In-vivo phenotyping of CYP3A using midazolam as probe drug: Development of novel approaches based on highly sensitive LC-MS/MS methods

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Für meine Familie

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Abbreviations

APCI	Atmospheric pressure chemical ionisation
API	Atmospheric pressure ionisation
AUC	Area under the curve
CAR	Constitutive androstane receptor
cDNA	Complementary Deoxyribonucleic acid
CE	Collision energy
CI	Confidence interval
CL	Clearance
C_{ss}	Steady-state concentration
C_{max}	Maximum drug concentration
CID	Collision induced dissociation
CNLS	Constant neutral loss scanning
CNS	Central nervous system
CV	Coefficient of variation
CYP	Cytochrome P450
DAD	Diode array detector
DIS	Daughter ion scan
ECD	Electron capture detector
EEG	Electroencephalogram
E_{hep}	Hepatic extraction ratio
EIA	Enzyme immuno assay
ELISA	Enzyme linked immunosorbent assay
EMBT	Erythromycin breath test
ESI	Electrospray ionization
F	Bioavailibility
f_u	Unbound fraction
FAB	Fast atom bombardment
FDA	Food and Drug Administration
FPIA	Fluorescent polarisation immunoassay
GABA	γ - amino butyric acid
GC	Gas chromatography
HLM	Human liver microsomes
HPLC	High performance liquid chromatography
IS	Internal standard
iv	Intravenous
k_e	Elimination rate constant
LC	Liquid chromatography
LLE	Liquid-liquid extraction
LLOQ	Lower limit of quantification
LOQ	Limit of quantification

LOD	Limit of detection
MDZ	Midazolam
MRM	Multiple reaction monitoring
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
m/z	Mass to charge ratio
NCSE	Nonconvulsive status epilepticus
NPD	Nitrogen phosphorous detector
1´-OHMDZ	1´-hydroxymidazolam
4-OHMDZ	4-hydroxymidazolam
ORF	Open reading frame
PAH	Polycyclic aromatic hydrocarbon
PB	Particle beam
PCR	Polymerase chain reaction
P-gp	P-gylcoprotein
po	Per oral
PXR	Pregnane X receptor
Q	Liver blood flow
QC	Quality control
r	Correlation coefficient
r^2	Coefficient of determination
RIA	Radioimmunoassay
Rif	Rifampicin
R_t	Retention time
SD	Standard deviation
SIM	Selected ion monitoring
SNP	Single nucleotide polymorphism
SPE	Solid phase extraction
SPME	Solid phase microextraction
SRM	Selected reaction monitoring
t_{max}	Time point of c_{max}
$t_{½}$	Elimination half-live
TFA	Trifluoro acetic acid
TOF	Time of flight
UDP	Uridine diphosphate
UDPGA	Uridine diphosphate glucuronic acid
UGT	Uridine diphosphate glucuronosyl transferase
ULOQ	Upper limit of quantification
UV	Ultra violet
V_d	Volume of distribution
V_{ss}	V_d at Steady-state

Summary

Cytochrome P450 (CYP) 3A is the most important subfamily of drug-metabolizing enzymes in humans. It exhibits broad substrate specificity and is responsible for metabolizing more than 50% of the currently marketed drugs, as well as endogenous substances and environmental chemicals. A huge number of studies on CYP3A-mediated drug metabolism in humans have demonstrated that the CYP3A activity exhibits marked interindividual variability, rendering the dosing and the therapeutic use of many CYP3A substrates difficult, especially those with narrow therapeutic ranges. Furthermore, a number of clinical drug-drug interactions have been ascribed to the induction and/or inhibition of CYP3A.
Assessing the functional CYP3A activity (phenotyping) might therefore be of clinical relevance to predict enzyme inhibition or induction and, as a consequence, to predict unwanted therapeutic outcomes such as lack of therapeutic efficacy or potentially harmful response due to sub-therapeutic or (severe) toxic drug levels.

Several methods for CYP3A phenotyping have been published in the past, showing that Midazolam (MDZ) is the most suitable probe drug available at present. However, these methods were using either therapeutic doses of MDZ, which may cause adverse reactions, or, if employing lower doses, were not able to quantify the two hydroxy-metabolites. In order to avoid these problems, the aim of the first project **(Chapter 3)** was to develop a sensitive and specific liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the quantitative determination of MDZ and two of its metabolites, 1´-hydroxymidazolam (1´-OHMDZ) and 4-hydroxymidazolam (4-OHMDZ), in human plasma using liquid-liquid extraction and a stable isotope derivative as internal standard. The validation data showed excellent results for all the parameters studied. The analytical method has been successfully applied to a pharmacokinetic study, showing that MDZ and, to our knowledge for the first time, both hydroxy-metabolites can be determined precisely in samples obtained *in vivo* following the administration of a single sub-therapeutic oral MDZ dose (2 mg). The method appears therefore to be useful for low-dose CYP3A phenotyping in plasma, resulting in minimized adverse reactions, but larger studies are needed to proof this assumption.

So far, CYP3A phenotyping with MDZ is based on the determination of the MDZ clearance, necessitating serial blood samples. Although the more recently proposed single point methods in plasma are less invasive, the use of oral fluid as a non-invasive matrix may be advantageous for assessing the CYP3A activity. Since MDZ is extensively bound to plasma

proteins (96%), concentrations of free drug are expected to be very low in oral fluid and therefore extremely sensitive analytical methods would be required. **Chapter 4** presents the development and validation of an LC-MS/MS method which allows for the first time the simultaneous determination of MDZ and two of its metabolites in human oral fluid. In a single subject pharmacokinetic study it has been shown that the assay is suitable for analyzing oral fluid samples after the administration of a therapeutic MDZ dose. MDZ oral fluid concentrations were roughly two orders of magnitude lower as compared to plasma. However, it could be shown that the kinetics of plasma and oral fluid are comparable with each other, resulting in a satisfactory correlation for MDZ (R^2=0.972). Therefore, the method seems to be useful for assessing the CYP3A activity in oral fluid.

To definitely confirm the results obtained in chapter 4, a randomized, two way cross-over study was conducted **(Chapter 5)** using the previously described analytical methods. Our main goal was to compare midazolam kinetics between plasma and saliva and to find out whether saliva is suitable for CYP3A phenotyping. Therefore, eight healthy subjects were treated with 2 mg midazolam intravenously (iv) or 7.5 mg orally (po) under basal conditions and after CYP3A induction with rifampicin. Our investigation demonstrated that under basal conditions and iv administration, midazolam and 1′-hydroxymidazolam (plasma, saliva), 4-hydroxymidazolam and 1′-hydroxymidazolam-glucuronide (plasma) were detectable. The correlation between the midazolam concentrations obtained in plasma and saliva, both under basal conditions and after induction, was significant. After treatment with rifampicin, the AUC of midazolam had decreased significantly. After po administration and basal conditions, midazolam, 1′-hydroxymidazolam and 4-hydroxymidazolam were detectable in plasma and saliva. Similar to iv administration, there was a significant linear correlation between MDZ plasma and saliva concentration, indicating that the kinetics of MDZ can be reliably assessed also in saliva. After treatment with rifampicin, the AUC of midazolam and 1′-hydroxymidazolam had decreased significantly. The results of the study also showed that after treatment with rifampicin, 1´-OHMDZ undergoes extensive glucuronidation, resulting in a decrease of 1´-OHMDZ AUC, instead of the expected increase. This is most likely due to an induction of UDP-glucuronosyltransferases by rifampicin. In conclusion, we provided evidence that MDZ and 1´-OHMDZ can be determined reliably in saliva, that the concentrations in saliva correlate well with those in plasma and that MDZ kinetics in saliva may be a useful tool to differentiate between constitutive and induced CYP3A activity. Therefore, saliva appears to be a suitable matrix for non-invasive CYP3A phenotyping using midazolam as a probe drug.

Chapter 6 describes a case report were we present a patient with refractory focal nonconvulsive status epilepticus who was treated with very high doses (up to 4 mg/min) of intravenous midazolam, phenytoin, carbamazepine, and other antiepileptics. From the literature it was known that the half-life of midazolam can increase at high dosage. Therefore, the kinetics of midazolam (MDZ), 1´-hydroxymidazolam, and 4-hydroxymidazolam were assessed at steady state (dosage 1 mg/min) and after stopping the treatment. The results showed that the total body clearance of MDZ and intrinsic hepatic clearance at steady state were both 5 to 10 times higher than after normal therapeutic doses, demonstrating hepatic CYP3A induction. Despite the high body clearance, we observed a substantially prolonged terminal half-life of midazolam, mainly due to an extraordinarily large volume of distribution caused by saturation of protein binding. The free fraction of MDZ at steady state was much higher as compared to normal therapeutic doses.

1 Introduction

1.1 General introduction: Drug biotransformation

During lifetime, most of us are exposed to a wide variety of drugs (and xenobiotics). Most of these pharmacologically active substances are lipophilic and require an enzymatic biotransformation into more polar derivatives before elimination can take place. The major organ for drug metabolism is the liver. Other sites include epithelial cells of the gastrointestinal tract, kidneys, lungs, and the skin.

Drug biotransformation reactions can be classified into two phases, phase I and phase II. In phase I, polar functional groups (e.g. hydroxyl groups) are either introduced or unmasked, which results in (more) polar metabolites of the original drug. Typical phase I reactions are oxidation, reduction or hydrolysis. If the metabolites of phase I reactions are sufficiently polar, they may be readily excreted at this point. Phase II involves the conjugation of the parent drug or its more polar phase I metabolite(s) with an endogenous substrate (such as glucuronic acid, sulphate, an amino acid, acetate or glutathione) rendering it more water soluble to assist excretion. Figure 1 shows the different phase I or II enzymes and their relative contribution on total drug metabolism.

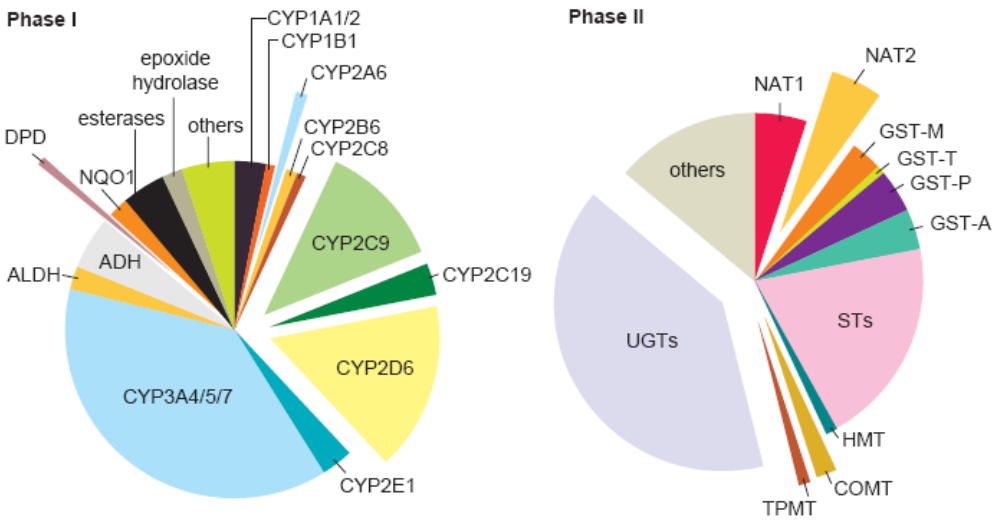

Figure 1. Drug metabolising enzymes in human: The relative contribution on total drug metabolism is estimated by the relative size of each section (Evans and Relling, 1999).

Phase I enzymes (modification of function groups, left): ADH: alcohol dehydrogenase; ALDH: aldehyde dehydrogenase; CYP: cytochrome P450; DPD: dihydropyrimidine dehydrogenase; NQO1: NADPH quinone oxidoreductase or DT diaphorase.

Phase II enzymes (conjugation with endogenous substituents, right): COMT: catechol *O*-methyltransferase; GST: glutathione *S*-transferase; HMT: histamine methyltransferase; NAT: *N*-acetyltransferase; STs: sulfotransferases; TPMT: thiopurine methyltransferase; UGTs: uridine-diphosphate-glucuronosyltransferases.

As mentioned introductorily, drug biotransformation leads in general to more polar, water-soluble metabolites that can be well excreted, resulting in the detoxification of a drug. In some cases however, formation of carcinogenic or toxic metabolites through biotransformation can also occur (toxification). Additionally, metabolism can convert inactive or less active xenobiotics (so-called prodrugs) into pharmacologically (more) active metabolites (e.g. conversion of levodopa to the active dopamine).
Orally administered drugs are mainly absorbed by the digestive system and enter the hepatic circulation through the portal vein. Some drugs (e.g. propranolol) are so extensively metabolised by the liver that only a small amount of unchanged drug may enter the systemic circulation, which results in a reduced bioavailability of the drug (so-called "first-pass effect") (Langguth et al., 2004; Mutschler, 1996).

1.2 Cytochrome P450 enzymes

The cytochrome P450 (CYP) superfamiliy is the major enzyme family responsible for the oxidative (phase I) biotransformation of a variety of xenobiotics including drugs and carcinogens (Omura and Sato, 1964; Wrighton et al., 1996). Beside involvement in drug metabolism, CYPs also play an important role in the biotransformation of endogenous substrates, such as cholesterol biosythesis and metabolism, bile-acid biosythesis, steroid synthesis and metabolism vitamin D3 synthesis and metabolism, and retinoic hydroxylytion (Nebert and Russell, 2002).
CYPs are heme-containing proteins that have been characterised in many species including bacteria, plants, fungi and mammals (Nebert and McKinnon, 1994). In mammalian cells, CYPs are predominantly localised along the membrane of the smooth endoplasmatic reticulum of the liver; but they are also expressed in extrahepatic tissues such as intestine, lungs, kidney, brain and placenta. The monooxygenases mediate oxidation reactions, where an incorporation of one oxygen atom from O_2 into the target molecule takes place, while the remaining oxygen atom is reduced to water:

$$R\text{-}H + \mathbf{O_2} + NAD(P)H + H^+ \rightarrow R\text{-}\mathbf{O}H + H_2\mathbf{O} + NAD(P)^+$$

The CYP enzymes are conveniently arranged into families and subfamilies on the basis of percentage homology in the amino acid sequence. Enzymes that share at least 40% identity are assigned to a particular family, denoted by an arabic numeral, whereas those sharing >55% homologies are grouped in the same subfamily, designated by a letter (Nebert et al., 1987). Subfamilies are often further divided into isoenzymes (arabic numeral), many of

which have specific drug substrates. To date, at least 18 CYP gene families have been identified in mammals (Table 1).

Table 1. Substrates and functions of human CYP gene families (Nebert and Russell, 2002)

Family	Number of subfamilies	Number of genes	Substrates and functions
CYP1	2	3	Foreign chemicals, arachidonic acid, eicosanoids
CYP2	13	16	Foreign chemicals, arachidonic acid, eicosanoids
CYP3	1	4	Foreign chemicals, arachidonic acid, eicosanoids
CYP4	5	12	Fatty acids, arachidonic acid, eicosanoids
CYP5	1	1	Thromboxane A_2 synthase
CYP7	2	2	Cholesterol, bile acid synthesis
CYP8	2	2	Prostacyclin synthase, bile-acid synthesis
CYP11	2	3	Steroidogenesis
CYP17	1	1	Steroid 17α-hydroxylase, 17/20-lyase
CYP19	1	1	Aromatase to form oestrogen
CYP20	1	1	Unknown
CYP21	1	1	Steroid 21-hydroxylase
CYP24	1	1	Vitamin D_3 24-hydroxylase
CYP26	3	3	Retinoic acid hydroxylation
CYP27	3	3	Bile-acid biosynthesis, vitamin D_3 hydroxylations
CYP39	1	1	24-hydroxycholesterol 7α-hydroxylase
CYP46	1	1	Cholesterol 24-hydroxylase
CYP51	1	1	Lanosterol 14α-desmethylase

Three main CYP gene families, CYP1, CYP2, and CYP3, are responsible for the biotransformation of many drugs and xenobiotics in humans (Nelson et al., 1996).
More than 90% of drug oxidation can be attributed to six main cytochromes: CYP1A2, 2C9, 2C19, 2D6, 2E1 and 3A4 (Table 2).

Table 2. Major human cytochrome P450 (CYP) enzymes and drug substrates

CYP	Drug substrate
1A2	Caffeine, clozapine, phenacetin, theophylline
2C9	Diclofenac, ibuprofen, losartan, phenytoin, S-warfarin
2C19	Diazepam, omeprazole, propranolol, progesterone, R-warfarin
2D6	Codeine, dextrometophan, fluoxetine, flecainide, imipramine, propaphenone, propranolol, sparteine
2E1	Ethanol, halotan
3A4(/5)	Atorvastatin, erythromycin, dapson, diazepam, felodipine, midazolam, nifedipine, ritonavir, tacrolimus, testosterone, verapamil

However, these CYP enzymes are often showing an overlapping substrate specificity, so that one specific substrate may be metabolised by more than one enzyme (Nelson et al., 1996).
Among the different cytochromes, CYP3A is the most important drug-metabolising subfamily in humans.

