de kans op toxiciteit hoger bij pasgeborenen, wanneer de dosis van het geneesmiddel niet wordt gecorrigeerd voor deze ontwikkelingsgerelateerde verschillen.

Hoofdstuk 6 beschrijft de populatiefarmacokinetiek en het metabolisme van midazolam toegediend als continu infuus aan 21 intensive care patiënten met een leeftijd van 2 dagen tot 17 jaar. De midazolam plasmaklaring bij deze patiënten blijkt lager te zijn dan eerder gerapporteerd voor pediatrische intensive care patiënten. Een mogelijke verklaring voor de lagere klaring is lagere CYP3A4/5 activiteit ten gevolge van de jongere leeftijd van de patiënten in onze studie populatie. 1-OH-midazolam-glucuronide, de glucuronide conjugaat van 1-OH-midazolam, kon bij alle patiënten gedurende de gehele onderzoeksduur worden aangetoond. Deze bevinding suggereert dat de tot nu toe niet gekarakteriseerde UGTs, die verantwoordelijk zijn voor de vorming van 1-OH-midazolam-glucuronide, bij pasgeborenen reeds aanwezig zijn. Deze studie laat tevens

het belang zien van aandoeningen zoals bv. lever- en nierfalen en behandeling zoals bv. gelijktijdige toediening van andere CYP3A4 geneesmiddelen op de farmacokinetiek van midazolam.

Farmacogenetica

Hoofdstuk 7 beschrijft een nieuwe detectiemethode voor een mutatie in het CYP3A4 gen (het CYP3A4*1B allel) en de frequentie van deze mutatie bij 199 Caucasische Nederlanders. Een eenvoudige en specifieke polymerase ketting reactie (PCR) met restrictie fragment lengte polymorfisme (RFLP) werd ontwikkeld. Allelfrequentie voor deze mutatie was 5.3%. Alle individuen met het allel waren heterozygoot, hetgeen leidt tot een heterozygote frequentie van 10.6%. Geconcludeerd wordt dat deze analysemethode studies naar het effect van dit polymorfisme op endogene processen, de gevoeligheid voor kanker en de individuele mogelijkheid om CYP3A4/5 geneesmiddelen te metaboliseren, kan ondersteunen.

Een nieuwe analysemethode (PCR-RFLP) voor de detectie van een andere CYP3A4 gen mutatie (het CYP3A4*3 allel) wordt beschreven in hoofdstuk 8. De frequentie van deze mutatie werd bepaald bij 499 Caucasische Nederlanders en 66 pediatrische patiënten. Dertien van de 499 en één van de 66 pediatrische patiënten waren heterozygoot voor het allel, hetgeen een allelfrequentie van ongeveer 1% oplevert. Dit betekent dat het CYP3A4*3 allel een voorkomend genetische polymorfisme blijkt te zijn en niet slechts een zeldzaam allel zoals eerder gesuggereerd is. Indien dit allel geassocieerd is met veranderde enzymactiviteit kan dit leiden tot veranderde farmacokinetiek van CYP3A4 geneesmiddelen. Voorgesteld wordt om verder onderzoek naar het klinische effect van dit polymorfisme te verrichten.

Hoofdstuk 9 beschrijft een pilot studie naar de mogelijke associatie tussen het CYP3A4*1B allel en de farmacokinetiek van midazolam bij premature neonaten werd onderzocht. Deze studie laat zien dat het CYP3A4*1B allel niet geassocieerd is met verschillen in intraveneuze of orale klaring van midazolam bij premature neonaten tot 14 dagen oud. De geringe studie populatie in ogenschouw nemend, een beperking van deze studie, wordt geconcludeerd dat verder onderzoek nodig is om het mogelijke effect van het CYP3A4*1B allel en andere allel variaties van CYP3A4 en CYP3A5 op de farmacokinetiek van CYP3A4/5 geneesmiddelen bij kinderen op te helderen.