1.3 CYP3A subfamily

Four members of the CYP3A subfamily, CYP3A4, CYP3A5, CYP3A7 and CYP3A43, have been identified so far. Their genes are arranged in tandem on chromosome 7q21 (Gellner et al., 2001; Krishna and Shekar, 2005). Members of this subfamily are involved in many clinically important drug-drug interactions (Slaughter and Edwards, 1995). The wide substrate spectrum of CYP3A is the reason behind.

CYP3A4 is the major phase I metabolising enzyme in humans. It exhibits broad substrate specificity and is responsible for metabolising more than 50% of the currently marketed drugs, as well as steroids and environmental chemicals. CYP3A4 also activates procarcinogens such as aflatoxin B1 and polycyclic aromatic hydrocarbons (Guengerich, 1999). CYP3A4 is the major form present in the liver and the small intestine, accounting for 30% and 70% of the total amount of CYP enzymes, respectively (Shimada et al., 1994; Thummel and Wilkinson, 1998). The location of CYP3A4 in the intestine and liver makes it well suited to play a significant role in first-pass drug metabolism. CYP3A protein amount and catalytic activity decreases longitudinal along the small intestine (Doherty and Charman, 2002). In the kidney, CYP3A4 is present in only about 30% of renal tissue samples. Typical substrates of this enzyme are shown in Table 2. Marked interindividual variations in CYP3A4 activity (up to 20-fold) are seen in the human population (Shimada et al., 1994; Wilkinson, 1996) which does not appear to be caused by genetic polymorphism (see 1.3.1.1).

CYP3A5 was isolated from a liver cDNA library and was first termed PCN3, sharing 85% sequence similarity with CYP3A4 (Aoyama et al., 1989). It is a polymorphic expressed form, which is present in the liver of about 20% of Caucasians and in more than 50% of African-American subjects (Daly, 2006; Kuehl et al., 2001). In individuals who express CYP3A5 in the liver, it represents up to 50% of the total hepatic CYP3A content. CYP3A5 is the most common CYP3A isoform in the human kidney and is also distributed in other organs and

tissues such as lung, prostate, colon or the intestine (Daly, 2006; Thummel and Wilkinson, 1998).

CYP3A7 was originally found in the fetal liver and accounts for between 30% and 50% of the total CYP content in this tissue (Shimada et al., 1996). It has been shown that CYP3A7 is expressed in placental and endrometrial microsomes that increases dramatically from the first to the second trimester of pregnancy (Schuetz et al., 1993). The level of expression of CYP3A7 varies with gestational age and is higher in the 20-week fetus than compared to the fetus at 40 weeks of age, and decreases dramatically after birth (Kitada and Kamataki, 1994; Kitada et al., 1987). It is now apparent that CYP3A7 is also expressed in adult livers (Koch et al., 2002). Other tissues expressing CYP3A7 in adults include intestine, endometrium, adrenal gland and prostate (Burk et al., 2002; Koch et al., 2002; Schuetz et al., 1993).

CYP3A43 is the most recently discovered member of the CYP3A family (Domanski et al., 2001; Gellner et al., 2001). Although the expression of CYP3A43 in fetal as well as in adult liver is low, there is significant extrahepatic expression, with relatively high mRNA levels being observed in the prostate and testis (Gellner et al., 2001; Westlind et al., 2001). CYP3A43 is expressed at 0.1% and 2% of the levels of CYP3A4 and CYP3A5 (Westlind et al., 2001).

1.3.1 Factors which may influence the CYP3A activity

Currently, most medications are dosed based upon the assumption that each individual in the general population metabolises drugs at approximately the same rate. However, it has been demonstrated that the activities of CYP enzymes show marked interindividual variations (up to the 100 fold for CYP3A activity) (Lin and Lu, 2001), which may lead to unwanted therapeutic outcomes such as ineffectiveness or potentially harmful response due to sub-therapeutic or toxic drug levels.
Many different factors may influence the hepatic and intestinal CYP3A activity which makes the dosing and therapeutic use of many CYP3A substrates difficult, especially those with a narrow therapeutic range.

1.3.1.1 Genetic variability

A genetic polymorphism, which is a monogenetic trait, expressed in at least two distinct phenotypes, each in a frequency >1% within a population, may account for a considerable variability (20-95%) in drug metabolism. The functional consequence of genetic variants

ranges from no effect to altered expression of the encoded protein and can thus result in treatment failure or toxic effects.

Data from twin studies suggest that the interindividual differences in CYP3A activity are mainly (60-90%) determined by genetic factors rather than by environmental factors (Ozdemir et al., 2000). Several allelic variants have been characterised for CYP3A4 (*www.imm.ki.se/CYPalleles*), most of them are single nucleotide polymorphisms (SNP) (Lamba et al., 2002). But these variants predict only to a very limited extent the observed wide interindividual variability of CYP3A4 activity (Eiselt et al., 2001). In general, with the exception of the CYP3A4*1B polymorphism (associated with progression of prostate cancer; frequency of 3.6%-9.6% of whites and 53%-67% of Africans), the SNPs described so far are too rare (<1% allele frequency) to be of clinical relevance (Krishna and Shekar, 2005; Mathijssen and van Schaik, 2006; Tang et al., 2005; Wojnowski, 2004; Wojnowski and Kamdem, 2006).

Several allelic variants have also been described for CYP3A5. The large interpopulation variation in CYP3A5 expression is largely due to a common SNP in the intron 3 of CYP3A5 resulting in a non-functional protein. The non functional allele CYP3A5*3 frequency varies from 27% to 50% in blacks and 85% to 95% in whites. The CYP3A5*6 and CYP3A5*7 allel also translate into non-functional CYP3A5 protein and are found only in African populations and African-American (Jin et al., 2007). Only people with at least one CYP3A5*1 allele (wild-type) express large amounts of CYP3A5 (Daly, 2006; Kuehl et al., 2001). Kuehl *et al.* also showed that CYP3A5 represents up to 50% of total hepatic CYP3A content in some individuals. CYP3A5 might thus be an important contributor to interindividual and interethnic differences in CYP3A dependent drug metabolism. However, studies that have related CYP3A5 genotype to pharmacokinetics of midazolam (MDZ), a substrate of both CYP3A4 and CYP3A5, show discrepancies in the outcome. Two studies suggest that CYP3A5*1 heterocygotes show faster MDZ clearance than CYP3A5*3 homozygotes, while results of other studies indicate that the genetic variants identified so far in CYP3A4 and CYP3A5 genes have only a limited influence on *in-viv*o MDZ clearance (Eap et al., 2004c; Floyd et al., 2003; He et al., 2005; Shih and Huang, 2002; Wong et al., 2004). Further studies are necessary to prove the impact of genetic variants on CYP3A mediated drug metabolism *in-vivo.* A few studies have suggested an association between CYP3A5 genotype and blood pressure and/or risk of hypertension; but the data were inconsistent. In a recent study, Langaee *et al.* has shown that CYP3A5 polymorphism does not appear to contribute importantly to blood pressure or risk of hypertension, but may influence response to calcium channel blockers in some populations (Langaee et al., 2007). Jin *et al.* has demonstrated that CYP3A5 plays an important role in the metabolism of verapamil *in vivo*, which has been

used clinically for the treatment of hypertention. CYP3A5 genotype is associated with variations in verapamil disposition and response in healthy volunteers (Jin et al., 2007).

1.3.1.2 Concurrent exposure to drugs or other environmental chemicals

A drug interaction occurs when the disposition of one drug is altered by another drug, food, drink or environmental chemical agent. This can lead to changes in drug metabolism which may affect pharmacokinetics of the drug and its clinical effects.

Induction and inhibition of metabolising enzymes are the two main mechanisms for interactions between drugs. Enzyme induction usually may lead to decreased plasma drug concentration and reduced pharmacological effect due to an increase in drug metabolism. In some cases, the metabolites formed during biotransformation may be chemically reactive, so that enzyme induction also may result in increased toxicity caused by the increased production of toxic metabolites. Enzyme inhibition may lead to an enhancement in systemic drug exposure and therefore potentially to toxic effects. Inhibition, the most important mechanism for drug interactions, is especially of great importance when patients receive drugs with narrow therapeutic ranges.

CYP3A activity can be inhibited and induced by a wide variety of drug substances. The clinically most relevant of these are listed in Table 3 (Shapiro and Shear, 2002; Sikka et al., 2005).

Several mechanisms of **inhibition** are possible. Azole antifungals, such as ketoconazole, act via competitive inhibition (most common inhibition mechanism) by rapid, reversible binding of the inhibitor to the active site of CYP3A. This type of inhibition is transient and normal activity returns once the inhibitor has been cleared. A well-known example is the interaction observed in some patients on co-administration of the antihistamine terfenadine and an inhibitor of CYP3A such as ketoconazole. Inhibition of this enzyme markedly elevates the terfenadine levels, leading to the inhibition of cardiac potassium levels and, in consequence, to a sometimes fatal cardiac arrhythmia (Wilkinson, 1996). Irreversible, mechanism-based inhibition occurs if a substrate binds to CYP3A and alters its structure (e.g. ethinylestradiol) (Thummel and Wilkinson, 1998).

Table 3. Inhibitors and inducers of CYP3A

Inhibitors	Inducers
Antibiotics	**Anticonvulsants**
Erythromycin	Phenobarbital
Clarithromycin	Carbamazepine
Norfloxacin	Phenytoin
Metronidazole	Ethosuximide
Quinupristin/dalfopristin (Synercid)	Primidone
Troleandomycin	**Antituberculous agents**
	Rifampin
	Isoniazid
Azole antifungals	
Fluconazole (> 200 mg/day)	**Miscellaneous**
Itraconazole	Dexamethasone
Ketoconazole	Griseofulvin
	St John's wort
HIV-1 protease inhibitors	
Ritonavir	
Saquinavir	
Indinavir	
Nelfinavir	
Calcium channel blockers	
Diltiazem	
Nifedipine	
Verapamil	
SSRIs	
Fluoxetine	
Paroxetine	
Fluvoxamine	
Sertraline	
Others	
Antiprogestins	
Amiodarone	
Cannabinoids	
Cimetidine	
Grapefruit juice	
Imatinib	
Interferon-γ	
Quinine	
Tacrolimus	

CYP3A **induction** is generally considered clinically less important than its inhibition because it is expected to reduce the plasma concentration of co-administerd CYP3A substrate drugs rather than to impair their safety (Burk and Wojnowski, 2004). However, the loss of efficacy also has a divesting effect on the treatment of life-threatening diseases, such as cancer, organtransplatation, and so on. CYP3A undergoes induction in the liver and also in the small intestine (Kolars et al., 1992; Schuetz et al., 1984). Enzyme induction refers to an increase in enzyme activity, which results usually from increased enzyme production through enhanced translation and transcription. One of the most effective inducers of CYP3A expression, both *in-vitro* and *in-vivo*, is the macrocyclic antibiotic rifampicin. In contrast to the immediate response of CYP inhibition, induction is a gradual process over time (Goodwin et al., 2002; Thummel and Wilkinson, 1998). The inducibility of

CYP3A expression, coupled with the remarkable versatility of CYP3A catalytic activities, creates the potential for drug-drug interactions.

On the transcription regulation side, the pregnane X receptor (PXR), a member of the nuclear receptor superfamily, has been identified as a major regulator of CYP3A expression and the mediator of CYP3A induction by xenobiotics. PXR is highly expressed in the liver and intestine. The mechanism proposed for rifampicin (ligand of PXR) induced CYP3A induction is shown and explained in Figure 2 (Chen and Raymond, 2006; Goodwin et al., 2002). CYP3A turned out to be inducible also by the constitutive androstane receptor (CAR) (Wojnowski, 2004). (Note: Rifampicin also acts as an inducer of P-glycoprotein (P-gp) and UDP-glucuronosyltransfease 1A through PXR, (Chen and Raymond, 2006))

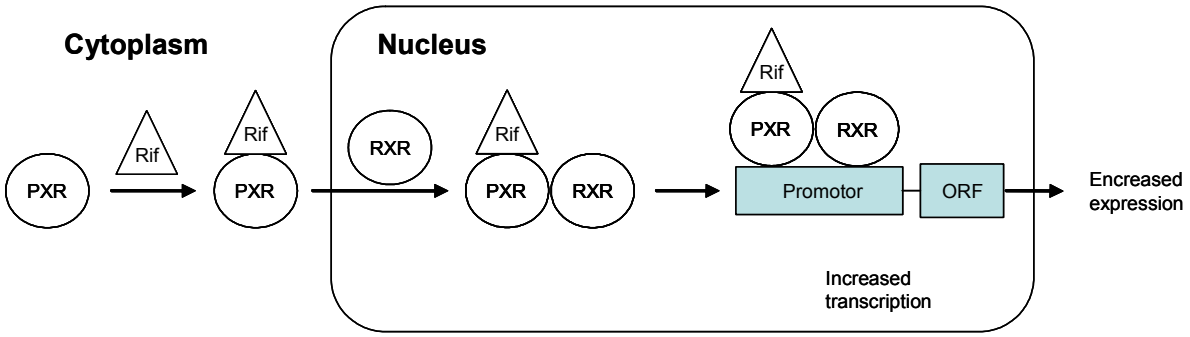

Figure 2. Transcriptional activation of rifampicin target genes.

Rifampicin binds with PXR in the cytoplasm (activation), which induces a conformational change within the receptor, and enters into the nucleus to form a heterodimer with the retinoic acid receptor RXR (co-activator protein). The heterodimer binds to the promoter of the target gene to activate the transcription of its open reading frame (ORF).

Although the majority of CYP3A4 mediated interactions occur within the liver, the enzymes in the intestine may also play a significant role in this process. For instance, grapefruit juice has been shown to be an important dietary component inhibiting enteric CYP3A4. Kupferschmidt *et al.* has shown that the bioavailability of MDZ increased by 46% after ingestion of grapefruit juice (Kupferschmidt et al., 1995).

Finally, CYP3A4 is the most important CYP3A member with regards to involvement in clinically significant drug interaction. CYP3A5 mediated drug metabolism is unlikely to be of relevance in individuals exposed to inducers (Daly, 2006).

1.3.1.3 Expression levels of nuclear receptors

In human liver, high correlations have been descibed between mRNA levels of PXR and CYP3A4 (r^2 = 0.68), between CAR and CYP3A4 (r^2 = 0.89) and between CAR and PXR (r^2 = 0.71 to 0.86) (Chang et al., 2003; Pascussi et al., 2001). These observations suggest that the expression levels of CAR and PXR might be a major determinant in the individual CYP3A4 expression. PXR polymorphisms may play a role in the observed interindividual CYP3A variability (Hustert et al., 2001).

1.3.1.4 Others

Inflammatory bowel **disease** and cirrhotic liver disease are known to decrease the content and activity of CYP3A in these tissues (Iqbal et al., 1990; McConn et al., 2009; Morgan, 2009; Paintaud et al., 1996). Cotreau *et al.* have reviewed studies on **age** dependent pharmacokinetics of CYP3A substrates and found a decreased clearance of CYP3A substrates in elderly people (Cotreau et al., 2005). Furthermore, aging affects many physiological factors that influence drug disposition, such as liver mass, liver blood flow and plasma protein binding (Kinirons and O'Mahony, 2004). The majority of in vivo-studies investigationg **sex**-based differences indicate that woman exhibits faster clearance for drugs that are CYP3A (and P-glycoprotein) substrates than men. Noumerous compounds have been utilized for that purpose (for review (Scandlyn et al., 2008)) (Chen et al., 2006; Gandhi et al., 2004). However results have been conflicting. Drugs that are only CYP3A, but not P-gp, substrates, did not exhibit gender dependent differences in pharmacokinetics. Overall, those findings suggested a sex-based differences may actually caused by P-gp (Cummins et al., 2002). Gorski *et al.* has reported a sex-related difference in the extent of intestinal and hepatic induction by rifampicin. Women had a significantly higher clearance than men, whereas men had a significantly greater induction of oral clearance than women (Gorski et al., 2003). During **pregnancy** hepatic and/or intestinal CYP3A and P-gp activities are increased (Hebert et al., 2008).

1.4 Phenotyping

Pronounced interindividual differences in drug disposition are mainly caused by differences in the activity of drug metabolising enzymes in the liver and the small intestine. As mentioned before, the variable activities have been ascribed to genetic variations, environmental factors and physiological factors such as inflammation or hepatic diseases. Patients with unusually high or low activity of metabolising enzymes might be of increased

risk for subtherapeutic or toxic response to many commonly used medications. Therefore, characterising of drug metabolising enzymes in an individual patient is important to maximise clinical drug safety and to minimise the occurrence of adverse drug reactions (Bachmann, 1996).

Using modern techniques, such as polymerase chain reaction (PCR), single base primer extension or gene sequencing, genotyping allows the characterisation of many significant CYP genes and the identification of SNPs (Bachmann, 2002; Tribut et al., 2002). However, genotyping alone does not succeed to predict the variable CYP expression enough. The optimal method to measure the real-time activities (functional expression) of drug metabolising enzymes is phenotyping, where the metabolism of a specific probe-drug (which is ideally a simple, fast, cheap, easily to administer and detectable compound and which is metabolised by the enzyme of interest) is used to estimate the activity of the enzyme involved in its metabolism. The individual ability to metabolise this probe-drug is measured by determining the circulating or excreted concentrations of the drug or its metabolites. Although it is often more time-consuming compared to genotyping, phenotyping provides the most clinically relevant information because it reflects the combined effects of genetic, environmental and endogenous factors on the activity of CYPs. Criteria for the validation of probe-based phenotyping are shown in Table 4 (Bachmann, 2002; Watkins, 1994; Zaigler et al., 2000).

Table 4. Criteria used for the validation of probe-based methods for phenotyping CYP activity

• Correlation with the activity of the target enzyme determined in human liver biopsies
• Correlation with the content of the target enzyme determined in human liver biopsies
• Correlation with the fractional clearance of the probe drug mediated by the target enzyme
• Correlation with other validated phenotyping procedures for the enzyme
• Reduction in enzyme activity in subjects treated with known inhibitors of the target enzyme
• Increase in enzyme activity in subjects treated with known inductors of the target enzyme
• Reduction in the *in-vivo* measure of enzyme activity in patients with severe liver disease
• Marked reduction during the anhepatic phase of liver transplant phase
• Proven *in-vitro* specificity of the metabolic step used
• Reproducibility
• Results reflect genetic polymorphism (if present)
• Lack of dependence on confounding factors (e.g. urinary flow, creatinine clearance, activity of other enzymes)
• Adherence of analytical assays for the substances to general validation criteria
• Low invasiveness (drug well tolerable, no blood sampling, no radioactivity)
• Technical ease of the procedure
• Inexpensive procedure

Using the probe-drug approach, the activity of the specific enzyme of interest *in-vivo* is best estimated by determining the clearance of the probe. If the probe has multiple metabolic pathways involving several enzymes, the partial clearance corresponding to the pathway specifically catalysed by the enzyme phenotyped should be an appropriate measure. However, to determine the clearance precisely, multiple blood samples are required. In order to minimise sampling, metabolic ratios based on single-point measurements of the parent drug and its respective metabolites at an appropriate time is an alternative approach. If the concentrations of parent drug and metabolite measured in oral fluid or urine are correlated with those in plasma, the use of these non-invasive matrices could be advantageous (Zaigler et al., 2000).

1.4.1 Phenotyping of CYP3A

As CYP3A is the most abundant CYP enzyme in humans and is known to be responsible for metabolising more than 50% of the currently marketed drugs, CYP3A phenotyping has been the subject of numerous investigations; especially because CYP3A4 is the most important CYP3A member with regards to involvement in clinically significant drug interactions.

The CYP3A probe is defined as any drug whose metabolism is principally or uniquely catalysized by CYP3A. Thus, the amount of drug's metabolite(s) generated through CYP3A-mediated metabolic pathway(s) can be used to reflect the level of the enzyme activity (Liu et al., 2007). Several CYP3A probes have been proposed so far, including midazolam, triazolam, testosterone, ^{14}C-erythromycin, cortisol, dapsone, alfentanil, dextromethorphan, nifedipine and lidocaine (Liu et al., 2007; Streetman et al., 2000). The advantages and disadvantages of some probe drugs are summarised in brief in Table 5 (Streetman et al., 2000). Unfortunately, none of these test probes meet all the criteria listed in Table 4 and, in addition, the correlation between the test procedures is not satisfactory (see 1.4.2)

The erythromycin breath test and the MDZ plasma clearance seems to be the best methods for phenotyping CYP3A at present. These two phenotyping procedures will be described in more detail in the next two chapters; an excellent review of other proposed CYP3A probes is given by Streetman *et al.* or Watkins (Streetman et al., 2000; Watkins, 1994).

Table 5. Summary of advantages and disadvantages associated with various proposed CYP3A probes

Probe	*Reaction(s)*	*Advantages*	*Disadvantages*
Midazolam	Midazolam 1′- and 4-hydroxylation	Considerable in-vitro and in-vivo evidence supporting its use. Not a *p*-glycoprotein substrate	Sedative effects and FDA Schedule III controlled drug status. Clearance may be related to hepatic blood flow at high levels of CYP3A activity. Multiple blood samples required to determine clearance
^{14}C-Erythromycin	Erythromycin *N*-demethylation	Considerable in-vivo evidence supports its use. Requires only single breath sample. Immediate results. Commercially available	Requires IV administration. Results may vary with volume of distribution and protein binding. Possibly lower sensitivity with high CYP3A activity. *P*-glycoprotein substrate
Cortisol	Cortisol 6β-hydroxylation	Endogenous substrate	Data suggest only use is to detect induction. Possible extrahepatic metabolism
Dapsone	Dapsone *N*-hydroxylation	Orally administered. May also be used to measure NAT2 activity	Poor correlations with other probes. Metabolism may be CYP2E1-mediated. Possible extrahepatic metabolism
Dextromethorphan	DM and dextrorphan *N*-demethylation	Orally administered and widely available. Also used to measure CYP2D6 activity	Poor correlations with other probes. Possible role of CYP2E1 in metabolism
Lidocaine	Lidocaine *N*-deethylation		Clearance is largely dependent on hepatic blood flow. Limited in-vivo evidence
Alfentanil	Piperidine *N*-dealkylation	Clearance relatively independent of hepatic blood flow	Few in-vivo data. IV administration. FDA Schedule II controlled drug status
Nifedipine	Nifedipine dehydrogenation	Orally administered	Limited in-vivo evidence

1.4.1.1 [^{14}C N-methyl] erythromycin breath test (EMBT)

The EMBT has been developed by Watkins (Watkins et al., 1989). Subjects were given a small dose (< 0.1μmol) of [^{14}C] N-methyl erythromycin intravenously which undergoes N-demethylation, mediated exclusively by CYP3A4 (not CYP3A5). Because the carbon atom in the cleaved methyl group largely appears in breath as $^{14}CO_2$, the activity of CYP3A4 is reflected in the rate at which breath $^{14}CO_2$ is produced (Baker et al., 1983; Watkins et al., 1989; Wrighton et al., 1990). Currently, the EMBT uses a single 20 min breath sample for estimating the enzyme activity (Turgeon et al., 1994). This offers the advantage of an indirect but rapid measure of CYP3A4 activity.

The EMBT has been validated according to most of the criteria given in Table 4. The results of EMBT phenotyping show a significant correlation with the concentration of CYP3A4 protein from liver biopsy samples, but not with the hepatic content of other CYP proteins (Lown et al., 1992). The breath test has shown to predict steady-state concentrations and clearance of the CYP3A4 substrate cyclosporine A (Turgeon et al., 1994; Watkins et al., 1990). Administration of the known inhibitor troleandomycin reduced the EMBT results and CYP3A inducers such as rifampicin increased EMBT results (Watkins et al., 1989), reflecting increased CYP3A activity. It is assumed that the EMBT responds predictably to CYP3A4 induction or inhibition and drug-drug interaction in human (DeVane et al., 2004; Nicandro et al., 2007). The mean EMBT results in patients with severe liver diseases is far

below that observed in subjects with normal liver function (Lown et al., 1992). Changes in volume of distribution (V_d), hepatic extraction ratio as well as protein binding can affect the results of EMBT (Lane and Parashos, 1986; Watkins, 1994).

Beside the high expression of CYP3A in the liver, the small intestine is the second major site of CYP3A expression in humans, where the enzyme mediates the first-pass metabolism of orally administered drugs. The intravenous EMBT therefore reflects only hepatic CYP3A4 activity and is not able to fully predict the metabolic fate of oral substrates. The intravenous administration should avoid significant metabolism by the gut. Indeed, little CO_2 was produced during the anhepatic phase of the liver transplant operation (Watkins et al., 1992).

Paine *et al.* developed an oral stable-labeled (^{13}C) formulation of the test with regard to measure aggregate liver and intestinal CYP3A4 activity (Paine et al., 2002). However, the oral EMBT has shown to be not suitable as an *in-vivo* phenotyping probe. In addition, erythromycin is not a "pure" CYP3A probe as it has been shown to be a substrate of P-glycoprotein (Kim, 2002; Takano et al., 1998) a drug-efflux pump localised among others in the intestinal wall.

The major disadvantage of the test is that the EMBT uses radiolabeled ^{14}C. Although the required doses are low, the very long half-live of ^{14}C raises theoretical health concerns.

1.4.1.2 MDZ as phenotyping probe

MDZ is recognised as one of the preferred *in-vivo* probes by the Food and Drug Administration (FDA) and the Pharmaceutical Research and Manufacturers in America (Bjornsson et al., 2003).

MDZ as CYP3A phenotyping probe has been validated by a number of different criteria (Table 4). MDZ is chiefly oxidised by both, CYP3A4 and CYP3A5 (Patki et al., 2003), to mainly 1´-hydroxymidazolam (1´-OHMDZ). Minor metabolites are 4-hydroxy- (4-OHMDZ) and 1´,4-dihydroxymidazolam (see also 1.5.1). A study with human liver biopsies from transplant recipients has demonstrated that the total MDZ clearance *in-vivo* (after intravenous MDZ application) is highly correlated with the hepatic CYP3A content *in-vitro* and with the *in-vivo* clearance of a known CYP3A substrate, cyclosporine A (Thummel et al., 1994b). Kronbach *et al.* have shown that the MDZ clearance correlates with the amount of CYP3A4 protein, recognised by mono- and polyclonal antibodies (Kronbach et al., 1989). In addition, validation by other criteria has been accomplished, including the finding of appropriate changes in MDZ clearance after pre-treatment with potent CYP3A inhibitors (Olkkola et al., 1993; Olkkola et al., 1994), inducers (Backman et al., 1996a; Backman et al.,

1996b) and in patients with severe liver disease (Pentikainen et al., 1989). Moreover, MDZ clearance can be influenced by hepatic blood flow (Rogers et al., 2003).

Since CYP3A is abundant in the intestine, orally administered MDZ is subject to both intestinal and hepatic CYP3A metabolism. In humans, it is therefore used to measure the total CYP3A activity. Studies during the anhepatic phase of a liver transplant operation (Paine et al., 1996) have shown that the small intestine contributes significantly to the first-pass metabolism of MDZ by mucosal CYP3A. Furthermore, in S-9 fraction prepared from small intestine biopsies, Lown *et al.* found that the rate of 1´-OHMDZ formation correlates significantly with CYP3A protein content (Lown et al., 1994).

In summary, MDZ possesses many of the characteristics of an ideal CYP3A probe in humans: it is exclusively metabolised by CYP3A4/3A5 to a primary metabolite, 1´-OHMDZ (Gorski et al., 1994); the short half-life allows to estimate the area under the concentration time-curve (AUC) and other easily measurable pharmacokinetic endpoints with sampling over a limited time period; its metabolism is sensitive to altered CYP3A activity (Gorski et al., 2003), and it is not a substrate of P-glycoprotein (Kim et al., 1999). In contrast to EMBT, which only measures the hepatic CYP3A4 activity, MDZ can be administered either intravenously to assess hepatic CYP3A activity (systemic clearance) or orally to assess combined intestinal and hepatic CYP3A activity (Thummel et al., 1996; Tsunoda et al., 1999). Furthermore, no radioisotopes are required, making it to a safer approach as compared to EMBT.

MDZ clearance has proven to be an effective marker for CYP3A activity; as such, it is widely used for predicting CYP3A-mediated drug-drug interactions (Streetman et al., 2000). However, there are some disadvantages in using MDZ as phenotyping probe including the production of sedative or amnesic effects in some subjects, the need of relatively complex analytical techniques and the requirement of multiple intravenous blood samples over a time frame of several hours, which can be inconvenient and cost-intensive. Several limited sampling strategies have been developed to simplify sampling and minimising costs and labor. A single time point measurement of MDZ plasma concentration at 4 h after intravenous or oral MDZ administration was suggested to represent an accurate marker of CYP3A phenotype under constitutive and modified states (Lin et al., 2001). Limited sampling models using MDZ plasma concentrations at one, two or three time points after intravenous administration using multiple linear regressions have shown to predict the AUC of MDZ (Kim et al., 2002). Several studies have assessed the usefulness of the ratio 1´-OHMDZ to MDZ plasma concentrations to predict the CYP3A activity, but results from these investigations have shown inconsistencies (Eap et al., 2004b; Penzak et al., 2008; Rogers et al., 2002; Thummel et al., 1994b; Zhu et al., 2001). In a recently published study (Lee et

al., 2006), the investigators could not find any time point of 1´-OHMDZ/MDZ plasma concentration ratio that would accurately predict the MDZ AUC, which is, beside the determination of MDZ clearance, a validated marker for CYP3A activity (Streetman et al., 2000). In addition, they have shown that limited sampling models with two (0.5 and 6 hours) and three (0.5, 2 and 6 hours) time points following oral administration can be used in healthy subjects to evaluate CYP3A baseline activity (Lee et al., 2006).

The use of urine samples to predict *in-vivo* CYP3A activity would be an attractive, non-invasive alternative to plasma sampling. Streetman *et al.* have investigated the utility of MDZ urinary metabolic ratios for assessing the CYP3A activity (Streetman et al., 2001). However, they found only a poor correlation to the total MDZ clearance, suggesting that these metabolic ratios are not an accurate measure of hepatic CYP3A activity. As MDZ is also metabolised by CYP3A5 (Patki et al., 2003), which is highly expressed in the human kidney, urine measurements can be problematic. Metabolites measured in this matrix might reflect not only hepatic but also in part renal CYP3A activity, which might therefore lead to confounding results.

So far, no data has been published on the correlation of MDZ measured in oral fluid with MDZ plasma clearance or other CYP3A phenotyping probe. In comparison, for assessing the CYP1A2 activity *in-vivo*, the determination of paraxanthine/caffeine ratio in saliva has been shown to be highly correlated to paraxanthine/caffeine ratio in plasma and to total caffeine clearance (Carrillo et al., 2000; Faber et al., 2005). If the MDZ concentrations in oral fluid would be also significantly correlated to MDZ concentrations in plasma, the quantification of MDZ (and metabolites) in oral fluid may offer the possibility to conduct simple, non-invasive and less expensive CYP3A phenotyping.

1.4.2 Problems and perspectives of CYP(3A) phenotyping

One might expect that characterisation of CYP3A activity using any or all probe drugs ought to give similar results. However, the correlations made between two probe-based measures of CYP3A activity have, in general, been poor or controversial (Hunt et al., 1992; Kinirons et al., 1993; Kinirons et al., 1999; Lown et al., 1995; Stein et al., 1996; Watkins et al., 1992). The disappointing correlations might be due to the following phenomena:

- Dietary factors that modify CYP3A activity
- More than 1 binding site within the CYP3A
- Different routes of drug administration
- Overlapping substrate specificity of probes (ie, processing by multiple CYPs)
- Extrahepatic processing of probes in gastrointestinal epithelium and kidney

- Metabolism by CYP3A5 or CYP3A7 in addition to CYP3A4
- Co-processing of a probe by transport proteins such as P-glycoprotein

Because of pronounced interindividual differences in drug disposition, optimal therapy requires drug administration according to each patients need. Assessing the metabolic capacity of an individual patient before or during drug therapy might help to find the optimal individual dose or to give hints at the cause of adverse events or non-response. Since *in-vitro* tests suggest that different CYPs may play a role in the bioactivation of different environmental chemicals with carcinogenic potential, for example activation of aflatoxins though CYP3A4, phenotyping might be important in risk assessment, as the increased activity of these CYPs have been implicated in increased cancer risk (Bachmann, 1996; Guengerich, 1990)

Despite the potential benefits, phenotyping of CYP enzymes is not used in routine clinical practice so far. More prospective studies, demonstrating the clinical benefit and/or cost-effectiveness of phenotyping have to be done with respect to predict ineffectiveness of treatment and/or toxic effects.

1.5 Midazolam

MDZ (Figure 3) is a short-acting imidazobenzodiazepine with hypnotic, muscle-relaxant, anticonvulsant and anxiolytic properties. In clinical practise, it is used for the induction of anaesthesia, sedation and the treatment of generalised seizures and status epilepticus (Nordt and Clark, 1997). Compared to other benzodiazepines, it is characterised by a more rapid onset of clinical effects and shorter duration of action. In acidic solutions, the diazepine ring opens between position 4 and 5, producing a polar, water-soluble primary amine derivative. However, at physiological pH, the drug is completely ring-closed, resulting in increased lipophilicity (Dundee et al., 1984; Kanto and Allonen, 1983; Smith et al., 1981).

1.5.1 Pharmacokinetics and metabolism

Following oral administration, MDZ is absorbed rapidly from the gastrointestinal tract with maximum plasma concentrations occurring usually within 30-90 min (Klotz, 1989; Reves et al., 1985). Due to the significant first-pass metabolism in the liver and the intestinal wall, only 40-50% of the usually orally administered dose reaches the systemic circulation. A higher bioavailability was observed after the administration of a higher (40 mg) dose (Heizmann et al., 1983). This may be explained by the fact, that the hepatic first-pass

metabolism is saturable (Bornemann et al., 1985). An increased bioavailability was also observed in patients with liver cirrhosis due to the impaired first-pass effect (Pentikainen et al., 1989). After intramuscular injection, the absorption of MDZ is rapid and nearly complete (absolute bioavailability >90%) and the pharmacological effects usually appear within 5-15 min. Following intravenous administration, the onset of pharmacological action occurs within 1-5 min (Klotz, 1989). MDZ undergoes intermediate hepatic extraction.