Farmacodynamiek

Hoofdstuk 10 beschrijft de farmacodynamiek van midazolam toegediend als een 30-minuten durend infuus of als orale bolus bij premature neonaten. Het effect (farmacodynamiek) werd gemeten met behulp van de COMFORT score; een sedatiescore specifiek ontwikkeld voor pediatrische intensive care patiënten. De resultaten suggereren dat midazolam effectief is bij de meerderheid van de patiënten. Dit blijkt uit de significante daling van de gemiddelde COMFORT score (=meer gesedeerd) binnen 30 minuten na intraveneuze en 60 minuten na orale toediening van midazolam. Dit gewenste farmacologische effect (midazolam werd toegediend om sedatie rondom een vervelende procedure te bewerkstelligen) werd geobserveerd zonder een klinisch belangrijke verandering van hemodynamische of andere veiligheidsparameters. Echter,

een belangrijk deel van de patiënten leek niet te reageren op midazolam. Gesuggereerd wordt dat dit gebrek aan respons op midazolam veroorzaakt kan worden door therapeutisch falen inherent aan immaturiteit van de geneesmiddel-receptor interactie en/of is geassocieerd met falen van de COMFORT score om sedatie adequaat weer te geven bij premature pasgeborenen. Dit laatste kan veroorzaakt worden doordat deze groep pasgeborenen soms zo ziek is dat ze niet kunnen reageren op vervelende, pijnlijke gebeurtenissen.

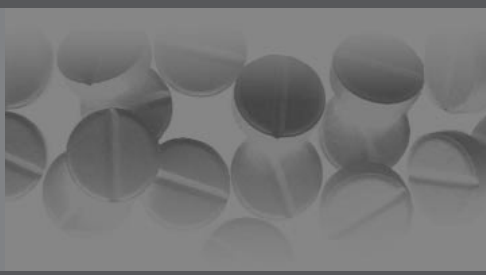
Hoofdstuk 11 beschrijft de farmacodynamiek van een continue midazolam infusie bij 21 intensive care patiënten. De data laten zien dat de dosering van midazolam succesvol getitreerd kan worden ten einde adequate sedatie te verkrijgen. Echter, de noodzaak tot verschillende niveaus van sedatie bij verschillende patiëntengroepen beperkt de mogelijkheid van een enkele COMFORT score range (bv. 17-26) die gebruikt kan worden als indicator voor adequate sedatie. Daarom wordt voorgesteld dat individuele COMFORT score ranges bepaald worden voor elke patiënt.

In hoofdstuk 12 worden de beperkingen van de studies besproken en worden voorstellen gedaan voor verder onderzoek.

Dit proefschrift illustreert dat leeftijd een belangrijke determinant is van de farmacokinetiek en de farmacodynamiek van midazolam bij premature neonaten en oudere kinderen. Daarnaast dragen fysiologische veranderingen (inherent aan ontwikkeling, zoals bv. een open ductus Botalli) en lever- en nierfalen bij aan de variabiliteit in farmacokinetiek en farmacodynamiek van midazolam.

De verkregen kennis over de ontwikkeling van de farmacokinetiek van midazolam kan worden geëxtrapoleerd naar andere geneesmiddelen die ook door CYP3A en dezelfde, nog onbekende, UGTs worden gemetaboliseerd. De afhankelijkheid van veel geneesmiddelen van metabolisme als belangrijkste eliminatie route benadrukt de noodzaak om specifieke gegevens te verkrijgen bij pediatrische patiënten. Leeftijdspecifieke dosering kan dan de kans op therapeutisch falen of toxiciteit bij kinderen sterk verminderen.

Dankwoord
Curriculum vitae
List of publications



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Curriculum vitae

Saskia N. de Wildt was born on September 12th, 1969 in Smallingerland (Friesland). She graduated from High School (VWO, Stedelijke Scholengemeenschap Leeuwarden) in 1988. In the same year she started medical school in Maastricht at the Rijksuniversiteit Limburg. As a clinical student she visited the Department of Pediatric Pulmonology of the University of Budapest in Hungary. From October 1992 till September 1993 she was a research student at the Reproductive Medicine Research Group of the University of Calgary in Canada (supervisor dr. F.G. Smith), working on the ontogeny of renal function in newborn lambs. After obtaining her medical degree in 1996, she worked as a resident at the department of Pediatric Surgery of the Wilhelmina Kinderziekenhuis in Utrecht (January-February 1996) and at the Department of Pediatrics of the Sint Franciscus Gasthuis in Rotterdam (March 1996-April 1997). From May 1997 until April 2001 she worked as a research fellow/Ph.D. student at the subdivision of Neonatology of the Sophia Children's Hospital in Rotterdam (promotor prof.dr. J.N. van den Anker), working on the research described in this thesis. As part of her Ph.D. training she worked for three months in the laboratory of the Department of Clinical Pharmacology and Experimental Toxicology of the Children's Mercy Hospital in Kansas City, US (supervisors prof.dr. G.L. Kearns and prof.dr. J.S. Leeder). Starting May 2001 she is working as a resident at the department of Pediatrics of the Sophia Children's Hospital in Rotterdam.

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