Midazolam has a V_d of 1-2.5 l/kg in normal healthy volunteers. The V_d of MDZ is increased in obese patients, elderly persons, during pregnancy and in patients with chronic renal failure. Approximately 96% of MDZ is bound to plasma proteins (mainly to albumin); protein binding of the drug is decreased in patients with chronic renal failure (Dundee et al., 1984; Reves et al., 1985; Vinik et al., 1983).

As mentioned before, MDZ is extensively metabolised mainly in the liver, but also in the small intestine, by CYP3A (Gorski et al., 1994). The main metabolite is 1´-OHMDZ; it accounts for 95% of net intrinsic clearance in human liver microsomes (von Moltke et al., 1996). This metabolite is pharmacologically active and has sedative properties equivalent to MDZ. 1´-OHMDZ is produced in higher concentrations following oral administration as a result of the first-pass metabolism. Minor metabolites are the pharmacologically inactive 4-OHMDZ and 1´,4-dihydroxymidazolam. All metabolites undergo rapid glucuronidation, one of the major phase II conjugation reactions, in the liver and are then excreted into the urine as glucuronide conjugates (Heizmann et al., 1983; Nordt and Clark, 1997). Nearly 90% of an orally administered dose of radiolabeled MDZ is excreted within 24 h. In humans, urinary recovery of 1´-OHMDZ-glucuronide accounted for 60-70% of an administerd dose of [^{14}C]midazolam (Heizmann and Ziegler, 1981). The conjugation with glucuronic acid is catalyzed by uridine diphosphate-glucuronosyl-transferases (UGTs), a family of enzymes, which transfers glucuronic acid from uridine diphosphate-glucuronic acid (UDPGA) to the aglycone substrate. UGT's comprise two subfamilies, UGT1 and UGT2. Until recently, glucuronidation of 1´-OHMDZ was not well characterized. Zhu *et al.* demonstrated *in vitro*, using human liver microsomes and recombinant human UGTs, that O-glucuronidation of the 1´-OH metabolite was catalyzed by UGT2B4 and UGT2B7. (Zhu et al., 2008) The metabolic pathway of MDZ is shown in Figure 3.

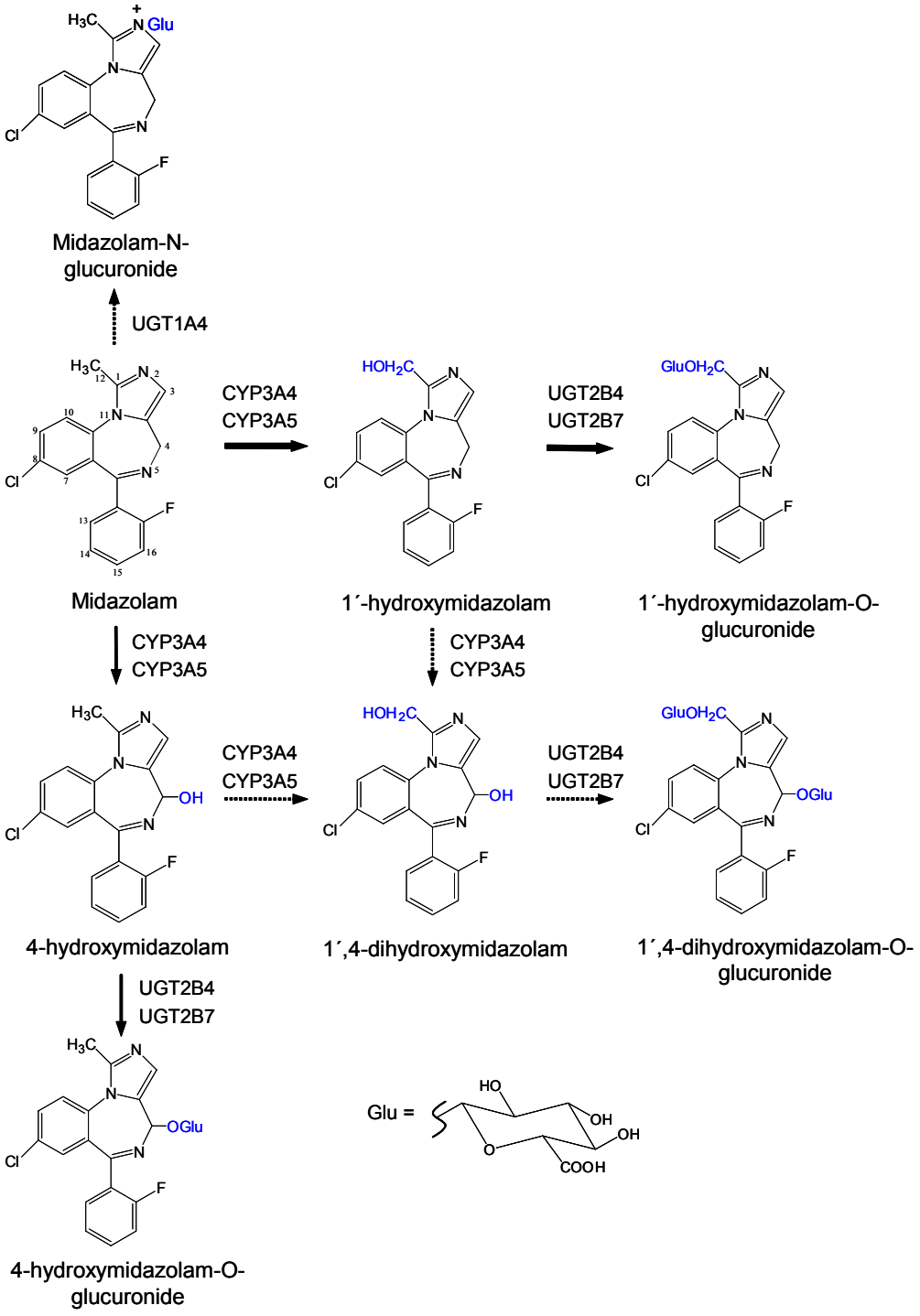

Figure 3. Metabolism of midazolam.

Zhu *et al.* also reported that in addition to the 1´-OHMDZ O-glucuronide (Figure 3), a quaternary N-glucuronide of this metabolite has been identified from incubations of 1´-OHMDZ in human liver microsomes. The N-glucuronidation was catalyzed by UGT1A4.

When MDZ was incubated with human liver microsomes (HLMs) enriched with UDPGA, a direct quaternary N-glucuronide of MDZ (N-2 position) was detected (Zhu et al., 2008). Similar results have been obtained in a recently published *in vitro* study (Klieber et al., 2008). Following incubation of MDZ with HLMs supplemented with UDPGA, two metabolites (which could be considered as two different conformers of the same molecule), were identified as quaternary direct N-glucuronides of MDZ (under control conditions but also in the presence of ketoconazole), thus revealing an additional metabolic pathway for MDZ. Very recently, it could be demonstrated by Hyland *et al*. that direct N-glucuronidation of MDZ also occurs *in vivo* (Hyland et al., 2009). Quantification of the N-glucuronide in urine samples obtained from individuals receiving iv and oral MDZ has shown that this metabolite accounted for 1-2% of the administered dose. NMR data confirmed a conjugation on the N-2 position and, as reported by Klieber *et al*. (Klieber et al., 2008) two forms of this glucuronide have been found. Furthermore, incubation of MDZ with recombinant human UGTs showed, that for MDZ N-glucuronidation, UGT1A4 was clearly favoured over UGT2B4 and 2B7 (>100-fold and >400-fold, respectively). From incubations with HLMs and recombinant human UGT enzymes, N-glucuronidation of 1´-OHMDZ and 4-OHMDZ is also inferred (Hyland et al., 2009).

In-vitro studies using recombinant CYP3A4 and CYP3A5 have shown that both, rCYP3A4 and rCYP3A5, contribute to MDZ metabolism producing 1´-OHMDZ and 4-OHMDZ (Patki et al., 2003). In human liver microsomes, some studies have demonstrated that the rate of 1´-OHMDZ formation correlated well with CYP3A protein content (r=0.92) and, to a lower extend, with CYP3A5 content (r=0.6) (He et al., 2006). However, other studies had inconsistent results. Yamaori *et al.* found that the correlation of the rate of 1´-OHMDZ formation with the CYP3A5 content was higher than that with CYP3A4 content, whereas correlation of the rate of 4-OHMDZ formation with CYP3A5 content was similar to that with CYP3A4 in HLMs from Japanese subjects (Yamaori et al., 2004).

Initial studies found that livers expressing CYP3A5 had an increased rate of 1´-OHMDZ formation (Gellner et al., 2001; Gorski et al., 1994). Further investigations using recombinant systems have also suggested that CYP3A5 is efficient in forming the 1´-OH metabolite compared with 4-OHMDZ and that the overall catalytic activity for 1´-OHMDZ formation is higher for CYP3A5 compared with CYP3A4 (Huang et al., 2004). These findings are also in accordance with results of an *in-vitro* study carried out in our laboratory. An *in-vivo* study has shown significantly higher total 1´-OHMDZ AUC / total 4-OHMDZ ratios in CYP3A5 as compared to poor metabolisers (Eap et al., 2004c). These results are in agreement with the *in-vitro* data showing that microsomal samples containing CYP3A5 and

CYP3A4 exhibit a greater ratio 1´-OHMDZ/4-OHMDZ compared with samples containing CYP3A4 alone (Gorski et al., 1994).

The 1´-OHMDZ-glucuronide also appears to have sedative properties when concentrations are high, as has been observed in adult patients with renal failure. The accumulation of the 1´-OHMDZ conjugate of MDZ may explain the prolonged sedation after cessation of MDZ (Bauer et al., 1995).

Compared with other benzodiazepines, MDZ has a short elimination half-life ($t_{1/2}$) of 1.5-3 h. During pregnancy, the $t_{1/2}$ decreases to 30-120 min, whereas the $t_{1/2}$ seems to be prolonged in obese patients, geriatric individuals and patients with impaired hepatic function (Greenblatt et al., 1984; Nordt and Clark, 1997). The elimination half-life of the 1´-hydroxy metabolite is approximately 1 h (Kanto, 1985). The total plasma clearance of MDZ is 5.8-9 mL/min/kg in healthy subjects but is decreased in elderly persons (Dundee et al., 1984).

1.5.2 Mechanism of action and potential adverse side effects

MDZ binds with high specificity to a subunit of the γ-amino butyric acid ($GABA_A$) receptors (which is not identical with the GABA binding site) in the central nervous system (CNS) which leads to the activation of these $GABA_A$ receptors. In contrast to barbiturates, which stimulate the GABA receptors directly, benzodiazepines act indirect by enhancing the inhibitory action of the neurotransmitter GABA. The binding of GABA to the receptor increases the flow of chloride ions into the cell, which than leads to a decreased ability of the cell to initiate an action potential. Therefore, the presence of GABA is required for the effectiveness of benzodiazepines. The effects of the benzodiazepines can be reversed by the specific competitive receptor antagonist flumazenil (Krähenbühl, 2003; Nordt and Clark, 1997).

Long-term use of MDZ may lead to addiction and to tolerance concerning the hypnotic effects. Furthermore, MDZ has been associated with anterograde amnesia, respiratory depression and cardiac arrest. Patients with hepatic or renal insufficiencies may be at risk for prolonged sedation. Elderly patients (age >60) have an increased risk for suffering adverse drug reactions because of a possible prolonged elimination half-life of MDZ. Synergy occurs when used in combination with anaesthetics, alcohol or other CNS depressants (Bauer et al., 1995; Nordt and Clark, 1997). MDZ may also interact with several concomitantly administered medications that may inhibit (e.g. erythromycin, azole antifungals, verapamil or cimetidine) or induce (e.g. carbamazepine, rifampicin or phenytoin) CYP3A activity (Backman et al., 1996a; Backman et al., 1996b; Nordt and Clark, 1997; Olkkola et al., 1993).

1.6 Determination of midazolam and its metabolites

1.6.1 Nonchromatographic techniques

Several non-chromatographic techniques are available for the analysis of midazolam (and metabolites) in urine and blood/plasma. These assays are often used to provide an initial test or to screen biological matrices for the presence of the benzodiazepine. Numerous commercial kits are existing for this purpose. Midazolam and its two major metabolites can be detected in urine by fluorescent polarisation immunoassay (FPIA) (e.g. Abbot TDx and ADx) and by the enzyme immunoassay (EIA) EMIT (Fraser et al., 1991). With FPIA, MDZ has also been determined in plasma and blood samples (Bourget et al., 1996; Huang and Moody, 1995). Other methods for determining MDZ in plasma or human tissues include enzyme linked immunosorbent assay (ELISA) (Gorczynski and Melbye, 2001) and radioimmunoassay (RIA) (Dixon et al., 1982). However, these techniques are not relevant for the quantitative determination of MDZ and its metabolites with regard to CYP3A phenotyping, and will therefore not be described in more details in this work.

1.6.2 HPLC and GC(-MS) analysis

Numerous high performance liquid chromatography (HPLC) and gas chromatography (-mass spectrometry) (GC-MS) methods have been published for the identification and quantitative determination of MDZ and its metabolites in different matrices.

A suitable sample preparation is an important prerequisite for the chromatography of biosamples (e.g. urine, plasma, blood or oral fluid). Mostly, the isolation of MDZ and its metabolites has been performed by either liquid-liquid extraction (LLE) or solid phase extraction (SPE). As MDZ is a weakly basic drug, LLE is most commonly conducted at a slightly alkaline pH, where the analyte is uncharged, using for example ethyl acetate, acetone, chloroform, toluene, hexane, dichloromethane, butylacetate, diethyl ether or mixtures of them as organic phases. The general pros and cons of using either LLE or SPE are described by Franke and De Zeeuw (Franke and de Zeeuw, 1998).

The most important published HPLC and GC(-MS) methods for the quantitative determination of MDZ and its metabolites are summarised in Table 6 and 7.

Although HPLC has been used for decades, for the quantification of MDZ and its major metabolite(s), theses methodologies typically represent relatively poor sensitivity and low throughput. As shown in Table 6, the limit of quantification (LOQ) is either in the ng/ml or in the high pg/ml range. Focusing on CYP3A phenotyping, the administration of therapeutic

MDZ doses (about 4-7.5 mg per oral) is often required, due to the lacking sentitivity, which may lead to adverse drug reactions such as sedation or amnesia.

Table 6. Selection of published HPLC methods

Reference	Matrix	Drugs	Extraction	Detection method	LOQ (ng/ml)
(Ha et al., 1993)	Plasma Urine	MDZ 1´-OHMDZ	LLE	UV (245 nm)	5
(Mastey et al., 1994)	Plasma	MDZ 1´-OHMDZ 4-OHMDZ	SPE	UV (254 nm)	15
(Lehmann and Boulieu, 1995)	Plasma	MDZ 1´-OHMDZ	LLE	UV (215 nm)	2
(Carrillo et al., 1998)	Plasma	MDZ 1´-OHMDZ 4-OHMDZ	LLE	UV (254 nm)	7
(Eeckhoudt et al., 1998)	Plasma	MDZ 1´-OHMDZ	LLE	UV (240/300 nm)	1 / 0.5
(ter Horst et al., 2003)	Plasma	MDZ 1´-OHMDZ	LLE	DAD	23.4
(Yasui-Furukori et al., 2004)	Plasma	MDZ 1´-OHMDZ	LLE	UV (254 nm)	0.5

DAD, Diode array detector
UV, Ultra violet
LOQ, Limit of quantification

Table 7. Selection of published GC(/MS) methods

Reference	Matrix	Drugs	Extraction	Detection method	LOQ (ng/ml)
(De Kroon et al., 1989)	Plasma	MDZ 1´-OHMDZ 4-OHMDZ	LLE	GC ECD	2
(Thummel et al., 1994a)	Plasma	MDZ 1´-OHMDZ	LLE	GC-MS	0.3
(Backman et al., 1996a)	Plasma	MDZ	LLE	GC NPD	0.1
(Martens and Banditt, 1997)	Serum	MDZ 1´-OHMDZ 4-OHMDZ	LLE	GC-MS	0.2
(Frison et al., 2001)	Plasma	MDZ	SPME	GC-MS	1.5
(Eap et al., 2004a)	Plasma	MDZ 1´-OHMDZ 4-OHMDZ	LLE	GC-MS	0.01
(Gunnar et al., 2006)	Blood	MDZ 1´-OHMDZ	LLE	GC/NICI-MS	10 / 2

ECD, Electron- capture detector
NICI, negative-ion chemical ionisation
NPD, Nitrogen-Phosphorus detector
SPME, Solid phase micro extraction

GC(-MS) methods (Table 7), in general, appear to be more sensitive compared to HPLC methods for the quantification of MDZ and its metabolites in biological fluids, yielding LOQs in the lower pg/ml range. The sensitive GC-MS method published by Eap *et al.* have shown (Eap et al., 2004c) that MDZ and two of its metabolites can be determined in human plasma after oral administration of 75 µg MDZ. The use of such low doses of the probe drug would be advantageous in order to minimise MDZ related central nervous side effects particularly in patients, who are more sensitive to benzodiazepines (such as elderly persons). However, the disadvantage of using GC is that this chromatographic method often requires extensive sample pre-treatment such as clean-up and/or derivatisation for analysis.
Coupling HPLC to mass spectrometry has shown to be a good alternative to GC-MS with regard to assay sensitivity. In addition, the usage of LC-MS(/MS) offers the opportunity to analyse also non-volatile, polar and thermal labile molecules.

1.6.3 LC-MS and LC-MS/MS analysis

In recent years, liquid chromatography coupled with single-stage (LC-MS) or tandem mass spectrometry (LC-MS/MS) is becoming increasingly important in routine analysis, especially for quantification of an analyte of interest. The advantages in using LC-MS(/MS) compared to HPLC and some of the GC(-MS) methods are better sensitivity, selectivity and specificity. Based on these characteristics, theoretically, sample clean-up can be reduced and chromatographic separation can be minimised, which would lead to higher throughput in routine analysis. However, LC-MS(/MS) is not completely without limitations which will be discussed in chapter 1.6.3.2.

1.6.3.1 Theoretical background

While GC can easily be coupled to MS, LC can only be coupled with MS via sophisticated interfaces to remove the mobile phase and ionise the analyte (Ardrey, 2003). Today, two relatively robust LC-MS interfaces have become the ‘golden standard’ of LC-MS: the atmospheric pressure ionisation (API) techniques, electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI). Although both interfaces perform so-called ‘soft-ionisation’ and generate ions at atmospheric pressure, each is characterised by a specific ionisation mechanism which both are described by Bruins and Kerbarle (Bruins, 1994; Kerbarle and Tang, 1993). ESI as well as APCI can be used in positive or negative polarity mode, dependent on the structure of the analyte of interest. Other interfaces such

as moving belt, fast atom bombardment (FAB) or particle beam (PB) are nowadays of minor relevance.

Mass analysis of ionised substances is performed using one or two mass analysers (MS/MS), which consist predominantly of ion traps and quadrupoles, sometimes of sector field and time-of-flight instruments (TOF). MS/MS can be performed either in space, by placing one mass spectrometer after another (e.g. triple quadrupole MS) or by carrying out successive mass-selective operations in time in a quadrupole ion trap. A detailed introduction to the latter is given by March (March, 1997).

MS/MS is used to produce structural information about a compound by fragmenting specific sample ions inside the mass spectrometer and identifying the resultant fragment ions. Using tandem mass spectrometry, it is also possible to detect a specific compound in a complex mixture on account of its specific and characteristic fragmentation patterns. In this way, selectivity and sensitivity can be gained.

Tandem mass spectrometry (MS/MS) is the practice of carrying out one mass-selective operation after another. The objective of the first step is to isolate an ion species designated as *parent ion*, while that of the second operation is to determine the mass to charge ratio (m/z) of the *fragment or daughter ion,* formed by collision induced dissociation (CID) of the parent ions (March, 1997).

The analysers can operate either in the full scan mode or in the more sensitive selected-ion monitoring mode (SIM) detecting positive or negative ions. For MS/MS analysis four different operating combinations are possible: Parent-ion scanning (scan mode in the first, SIM in the second analyser), daughter-ion scanning (DIS; SIM in the first, scan mode in the second analyser), constant neutral-loss scanning (CNLS; scan mode in both analysers) or selected-reaction monitoring (SRM; SIM in both analysers) (Careri et al., 1996; Hoja et al., 1997). DIS is preferable for the identification of drugs and or their metabolites in complex matrices. Separation is performed on the LC and in the first mass analyser, while structural information is obtained by fragmentation in the second analyser. SRM (also named multiple-reaction monitoring MRM) is the most powerful technique for quantification of small amounts of analyte in complex matrices.

1.6.3.2 Matrix effect / Ion suppression

One limitation associated with LC-MS analysis is its susceptibility to matrix effect. Matrix effect is defined as the effect of co-eluting residual matrix components on the ionisation of the target analyte, which can lead either to an enhancement or suppression of the ion intensity (Benijts et al., 2004; Dams et al., 2003). For LC-MS(/MS) method development, it

is therefore very important to take this effect into consideration because interfering matrix components are often accompanied with diminished precision, sensitivity and accuracy of a quantitative analytical procedure (Annesley, 2003).

Of the two most currently used API sources, ESI is more likely to suffer from ion suppression than APCI (Dams et al., 2003). Non-volatile compounds, such as salts or ion pair agents (e.g. trifluoro acetic acid, TFA) but also endogenous compounds or drugs, can change the efficiency of droplet formation or droplet evaporation, which affects the amount of charged ions that reaches the detector. Molecules with higher masses can suppress the signal of smaller molecules and polar analytes are more susceptible to ion suppression (Annesley, 2003).

Sample clean-up has been shown to be a very important factor that may influence the amount of ionisation of a compound. Bonfiglio *et al.* demonstrated that ion suppression is considered to be more likely a problem when using a simple protein precipitation step for sample preparation as compared to the more exhaustive LLE or SPE methods (Bonfiglio et al., 1999).

There are several methods to overcome ion suppression. One solution would be, to use cleaner sample preparation whenever possible. Furthermore, the chromatographic conditions could be modified so that the compounds of interest elute in a region where ion suppression is not observed. In general, this involves an increase in chromatographic separation. Another possibility is the use of a suitable internal standard (IS), e.g. a stably labeled isotope of the analyte, which ideally co-elutes with the analyte of interest. In this way, the IS and the analyte should be affected by the matrix components to the same extent and peak area ratios should be therefore still reliable. For ion pairing, weaker acids, such as acetic or formic acid, can be used instead of TFA (Annesley, 2003; Bonfiglio et al., 1999; Schuhmacher et al., 2003; van Hout et al., 2003).

For reliable quantitative determinations it is preferable that the retention times of the test compounds are in the region of little or no ion suppression. The chromatographic profile and the extent of change in the analyte ESI response in the presence of interferences under assay conditions can be investigated by post column infusion (Bonfiglio et al., 1999). The analyte of interest is infused through a PEEK tee union into a chromatographic run of an extract or a blank matrix. This signal is then compared to the signal obtained with the post-column infusion of the same model analyte in a chromatographic run with mobile phase only. Another method to assess the matrix effect is described by Matuszewski *et al.* (Matuszewski et al., 2003). Three sets of samples are necessary. Set A consists of standard solutions in mobile phase. For Set B, blank matrix (after extraction) is supplemented with the same amount of standard as used for set A. Set C consists of blank

matrix extract supplemented with the same amount of standard added before extraction. For each of the following calculations, the resulting peak areas are needed: Matrix effect (%) = B/A x 100, recovery (%) = C/B x 100 and process efficiency (%) can be calculated by C/A x 100.

Because matrix effects may possibly have a great impact on assay sensitivity, accuracy and precision in quantitative LC-MS(/MS), it is very important to perform ion suppression studies during method development, especially when ESI is used.

1.6.3.3 Analysis of MDZ and metabolites by LC-MS(/MS)

Several LC-MS and LC-MS/MS methods for the quantititative determination of MDZ and metabolites have been published in recent years. A selection of the most important ones is presented in Table 8. It has been shown that in most cases, LC-MS is not as sensitive as GC-MS. However, LC coupled to tandem mass spectrometry yielded comparable LOQs.

Table 8. Selection of the recently published LC-MS and LC-MS/MS methods

Reference	Matrix	Drugs	Extraction	Detection method	LOQ (ng/ml)
(Marquet et al., 1999)	Serum	MDZ 1´-OHMDZ	LLE	LC-MS	0.2/0.5
(Shiran et al., 2003)	Plasma	MDZ 1´-OHMDZ	LLE	LC-MS	6.5
(Lepper et al., 2004)	Plasma	MDZ	LLE	LC-MS	1
(Smink et al., 2004)	Blood	MDZ	LLE	LC-MS	3.5
(Quintela et al., 2004)	Plasma Saliva	MDZ 1´-OHMDZ	LLE	LC-MS	1 (plasma) 0.2 (saliva)
(Muchohi et al., 2005)	Plasma	MDZ 1´-OHMDZ	LLE	LC-MS	5
(Kashuba et al., 1998)	Plasma	MDZ 1´-OHMDZ 4-OHMDZ		LC-MS/MS	0.25
(Yin et al., 2004)	Plasma	MDZ 1´-OHMDZ	SPE	LC-MS/MS	1
(Harris et al., 2004)	Plasma (dog)	MDZ	SPE	LC-MS/MS	0.1
(Jabor et al., 2005)	Plasma	MDZ 1´-OHMDZ	LLE	LC-MS/MS	0.1
(Li et al., 2007)	Plasma	MDZ 1´-OHMDZ	LLE	LC-MS/MS	0.1

In summary, for CYP3A phenotyping using MDZ as probe drug, the availability of very sensitive analytical methods is of great importance due to the following reasons:

- By improving the sensitivity of the analytical method, it should be possible to conduct phenotyping of CYP3A with a lower dose of the probe drug which would be less objectionable to patients
- Simple, non-invasive CYP3A phenotyping methods would be highly welcome, both for routine use and development, using e.g. saliva as biological matrix. Since MDZ is extensively bound to plasma proteins (96%), the concentrations of free drug are expected to be very low in this matrix and therefore poses a challenge for to the analytical method development.

As mentioned before, the use of LC-MS/MS compared to GC-MS has several advantages. Although numerous tandem mass spectrometry assays have been described in the literature, no LC-MS/MS method has been published so far, which is able to quantify MDZ and both metabolites, 1´-OHMDZ and 4-OHMDZ, in human plasma after the administration of a low oral MDZ dose. Furthermore, to our knowledge, no one has described an LC-MS/MS method for the simultaneous quantitative determination of MDZ and its hydroxymetabolites in oral fluid, which shows enough sensitivity to analyse *in-vivo* samples after MDZ administration to humans and to determine the pharmacokinetic profiles of the mentioned substances. To date, the impact of analysing oral fluid samples for non-invasive CYP3A phenotyping is therefore not yet known.

1.7 References

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2 Aims of the thesis

The major goal of this thesis was to develop less/non-invasive CYP3A phenotyping procedures using midazolam as a probe drug.

Among several phenotyping probes, MDZ plasma clearance appears to be the most reliable method to determine the “real-time” CYP3A activity. However, due to the limited sensitivity of most standard analytical methods, the use of therapeutic MDZ doses is often required, which may cause adverse reactions such as sedation or amnesia. To overcome this problem, the aim of the **first project (Chapter 3)** was, to develop and validate a very sensitive LC-MS/MS method, which allows the determination of midazolam and two of its metabolites at pg/ml levels in human plasma and, as a consequence, to minimize drug exposure. The method was applied to a pharmacokinetic study, where the administration of a sub-therapeutic oral MDZ dose has been studied in order to investigate the possibility of conducting low-dose CYP3A phenotyping.

Another disadvantage of CYP3A phenotyping using MDZ plasma clearance is that the procedure requires multiple blood samples over a time frame of several hours, which limits its application as screening method. Since non-invasive methods would be very advantageous for this purpose, **the second project (Chapter 4)** concentrates on the LC-MS/MS method development and validation in saliva. In a single subject study, the following questions were assessed:

- Since the MDZ concentration is expected to be very low in saliva, is it possible at all to detect and quantify MDZ and its principal metabolites in saliva? If yes,
- could the pharmacokinetics of MDZ in this matrix be determined and is a correlation of plasma and saliva MDZ concentrations existing?

Based on the results of before mentioned studies, an open-label, randomized, two way cross-over trial in eight healthy subjects was conducted to study the kinetics of MDZ after intravenous and oral administration of the drug both under basal conditions and after CYP3A induction with rifampicin **(Chapter 5)**. Our main interest was to find out whether:

- the kinetics of MDZ can be assessed reliably in saliva and
- saliva can be used as a matrix to determine CYP3A activity with MDZ as a probe drug.

A further focus of the thesis was to investigate the pharmacokinetics of midazolam and metabolites in a patient with refractory status epilepticus treated with extraordinary doses of midazolam **(Chapter 6)** and the value of plasma level monitoring.

3 Determination of midazolam and its hydroxy-metabolites in human plasma at pg/ml levels by liquid chromatography-electrospray ionisation ion trap tandem mass spectrometry

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3.1 Abstract

Midazolam (MDZ), a short-acting benzodiazepine, is a widely accepted probe drug for CYP3A phenotyping. So far published methods were using either therapeutic doses of MDZ, which may cause adverse reactions, or, if employing lower doses, were not able quantify the two hydroxy-metabolites. Avoidance of these problems necessitates sensitive analytical methods in order to minimise drug exposure. In the present study, a sensitive and specific liquid chromatography-electrospray ionisation tandem mass spectrometry method was developed and validated for the quantitative determination of MDZ and two of its metabolites, 1´-hydroxymidazolam (1´-OHMDZ) and 4-hydroxymidazolam (4-OHMDZ), in human plasma. After liquid-liquid extraction with hexane:dichlormethane (73:27, v/v), the analytes were separated on a Luna C18(2) (100 x 2.1 mm) analytical column using gradient elution. Detection was achieved using an ion-trap mass spectrometer operating in the tandem-mass spectrometry mode (MS/MS). Midazolam-d_6 was used as internal standard for quantification. Calibration curves were linear (R^2>0.998) between 0.05 and 20 ng/ml for MDZ and both metabolites. Using a 1 ml plasma sample, the limit of detection was 0.025 ng/ml and the limit of quantification 0.05 ng/ml for all analytes. Intra- and inter-day accuracies, determined at three different concentrations, were between 92.1 and 102.3% and the corresponding coefficients of variation were <7.3%. The average recoveries were 90.6%, 86.7% and 79.0% for MDZ, 1´-OHMDZ and 4-OHMDZ, respectively. The method was successfully applied to a pharmacokinetic study, showing that MDZ and both hydroxy-metabolites can be determined precisely in samples obtained *in vivo* following a single oral dose of 2 mg MDZ. The method appears therefore to be useful for CYP3A phenotyping in plasma using sub-therapeutic MDZ doses, but larger studies are needed to proof this assumption.

3.2 Introduction

Midazolam (MDZ) is a short-acting benzodiazepine, which is used in humans as a hypnotic, for the induction of anaesthesia, and for the treatment of patients with status epilepticus or generalised seizures (Nordt and Clark, 1997). MDZ is extensively metabolised by cytochrome P450 3A (CYP3A), mainly to its pharmacologically active metabolite 1´-hydroxymidazolam (1´-OHMDZ) and, to a smaller extent, to 4-hydroxymidazolam (4-

OHMDZ) and 1,4-dihydroxymidazolam. The metabolites are excreted as glucuronoconjugates in urine (Heizmann and Ziegler, 1981; Kronbach et al., 1989).

CYP3A, which usually includes CYP3A4 and CYP3A5 in adults, is the most important subfamily of drug metabolising enzymes in humans. More than 50% of clinical drugs and a variety of other xenobiotics and endogenous substances are metabolised by these enzymes (Maurel, 1996; Paine et al., 1996). They are expressed as the most abundant constituent in the liver CYP enzyme system and are also present in the intestinal epithelial cells. It is well known that CYP3A exhibits a marked interindividual variability in enzyme activity, rendering the dosing and therapeutic use of many CYP3A substrates difficult, especially of those with a narrow therapeutic range (Lin and Lu, 2001; Thummel et al., 1994a; Wilkinson, 1996). Therefore, the possibility to prospectively determine the CYP3A activity with phenotyping probes is potentially useful to identify individuals at risk for potential drug-drug interactions (resulting from inhibition or induction of the catalytic activity) and also to predict insufficient drug response or toxic effects from CYP3A substrates.

Several methods for CYP3A phenotyping have been proposed, including ^{14}C-labeled erythromycin, alfentanil, midazolam, nifedipine or endogenous cortisol (Kharasch et al., 2004; Streetman et al., 2000; Thummel et al., 1994b; Watkins, 1994). However, the MDZ plasma clearance appears to be the most reliable of the available methods and is therefore widely used to assess the activity of CYP3A (Streetman et al., 2000). MDZ can be administered by both, intravenous and oral routes, and therefore offers the opportunity to assess the hepatic as well as the combined intestinal and hepatic CYP3A activity (Gorski et al., 1998; Thummel et al., 1996; Tsunoda et al., 1999). In addition, MDZ is not a substrate of p-glycoprotein (Kim et al., 1999).

Numerous chromatographic methods have been reported for the determination of MDZ and its metabolites in human biosamples including high performance liquid chromatography (HPLC) with UV detection (Carrillo et al., 1998; Drummer, 1998; Eeckhoudt et al., 1998; Ha et al., 1993; Lehmann and Boulieu, 1995; Mastey et al., 1994; ter Horst et al., 2003), gas chromatography (GC) equipped with different detecting systems (Backman et al., 1996a; De Kroon et al., 1989; Frison et al., 2001; Martens and Banditt, 1997) and HPLC coupled to mass spectrometry (LC-MS) (Marquet et al., 1999; Muchohi et al., 2005; Quintela et al., 2004; Shiran et al., 2003) or tandem mass spectrometry (LC-MS/MS) (Jabor et al., 2005; Yin et al., 2004). For all of these methods, the limit of quantification for MDZ was either in the ng/ml range or in the high pg/ml range.

For CYP3A phenotyping, the administration of therapeutic MDZ doses (oral administration of 5 to 7.5 mg) is often required due to the limited sensitivity of most standard analytical methods used for this purpose. This may lead to adverse drug reactions such as sedation

or amnesia. To overcome these problems, more sensitive methods are needed to assess the CYP3A activity with safe and sub-therapeutic MDZ doses. Recently, Eap *et al.* published a very sensitive GC-MS method which allows the measurement of MDZ and two of its metabolites in human plasma after oral administration of 75 µg MDZ without production of any central nervous side effects (Eap et al., 2004). However, the pharmacokinetic profile of the 4-hydroxy-metabolite could not be assessed with such a low dose (Eap et al., 2004b) and, in addition, GC requires extensive sample pretreatment such as clean up and derivatisation for analysis.

To our knowledge, no LC-MS/MS method has been published so far that is able to quantify both, MDZ and its hydroxy-metabolites in human plasma after the administration of a low oral dose of MDZ. Since we plan to use CYP3A phenotyping in the future in special patient populations, such as elderly patient or patients with HIV infection, the purpose of this work was to develop and validate a very sensitive and selective LC-MS/MS method which allows the simultaneous quantification of MDZ, 1´-OHMDZ and 4-OHMDZ (Figure 1) in human plasma.

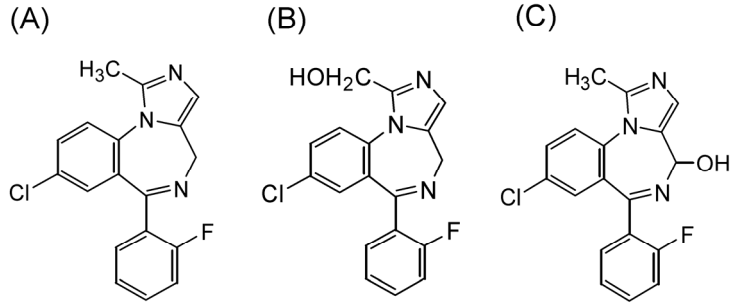

Figure 1. Chemical structures of (A) midazolam and two of its metabolites (B) 1´-hydroxymidazolam and (C) 4-hydroxymidazolam.

3.3 Materials and Methods

3.3.1 Chemicals

Midazolam hydrochloride (MDZ, purity 99.6%), 1´-hydroxymidazolam (1´-OHMDZ, purity 99.7%) and 4-hydroxymidazolam (4-OHMDZ, purity 99.1%) were purchased from Lipomed AG (Arlesheim, Switzerland). The internal standard (IS) midazolam-d_6 was kindly provided from F. Hoffmann-La Roche (Basel, Switzerland). Acetic acid (Suprapur®) as well as LiChrosolv® grade water, acetonitrile, hexane and dichlormethane were from Merck

(Darmstadt, Germany). Glycine was of ultra pure grade and purchased from Fluka (Buchs, Switzerland). All other chemicals and solvents were of analytical grade or better.

3.3.2 Preparation of stock solutions, standards and quality control samples

The primary standard stock solutions of MDZ, 1´-OHMDZ, 4-OHMDZ were prepared at 0.6 mg/ml in methanol and stored in the dark at -70°C for up to 6 months. Working solutions of each analyte were made by diluting the stock solutions with methanol. A mixed working solution containing both midazolam and metabolites was prepared from the individual working solutions. After subsequent dilution with methanol, a series of mixed standard spiking solutions, ranging from 2-800 ng/ml, were obtained, which were further used to prepare calibration and quality control (QC) standards. An IS stock solution was prepared at 12 μg/ml in methanol. A working solution containing 1.2 μg/ml midazolam-d_6 was obtained by diluting the IS stock solution with methanol. All standard solutions were stored at -70°C.
QC samples were made by spiking pooled blank plasma with the mixed standard QC spiking solution to give final concentrations of 0.6 (QC low), 3.7 (QC medium) and 15 ng/ml (QC high).

3.3.3 Plasma sample collection

Heparinised human blank plasma samples were obtained from the blood collection centre of the University Hospital of Basel (Basel, Switzerland) and stored frozen at -20°C. For the development and validation of the method, drug-free plasma from six different sources were pooled and used to prepare plasma calibrators and plasma quality control samples.

3.3.4 Preparation of samples and calibration standards

To 1 ml plasma, 10 μl IS working solution (1.2 μg/ml in methanol) and 1 ml 0.75 M glycine buffer (pH 9.2) were added. The extraction was carried out with 7 ml n-hexane:dichlormethane (73:27, v/v) by mixing on a flat-bed shaker (Infors AG, Basel, Switzerland) for 15 min. After centrifugation at 2500 x g for 5 min, the aqueous layer was frozen in a dry ice/acetone bath and the organic phase was transferred into a silanised glass tube. The organic solvent was evaporated to dryness at 40°C under a gentle stream of nitrogen using a TurboVap® evaporator (Zymark, Hopkinton, MA, USA). The residue was reconstituted in 150 μl acetonitrile:water (23:77, v/v) and 20 μl were injected onto the column for LC-MS/MS analysis.

Calibration standards were prepared freshly each day by spiking 1 ml blank plasma with 25 µl of the appropriate mixed standard spiking solution at concentrations of 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10 and 20 ng/ml and were extracted according to the above described procedure. To blank samples and samples containing only IS, the same volume of methanol was added as for standard solutions.

3.3.5 Instrumentation and chromatographic conditions

The analytes were separated on a HP 1100 system (Agilent Technologies, Palo Alto, CA, USA) which consisted of a degaser, a quaternary pump and an autosampler. Gradient elution was performed on a Luna C18(2) analytical column (100 x 2.1 mm i.d., particle size 3 µm) equipped with a corresponding guard column C18 ODS, 4 x 2.1 mm i.d. (Phenomenex, Torrance, CA, USA). The column oven temperature was set at 40°C and the autosampler was maintained at 15°C. Mobile phases consisted of 0.1% acetic acid in water:acetonitrile (90:10, v/v) (eluent A) and 0.1% acetic acid in acetonitrile (eluent B). Starting at 5% B, the gradient settings were as follows: 0-0.5 min linear increase to 17% B, 0.5-2 min 17% B, 2-3.5 min linear increase to 80% B. After 2.5 min at these conditions, the column was re-equilibrated with 5% B for 4.5 min. The flow rate was 0.24 ml/min.
The analytes were detected using a LCQ$^{DECA\,®}$ ion trap mass spectrometer (Thermo Fischer Scientific, Inc., Waltham, MA, USA) equipped with an electrospray ionization source (ESI) operating in the positive ion mode (4kV). The other main parameters were as follows: heated capillary temperature 255 °C, sheath gas (N_2) pressure 80 arbitrary units, auxiliary gas (N_2) pressure 6 arbitrary units and capillary voltage 3 kV. Helium was used as collision gas. Data acquisition and quantitative analysis were performed using Xcalibur 1.2 software (Thermo Fischer Scientific, Inc.). For the quantification of MDZ and its metabolites, the MS/MS product ion scan mode was chosen. A mass filter for the two most abundant product ions (Table 1) was used in the quantification software afterwards. Each compound was associated with a specific retention time segment, within which one precursor ion, $[M+H]^+$, was stored and further fragmented with a collision energy that allowed a sufficient fragmentation of the selected ion. As the IS is a stable isotope labeled version of MDZ, it elutes in the same segment. Detailed fragmentation parameters are shown in Table 1. Automatic gain control was employed using one microscan and a maximum injection time of 200 ms.

Table 1. LC-MS/MS parameters for the quantitative determination of midazolam and its metabolites in human plasma

Compound	Segment (min)	Scan range (*m/z*)	Precursor ion (*m/z*)	Product ions (*m/z*) used for quantification	CE (eV)	Isolation width (*m/z*)
4-hydroxymidazolam	1 (5.50-6.75)	120-400	342.2	325.2, 234.2	35	1.4
Midazolam-d_6 (IS)	2 (6.75-7.40)	180-405	332.3	297.4, 247.2	40	1.5
Midazolam	2 (6.75-7.40)	100-390	326.2	291.3, 244.2	40	1.5
1´-hydroxymidazolam	3 (7.40-8.10)	190-445	342.2	324.2, 203.2	35	1.4

CE, collision energy; IS, internal standard
The most abundant product ion is underlined

3.3.6 Method validation

Spiked plasma calibration standards at 9 concentrations ranging from 0.05 to 20 ng/ml for each analyte were prepared freshly and analysed in six independent analytical runs. Calibration curves were constructed using the analyte to IS peak area ratio versus the analyte concentration of the nominal standard, and were fitted by linear regression analysis with a weighting factor of 1/x. To assess linearity, deviations of the mean calculated concentrations over six runs had to be ≤15% of the nominal concentration, except for the limit of quantification (LOQ), where a deviation of 20% was permitted.

Intra-day precision and accuracy were determined at three concentrations (0.6, 3.7 and 15 ng/), by analysing on the same day six replicates of each QC level. Inter-day precision and accuracy were assessed by the analysis of QC samples at three concentrations each day for six days. Precision should not exceed 15% of the coefficient of variation (CV) at each concentration level, except for the LOQ for which 20% was acceptable. Accuracy was calculated by comparing the mean experimental concentration of assayed samples with their nominal values, and the percentage bias was used as the index. The acceptable bias was set at ≤ 15% of the theoretical values at each concentration level except for the LOQ, for which 20% was acceptable.

The validation of the analysis of samples with concentrations above the upper limit of quantification (ULOQ), was carried out by diluting additional QCs (100 ng/ml and 50 ng/ml) with human blank plasma by a factor of 10 or 5, respectively. Four replicates of each control sample were analyzed together with a calibration curve, after appropriate dilution of the samples. Accuracy and precision of the calculated QC concentrations, when multiplied with their respective dilution factors, had to be in the range of ±15% (Dadgar et al., 1995).

The specificity of the method was evaluated by analysing plasma samples from six different sources to investigate the potential interferences at the LC peak region for each analyte and

IS. In addition, drugs which could be used in combination with MDZ (alprazolam, diazepam, flunitrazepam, oxazepam, lorazepam, carbamazepine, rifampicin and ketokonazole,) were dissolved in methanol (500 ng), dried, reconstituted with 150 µl acetonitrile:water (23:77, v/v) and were then injected under assay conditions.

Recoveries at three concentrations (0.6, 3.7 and 15 ng/ml) were determined by comparing the peak areas of extracted samples (n=6) with those of pure QC standards prepared in mobile phase, which had not been extracted and were directly measured, containing the corresponding concentration of analytes.

Possible matrix effects on the ESI response were investigated via post column continuous infusion (Annesley, 2003; Bonfiglio et al., 1999; Muller et al., 2002). Mobile phase was delivered into the electrospray interface at a flow rate of 0.24 ml/min while analyte (1µg/ml) was being infused, post column, through a PEEK tee union using a syringe pump (5 µl/min). Twenty µl of mobile phase or reconstituted blank plasma samples, extracted as previously described, were then injected onto the analytical column. Effluent from the column, combined with the infused analytes, entered the ESI interface and were analysed.

The stability of MDZ and its metabolites in spiked human plasma was investigated under various storage conditions. In particular, QC samples (n=3) at two concentrations (0.6 and 3.7 ng/ml) were stored for 24h at ambient temperature, 48h at 4°C and at –70°C over a time period of 8 months. To asses the stability of processed samples, QC samples were extracted and placed in the autosampler at 15°C for 24h and 48h, and then injected for analysis. Freeze-thaw stability was also evaluated by three complete cycles of freezing (-70°C) and thawing (ambient temperature). Stability samples were analysed together with freshly prepared calibration samples. The percent deviation in concentration was used as indicator of stability. The analytes were considered to be stable, when the percent deviation was within ±15% of the expected concentration.

3.3.7 Method application

The developed method was used to investigate the plasma kinetic profiles of MDZ and its two metabolites 1´-OHMDZ and 4-OHMDZ. This study had been approved by the local ethics committee. After having given written informed consent, a healthy volunteer was treated with a single dose of 2 mg midazolam after an overnight fast. Blood samples were collected into heparinised tubes at 0, 30, 40, 60, 90, 120, 180, 240, 360 and 600 min after MDZ administration. Plasma samples were stored frozen at -70°C until analysis.

3.4 Results and Discussion

3.4.1 Liquid chromatography and mass spectrometry

Midazolam and its metabolites are basic compounds possessing a tertiary amine in their structure. Therefore, the positive ion mode was chosen for their determination. The electrospray ionisation process showed a predominant protonated molecule of $[M+H]^+$ for all analysed compounds (data not shown). The formation of these ions may also be supported by the presence of acetic acid in the mobile phase. These $[M+H]^+$ ions were selected as precursor ions to undergo collision-induced dissociation in the ion trap to produce the product ion spectra. Quantification was then carried out in the MS/MS product ion scan mode, from the $[M+H]^+$ ion as precursor ion by using the two most abundant product ions (Table 1).

In Figure 2A, a chromatogram (product ion scan) obtained from the analysis of a representative blank plasma sample is shown. No interfering peaks were observed in the extract. A chromatogram of a blank plasma sample spiked with 0.05 ng/ml MDZ and hydroxy-metabolites and 12 ng of IS is shown in Figure 2B. All compounds were well separated from each other, except the deuterated IS, which, as excpected, eluted at the same time as the unlabeled MDZ. The retention times for 4-OHMDZ, MDZ-d_6 (IS), MDZ and 1´-OHMDZ were 6.55, 6.99, 7.05 and 7.61 min. The total run time was 10.5 min.

Stably labeled isotopes of the analytes of interest are desirable as internal standard when the analysis is performed by mass spectrometry. They are essentially identical regarding their chemical and chromatographic properties to the respective unlabeled compound, while being easily distinguishable by mass spectrometry because of their mass difference. In this case, as shown in Figure 2B, MDZ-d_6 was used as IS.

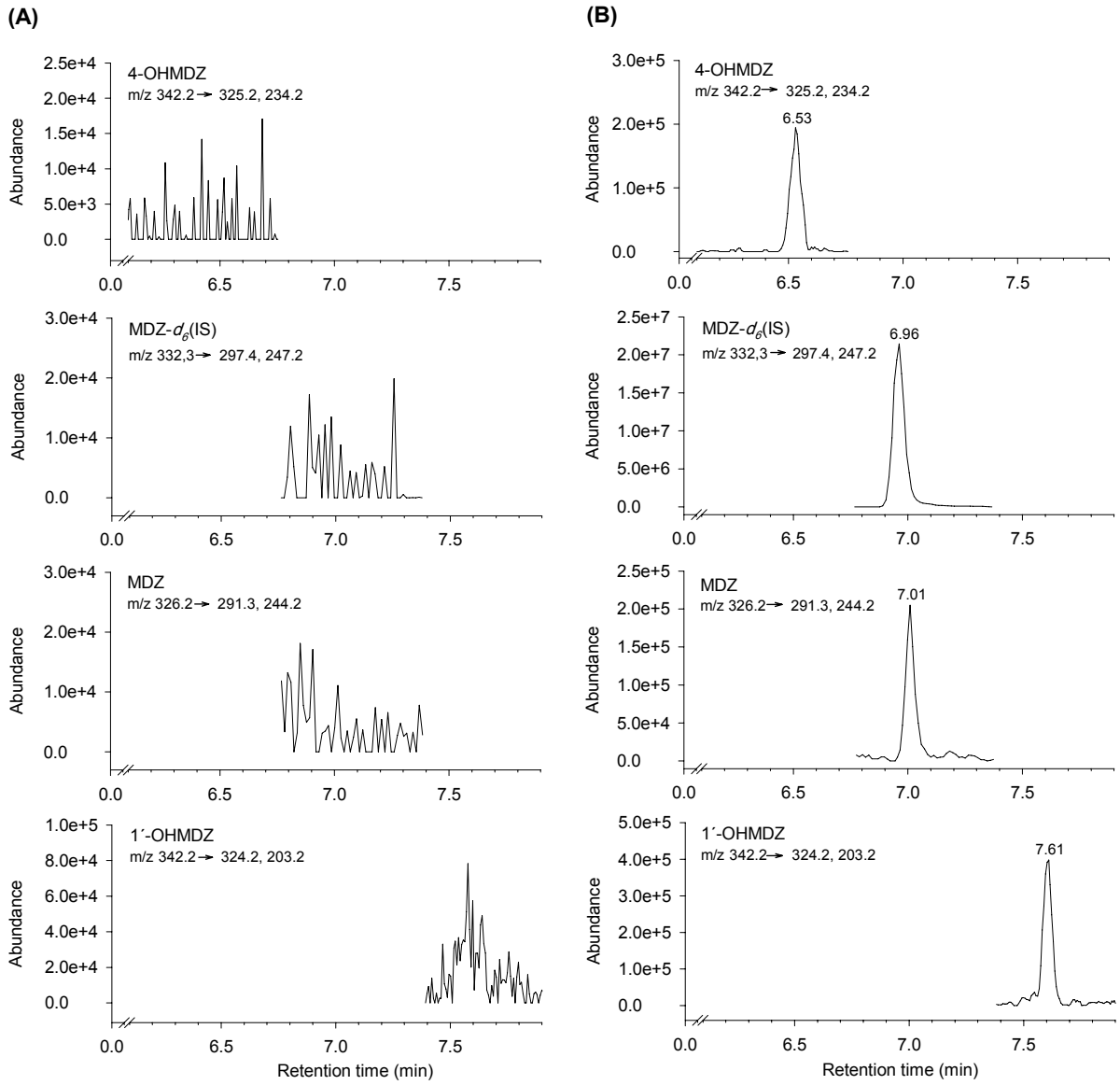

Figure 2. Chromatograms (product ion scan) of (A) an extract of a blank plasma sample and (B) an extract of blank plasma spiked with 0.05 ng/ml midazolam (MDZ) 1´-hydroxymidazolam (1´-OHMDZ), 4-hydroxymidazolam (4-OHMDZ) and 12 ng of MDZ-d_6 (IS). The product ions used for quantification are as follows: MDZ *m/z* 326.2 → 291.3, 244.2; 1´-OHMDZ *m/z* 342.2 → 324.2, 203.2 ; 4-OHMDZ *m/z* 342.2 → 325.2, 234.2 and MDZ-d_6 *m/z* 332.3 → 297.4, 247.2.

To maximise the response of the respective precursor ion $[M+H]^+$, the MS settings of the analyte were optimised while infusing solutions (1 µg/ml) of each compound at 8 µl/min into a flow of 240 µl/min of 45% mobile phase B. Various MS conditions (sheath and auxiliary gas flow, ESI probe temperature and capillary voltage) were investigated. The ESI probe

temperature was set at a minimum acceptable value (255°C) considering the flow rate of the mobile phase so as to avoid thermal degradation of the compounds.
In order to detect midazolam and its metabolites at very low concentrations, a sufficient number of data points for each peak are required. Because an alternate switching from the MS (full MS) to the MS/MS (product ion scan) mode would reduce the sensitivity, due to the resulting reduction in the number of scans per peak, the full-scan (MS) mode was not used in this method. Additionally, by dividing the chromatographic run into segments and due to baseline separation of all substances analysed, the sensitivity of the method has been optimised.

3.4.2 Validation data

Validation of the assay was conducted according to FDA guidelines (Administration, 2001). Quantification was carried out in the MS/MS product ion scan mode using the two most abundant product ions (Table 1). The calibration curves were constructed by plotting the analyte/IS peak area ratio against the nominal concentrations of MDZ or its two metabolites, respectively. Good linear relationships were observed between 0.05-20 ng/ml. The recalculated calibrator concentrations (recalculation performed with 1/x weighted linear regressions, n=6) were used to validate the linearity. The results for MDZ showed accuracies of 100.0±4.2% and CV of better than 6.8%. The metabolites 1´-OHMDZ and 4-OHMDZ had accuracies of 100.2±3.4% and 99.8±3.4% and CV between 3.2-9.3% and 2.7-10.4%, respectively. The coefficients of determination (R^2), obtained from six independent experiments, were better than 0.998. The validation data concerning linearity, LOD, LOQ and recovery are shown in detail in Table 2.

Table 2. Calibration and recovery data for midazolam (MDZ), 1´-hydroxymidazolam (1´-OHMDZ) and 4-hydroxymidazolam (4-OHMDZ) in human plasma

				Limits (ng/ml)		Recovery (n=6)		
Compound	Linear range (ng/ml)	Slope (CV%)	Mean R^2	LOD	LOQ	Nominal conc. (ng/ml)	Mean ± SD (%)	CV (%)
MDZ	0.05-20	0.199	0.9991	0.025	0.05	0.6	87.7 ± 6.2	7.1
		(2.2)				3.7	87.6 ± 5.9	6.7
						15.0	96.4 ± 4.9	5.1
1´-OHMDZ	0.05-20	0.207	0.9978	0.025	0.05	0.6	82.3 ± 2.2	2.7
		(3.0)				3.7	89.9 ± 1.3	1.4
						14.7	88.0 ± 2.6	2.9
4-OHMDZ	0.05-20	0.112	0.9986	0.025	0.05	0.6	74.6 ± 3.2	4.3
		(3.4)				3.7	82.3 ± 5.3	6.5
						14.9	80.2 ± 1.9	2.3

SD, standard deviation; CV, coefficient of variation; R^2, coefficient of determination; LOD, limit of detection ; LOQ, limit of quantification

The liquid-liquid extraction used in the assay yielded average recoveries of 90.6%, 86.7% and 79.0% for MDZ, 1´-OHMDZ and 4-OHMDZ, respectively. The limit of detection (LOD), defined as the lowest tested concentration yielding a signal-to-noise ratio higher than three, was 0.025 ng/ml in plasma, when a 1 ml sample volume was used. The LOQ was determined based on the criteria that (1) the signal-to noise ratio at the LOQ is at least 5 (shown in Figure 2B) and (2) that the analyte response at LOQ can be determined with a CV of ≤20% and an accuracy of 80-120% (n=6) (Administration, 2001). Using the present method, the LOQ were found to be 0.05 ng/ml for all three substances investigated, showing accuracies between 102.1 and 106.4% and CV between 5.8 and 10.4%. A recently published GC-MS method has yielded a lower LOD and LOQ than the present technique (Eap et al., 2004). However GC methods require in general time-consuming sample preparation and derivatisation procedures for analysis. Compared to previously published LC-MS and LC-MS/MS assays, our method was found to be 2-70 times more sensitive for the quantification of MDZ and 1´-OHMDZ in human plasma (Jabor et al., 2005; Muchohi et al., 2005; Quintela et al., 2004; Smink et al., 2004). Furthermore, the present method offers the opportunity to detect and quantify the 4-hydroxy metabolite of MDZ as well.

Intra- and inter-day precision and accuracy data were obtained at three different concentrations, covering the concentration range of the calibration standards used, and are summarised in Table 3. The CV ranged from 1.2 to 7.3% for MDZ, from 1.9 to 6.3% for 1´-

OHMDZ and from 3.2 to 6.2% for 4-OHMDZ. The accuracy, presented as the percentage bias against the nominal concentration, was between 92.1 and 102.3%.

Table 3. Intra-day and inter-day precision and accuracy for midazolam and metabolites in human plasma (n=6 determinations)

	Intra-day			Inter-day		
	Low	Medium	High	Low	Medium	High
Midazolam						
Nominal concentration (ng/ml)	0.6	3.7	15.0	0.6	3.7	15.0
Mean found concentration (ng/ml)	0.6	3.6	14.3	0.6	3.7	14.5
CV (%)	3.1	3.4	1.2	6.0	4.2	7.3
Accuarcy (%)	100.4	96.2	95.6	102.3	99.5	96.6
1´-hydroxymidazolam						
Nominal concentration (ng/ml)	0.6	3.7	14.7	0.6	3.7	14.7
Mean found concentration (ng/ml)	0.6	3.5	14.0	0.6	3.5	14.0
CV (%)	6.0	6.3	4.8	3.9	3.0	1.9
Accuarcy (%)	98.8	95.5	95.3	98.5	94.3	95.5
4-hydroxymidazolam						
Nominal concentration (ng/ml)	0.6	3.7	14.9	0.6	3.7	14.9
Mean found concentration (ng/ml)	0.6	3.6	13.7	0.6	3.6	14.2
CV (%)	4.4	6.1	4.6	6.2	3.5	3.2
ccuarcy (%)	96.9	97.2	92.1	95.6	95.9	95.1

CV, coefficient of variation

Extrapolation of the calibration curve for the measurement of concentrations above the ULOQ, is not acceptable (Dadgar et al., 1995). Therefore, the effect of dilution of concentrated samples with human blank plasma was tested. The validation of this procedure showed the following results: For all substances, the 1/10 and 1/5 dilution yielded accuracies of 97.0-104.4% and 95.8-99.9%, respectively, with CV <6.5%. All of these values fall within the defined precision and accuracy criteria (Administration, 2001; Dadgar et al., 1995).

Ion suppression, caused by co-eluting matrix components which can reduce the ionization efficiency of the analytes of interest, is often accompanied by diminished sensitivity, accuracy and precision of an analytical method. Due to the susceptibility of ESI to ion suppression, this matrix effect in plasma was evaluated by postcolumn continuous infusion, in order to observe the chromatographic profile and the analyte response in the presence of possible interferences (Annesley, 2003; Bonfiglio et al., 1999; Muller et al., 2002). No suppressive effects could be detected in the regions of interest under assay conditions.

The specificity of the assay was also evaluated**.** No interfering peaks were observed following the extraction of plasma samples from six different human controls who were not receiving any medication. The extracted ion chromatogram of a representative blank plasma sample is shown in Figure 2A. Interferences with common drugs, which may be ingested in combination with the analyte, were also tested and found not to interfere with the analytes on the basis of different retention times and mass spectra (data not shown).
All analytes were found to be stable in plasma when stored at -70°C for up to 8 months and at 4°C for 48h or after three freeze-thaw cycles. The mean deviations in the concentration of the analytes were -8.2 to 7.1%. The analytes were also stable for 24h at room temperature, except for 4-OHMDZ, where deviations in the concentration were up to -18.5%. However, if samples are analysed immediately after thawing, this limitation should be of minor importance. When dissolved in acetic solutions, the 4-hydroxy metabolite was not stable (data not shown), which has also been reported by other authors (Ha et al., 1993; Kuhn et al., 2003). A later eluting degradation product was present in the chromatogram under these conditions with a specific precursor ion (*m/z* 343,2) and product ion (*m/z* 315,2). To prevent 4-OHMDZ from degradation, we were using acetonitrile:water (23:77, v/v) without supplementation of acetic acid for the dissolution of plasma extract residues. No significant loss was observed when extracts were kept in the autosampler at 15°C for up to 48h. The deviations in concentration were -6.8 to 4.8% for MDZ and 1´-OHMDZ and up to -11.0% for 4-OHMDZ. Nearly no degradation product was observed under these conditions.

3.4.3 Method application

We have successfully used the described method for the quantitative measurement of MDZ and the two hydroxy-metabolites in plasma samples. Figure 3 shows an example of a chromatogram (product ion scan) obtained from a plasma sample drawn 90 min after the intake of a single oral dose of 2 mg midazolam by a healthy volunteer. The measured concentrations of MDZ, 1´-OHMDZ and 4-OHMDZ were 8.1, 2.2 and 0.3 ng/ml, respectively. The semi-logarithmic concentration-versus-time curves of MDZ and metabolites in this subject are shown in Figure 4. MDZ and 1´-OHMDZ could be quantified precisely in plasma until 10 h after the administration of a low oral MDZ dose. Since the 4-hydroxy-metabolite is produced to a much smaller extent compared to 1´-OHMDZ, the pharmacokinetic profile of 4-OHMDZ in plasma has shown to be not assessable in many of the reported *in-vivo* studies due to the limited assay sensitivities (Carrillo et al., 1998; Eap et al., 2004). The presented LC-MS/MS method however showed a sufficient sensitivity, which allows the reliable quantification also of this minor metabolite until 6 h after drug

administration. The method therefore appears to be useful for the assessment of the CYP3A activity in humans following sub-therapeutic MDZ doses, allowing to minimise MDZ related adverse reactions.

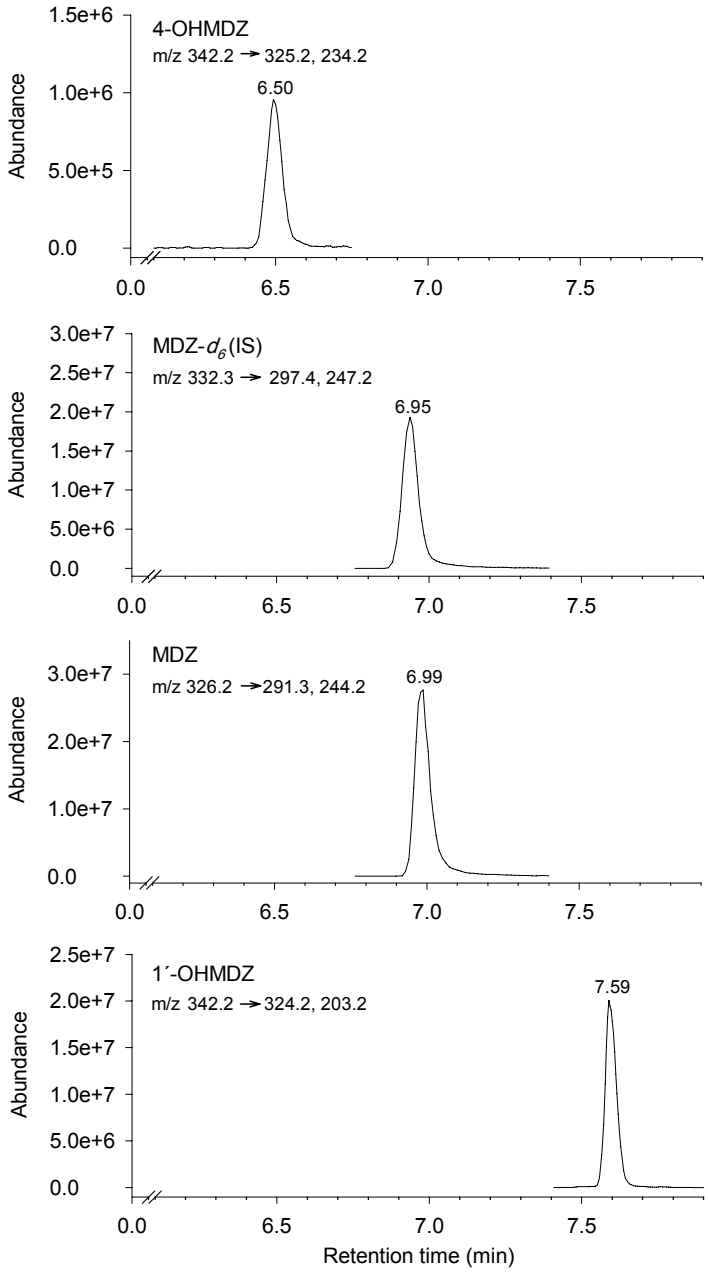

Figure 3. Chromatogram (product ion scan) of a plasma sample obtained after 90 min following the administration of a 2 mg single oral dose to a healthy volunteer. The measured concentrations were 8.1, 2.2 and 0.3 ng/ml for midazolam (MDZ), 1´-hydroxymidazolam (1´-OHMDZ) and 4-hydroxymidazolam (4-OHMDZ), respectively. The product ions used for quantification are the same as those shown in Figure 2.

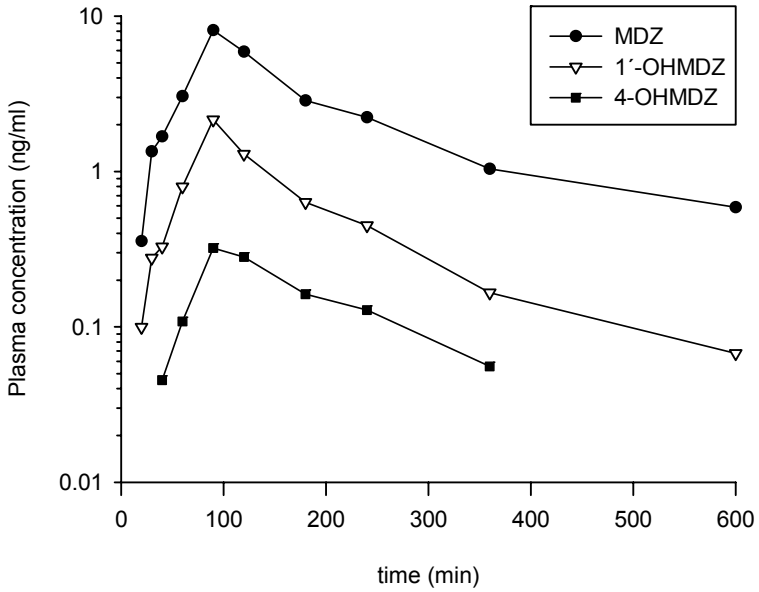

Figure 4. Semi-logarithmic plasma concentration-versus-time profiles of midazolam (MDZ), 1´-hydroxymidazolam (1´-OHMDZ) and 4-hydroxymidazolam (4-OHMDZ) following a single oral dose (2 mg) of midazolam in a healthy volunteer.

3.5 Conclusions

We have developed a very sensitive, selective and reliable LC-MS/MS method for the quantitative determination of MDZ and two of its hydroxy-metabolites in human plasma. The method has been completely validated, including dilution and ion suppression effects, showing excellent results for all the parameters studied. The present method has been successfully applied to a human pharmacokinetic study, showing sufficient sensitivity to determine plasma concentrations of MDZ, 1´-OHMDZ and 4-OHMDZ following the administration of a sub-therapeutic oral MDZ dose (2 mg). Further studies are necessary to proof that the method is useful for low-dose CYP3A phenotyping in special populations such as elderly patient or patients with HIV infection.

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4 Highly sensitive method for the simultaneous quantification of midazolam and two of its metabolites in oral fluid using liquid chromatography-electrospray ionisation ion trap tandem mass spectrometry

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4.1 Abstract

Midazolam (MDZ), a short-acting benzodiazepine, is widely used as a probe drug for CYP3A phenotyping. So far, MDZ and two of its metabolites, 1´-hydroxy and 4-hydroxymidazolam have been determined in human plasma for assessing the CYP3A activity. Since it may be of advantage to use oral fluid as a non-invasive matrix for this purpose, we developed and validated a sensitive liquid chromatography-electrospray ionisation tandem mass spectrometry (LC-MS/MS) method for the simultaneous detection and quantification of MDZ and its metabolites in oral fluid. The procedure includes liquid-liquid extraction with hexane:dichlormethane (73:27, v/v), which is followed by the separation on a Luna C18(2) (100 x 2.1 mm I.D.) column using gradient elution. The detector was an ion trap mass spectrometer and quantification was carried out in the MS/MS product ion scan mode using midazolam-d_6 as internal standard. Calibration curves were linear (R^2>0.999) between 0.05 and 20 ng/ml and the limit of quantification was 0.05 ng/ml for all analytes using a 1 ml oral fluid sample. Accuracies were between 96.6 and 108% and the coefficients of variation were <7.6%. The method was successfully applied to a pharmacokinetic study, showing that the assay is suitable for analysing oral fluid samples obtained *in vivo.* Furthermore, it could be shown that the kinetics of MDZ and its 1´-hydroxymetabolite in plasma and oral fluid are comparable with each other, resulting in a satisfactory correlation for MDZ (R^2=0.972). Therefore, the method appears to be useful for CYP3A phenotyping in oral fluid. But larger studies are needed for definitive proof of this assumption.

4.2 Introduction

Midazolam (MDZ) is a short-acting benzodiazepine with hypnotic, anticonvulsant, muscle-relaxant and anxiolytict properties. In clinical practise, it is used for the induction of anaesthesia, sedation and the treatment of generalized seizures or status epilepticus (Nordt and Clark, 1997). Furthermore, MDZ is a widely accepted probe drug for CYP3A phenotyping (Streetman et al., 2000). MDZ is rapidly metabolised by cytochrome P450 3A (CYP3A) to its primary, pharmacologically active metabolite 1´-hydroxymidazolam (1´-OHMDZ) and to a smaller extent, to 4-hydroxymidazolam (4-OHMDZ) and 1,4-dihydroxymidazolam. These metabolites are conjugated and then excreted as glucuronides in the urine (Bauer et al., 1995; Heizmann and Ziegler, 1981; Kronbach et al., 1989).

Oral fluid has been increasingly used as an alternative biological matrix for therapeutic drug monitoring, pharmacokinetic studies, and detection of illicit drugs (Aps and Martens, 2005; Choo and Huestis, 2004; Drummer, 2005; Kidwell et al., 1998; Kintz and Samyn, 2002; Maurer, 2005). As compared to more conventional biological matrices used for these purposes, such as urine or blood, specimen collection for oral fluid is non-invasive and therefore less objectionable to patients, and is easy to perform without medical supervision, possibly representing therefore a cost-effective approach for screening large populations. The disadvantages of oral fluid usage include insufficient production, rendering quantitative analysis impossible, and a shorter time span for the possible detection of substances than in plasma (Choo and Huestis, 2004; Langman, 2007).

The most common way of drug molecules to pass from blood to oral fluid is passive diffusion through lipid membranes, which is restricted to molecules that are lipophilic, uncharged, and not bound to plasma proteins (Aps and Martens, 2005; Choo and Huestis, 2004). Since the salivary drug concentration correlates with the free, pharmacologically active concentration of drugs in plasma, for certain drugs the concentration in oral fluid may reflect drug effects better than the total plasma concentration (Horning et al., 1977). Active transportation and ultrafiltration through pores in cell membranes are additional mechanisms for drugs and metabolites to reach oral fluid (Chiappin et al., 2007; Langman, 2007). Due to the extensive binding of benzodiazepines (MDZ 96%) to plasma proteins (Nordt and Clark, 1997), their concentrations in oral fluid are generally very low compared to that in plasma. For this reason, highly sensitive analytical methods must be used for their detection and quantification.

Numerous methods have been developed for the determination of MDZ (and its metabolites) in biological matrices, including gas chromatography (GC) with different detecting systems (De Kroon et al., 1989; Eap et al., 2004; Frison et al., 2001; Martens and Banditt, 1997), high performance liquid chromatography (HPLC) with UV detection (Carrillo et al., 1998; Eeckhoudt et al., 1998; Ha et al., 1993; Lehmann and Boulieu, 1995; Yasui-Furukori et al., 2004) and HPLC coupled to mass spectrometry (LC-MS) (Kratzsch et al., 2004; Marquet et al., 1999; Quintela et al., 2004) or tandem mass spectrometry (LC-MS/MS) (Jabor et al., 2005; Kapron et al., 2003; Yin et al., 2004).

However, there are only few reports about the determination of MDZ in oral fluid. Quintela *et al.* have developed a sensitive LC-MS method for the quantification of MDZ in oral fluid (Quintela et al., 2005; Quintela et al., 2004) and Kintz *et al.* described a screening method for benzodiazepines and hypnotics in oral fluid by LC-MS/MS after the extraction of 0.5 ml matrix (Kintz et al., 2005). Gunnar *et al.* have described a GC-MS method for the simultaneous determination of various drugs of abuse in oral fluid, including MDZ. However,

none of the three methods used in these studies determined the metabolites of MDZ (Gunnar et al., 2005). Very recently, a method has been reported for screening urine and oral fluid for benzodiazepines and benzodiazepine-like substances by LC-MS(/MS) and an immunoassay (Smink et al., 2006). For all of these methods, the limit of quantification for MDZ was either in the ng/ml range or in the high pg/ml range.

To our knowledge, no analytical method for the separation and quantification of MDZ and its 1´- and 4-hydroxymetabolites in oral fluid has been described so far. For this reason, the aim of our study was to develop and validate a very sensitive LC-MS/MS method, which allows the simultaneous detection and quantification of MDZ, 1´-OHMDZ and 4-OHMDZ in the pg/ml range in oral fluid. The validated method was applied to a pharmacokinetic study to determine the oral fluid concentrations of MDZ and its metabolites after the administration of a single intravenous dose of 2 mg MDZ to a healthy volunteer.

4.3 Experimental

4.3.1 Chemicals

Midazolam hydrochloride (MDZ, purity 99.6%), 1´-hydroxymidazolam (1´-OHMDZ, purity 99.7%) and 4-hydroxymidazolam (4-OHMDZ, purity 99.1%) were purchased from Lipomed AG (Arlesheim, Switzerland). The internal standard (IS) midazolam-d_6 was a kind gift from F. Hoffmann-La Roche (Basel, Switzerland). Acetic acid (Suprapur®) as well as LiChrosolv® grade water, acetonitrile, hexane and dichlormethane were from Merck (Darmstadt, Germany). Glycine was of ultra pure grade and purchased from Fluka (Buchs, Switzerland). All other chemicals and solvents were of analytical grade or better and were purchased either from Fluka, Sigma-Aldrich (Buchs, Switzerland) or from Merck.

4.3.2 Preparation of stock and standard solutions

Standard stock solutions of MDZ, 1´-OHMDZ, 4-OHMDZ were prepared in methanol at 0.6 mg/ml and stored light protected at -70°C for up to 6 months. From these stock solutions, methanolic working solutions of each compound at 60 µg/ml were made. A mixed working solution containing MDZ, 1´-OHMDZ and 4-OHMDZ was prepared from the individual working solutions and was used to prepare a series of mixed standard spiking solutions after subsequent dilution with methanol to give concentrations in the range 2-800 ng/ml. Three mixed standard QC spiking solutions of 600, 148 and 12 ng/ml were obtained by the dilution of an independently prepared mixed working solution with methanol.

An IS stock solution of 12 μg/ml midazolam-d_6 was prepared in methanol. A working solution containing 1.2 μg/ml IS was obtained by diluting the IS stock solution with methanol. All standard solutions were stored at -70°C.

4.3.3 Oral fluid collection

Human blank oral fluid samples were obtained from healthy volunteers by spitting into a polypropylene tube without any stimulation of the saliva production. The oral fluid donors were instructed not to eat or to drink 30 min before sampling and to rinse their mouth with fresh water before spitting. After centrifugation at 2500 x g for 10 min to remove potential food debris, the supernatant was stored frozen at -20°C. All blank samples were screened prior to use to ensure that they were free from endogenous interferences at the retention time of the analytes. For the development and validation of the method, drug-free oral fluid from seven different sources were pooled and used to prepare oral fluid calibrators and quality control (QC) samples.

4.3.4 Spiked oral fluid calibration standards and quality control samples

Oral fluid calibration standards as well as QC samples were prepared freshly each day with concentrations of 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10 and 20 ng/ml or of 0.3 (QC low), 3.7 (QC medium) and 15 ng/ml (QC high), respectively, by spiking blank oral fluid with the appropriately mixed (QC) standard spiking solution.

4.3.5 Sample preparation

To 1 ml oral fluid, 1 ml 0.75 M glycine buffer (pH 9.2), 10 μl IS working solution (1.2 μg/ml in methanol) and 25 μl of the appropriate mixed spiking solution (in case of the calibrators or QC samples) or methanol (for unspiked oral fluid samples) were added. Extraction was performed by adding 7 ml n-hexane:dichlormethane (73:27, v/v) to each tube and mixing on a flat-bed shaker (Infors AG, Basel, Switzerland) for 15 min. After centrifugation at 2500 x g for 5 min, the two phases were separated by freezing out of the water phase in a dry ice/acetone bath. The organic layer was then transferred into a silanised glass tube and evaporated to dryness under nitrogen at 40°C using a TurboVap® evaporator (Zymark, Hopkinton, MA, USA). The residue was reconstituted in 150 μl acetonitrile:water (23:77, v/v) and 20 μl were injected onto the column for LC-MS/MS analysis.

4.3.6 Instrumentation and chromatographic conditions

Separation of the analytes was performed on a HP 1100 system (Agilent Technologies, Palo Alto, CA, USA), which consisted of a degaser, a quaternary pump and an autosampler. Aliquots (20 µl) were injected onto a Luna C18(2) analytical column (100 x 2.1 mm I.D., particle size 3 µm) equipped with a corresponding pre-column C18 ODS, 4 x 2.1 mm I.D. (Phenomenex, Torrance, CA, USA) maintained at 40°C. The mobile phases consisted of 0.1% acetic acid in water:acetonitrile (90:10, v/v) (eluent A) or in acetonitrile (eluent B). Starting at 5% B, the gradient increased linear to 17% B in 0.5 min, was kept at 17% B for 1.5 min and was increased linear to 80% B within 1.5 min, which was followed by a plateau at 80% B for 3 min. The column was then re-equilibrated with 5% B for 4.5 min. The flow rate was 0.24 ml/min and the autosampler temperature was set at 15°C.

Detection was performed using a LCQ$^{DECA\ ®}$ ion trap mass spectrometer (Thermo Fischer Scientific, Inc., MA, USA). Ionisation was achieved using electrospray in the positive ionisation mode (ESI+, 4kV). In order to maximise the response of the respective precursor ion $[M+H]^+$, acquisition parameters were determined by the direct infusion of standard solutions (1 µg/ml) at 8 µl/min into a flow of 240 µl/min of 45% mobile phase B. Various MS conditions such as sheath and auxiliary gas flow, ESI probe temperature and capillary voltage were investigated. During the experiment, a mass range from m/z 100-500 was monitored and the acquisition was carried out in the selected ion monitoring (SIM) mode. The final operating conditions were chosen as follows: sheath gas (N_2) pressure was set at 80 arbitrary units, auxiliary gas (N_2) pressure was set at 6 arbitrary units and capillary voltage at 3 kV. The heated capillary temperature was kept at 255 °C so as to avoid thermal degradation of the compounds. Helium was used as collision gas.

Data acquisition and quantitative analysis were performed using Xcalibur 1.2 software (Thermo Fischer Scientific, Inc.). For quantification of MDZ and its hydroxymetabolites, the MS/MS product ion scan mode was chosen. Each compound was associated with a specific retention time segment. During this time window, one precursor ion $[M+H]^+$ was stored and further fragmented with a collision energy that allowed a sufficient fragmentation of the selected ion. MDZ-d_6 is a stable isotope of MDZ and therefore elutes in the same segment as its respective analyte. Detailed fragmentation parameters are given in Table 1. Automatic gain control was employed using one microscan and a maximum injection time of 200 ms.

Table 1. LC-MS/MS parameters for the quantitative determination of midazolam and two of its metabolites in oral fluid

Compound	R_t (min)	Segment (min)	Scan range (*m/z*)	Precursor ion (*m/z*)	Product ions (*m/z*) used for quantification	CE (eV)	Isolation Width (*m/z*)
4-hydroxymidazolam	6.55	1 (5.50-6.75)	120-400	342.2	325.2, 234.2	35	1.4
Midazolam-d_6 (IS)	6.99	2 (6.75-7.40)	180-405	332.3	297.4, 247.2	40	1.5
Midazolam	7.05	2 (6.75-7.40)	100-390	326.2	291.3, 244.2	40	1.5
1´-hydroxymidazolam	7.61	3 (7.40-8.10)	190-445	342.2	324.2, 203.2	35	1.4

CE, collision energy; R_t, retention time; IS, internal standard
The most abundant product ion is underlined

4.3.7 Method validation

Validation was performed based on Guidance for Industry, Bioanalytical method validation issued by the Food and Drug Administration (FDA) in May 2001 (Administration, 2001). Linearity of the developed LC-MS/MS method was investigated for MDZ and two of its metabolites in oral fluid in six independent analytical runs. To construct calibration curves, the peak area ratios of the appropriate standard to those of the IS were plotted against the analyte concentration of the standard. Linear regressions were calculated with a weighting factor of 1/x. Calibrations (nine points) were prepared in concentration ranges of 0.05 to 20 ng/ml. Oral fluid employed for the validation was screened prior to use to determine the absence of interfering peaks.

In order to evaluate the precision and accuracy of the method, QC samples were processed in six replicates at each of the three different concentrations covering the calibration range, on the same (intra-day variability) and on different days (inter-day variability). Precision should not exceed 15% of the coefficient of variation (CV) at each concentration level, except for the lower limit of quantification (LLOQ) for which 20% was acceptable. Accuracy was calculated by comparing the mean experimental concentration of assayed samples with their nominal values, and the percentage bias was used as the index. The acceptable bias was set at ≤15% of the theoretical values at each concentration level except for the LLOQ, for which 20% was acceptable.

The limit of detection (LOD) was calculated at a signal-to-noise ratio of higher than three. The LLOQ was determined based on the criteria that (1) the signal-to noise ratio at the LLOQ is at least 5 and (2) that the analyte response at LLOQ can be determined with CV of ≤20% and accuracy of 80-120% (n=6).

The efficiency of the extraction procedure was assessed by analysing six replicates of spiked oral fluid samples at each of the three different concentrations (0.3, 3.7 and 15

ng/ml). Recoveries were determined by comparing the peak areas of the extracted spiked samples with those of extracted blank sample spiked with the same amount of analyte after extraction.

Possible matrix effects on the ESI response were investigated via post column continuous infusion (Annesley, 2003; Bonfiglio et al., 1999; Muller et al., 2002). Mobile phase was delivered into the electrospray interface at a flow rate of 0.24 ml/min while analyte (1μg/ml) was being infused, post column, through a PEEK tee union using a syringe pump (5 μl/min). 20 μl of mobile phase or reconstituted blank plasma samples, extracted as previously described, were then injected onto the analytical column. Effluent from the column combined with the infused analytes entered the ESI interface and was analyzed.

Specificity of the method was also evaluated. Drugs which are often used in combination with MDZ (alprazolam, diazepam, flunitrazepam, oxazepam, lorazepam, carbamazepine, rifampicin and ketokonazole) were diluted in either methanol or ethanol, dried under nitrogen, reconstituted with 150 µl acetonitrile:water (23:77 v/v) and injected onto the column.

The stability of MDZ and its metabolites was investigated under various storage conditions using spiked oral fluid (n=3) prepared at two QC concentrations (0.3 and 3.7 ng/ml). Samples were stored for 24h at ambient temperature, 48h in the refrigerator and at –70°C over a time period of 10 months. For the evaluation of freeze-thaw stability, QC samples were analysed after three complete cycles of freezing (-70°C) and thawing (ambient temperature). To asses the stability of processed samples, QC samples were extracted and placed in the autosampler at 15°C for 24h and 48h, and then injected for analysis. Stability samples were analysed together with freshly prepared samples. The percent deviation in concentration was used as an indicator of stability. The analytes were considered to be stable, when the percent deviation was within ±20% of the expected concentration.

4.3.8 Method application

The developed LC-MS/MS method was used to investigate oral fluid kinetic profiles of MDZ and two of its metabolites 1´-OHMDZ and 4-OHMDZ in a pharmacokinetic study that was approved by the local ethics committee. After having given written informed consent, a healthy male volunteer (46 years, 74 kg) was treated with a single intravenous dose of 2 mg midazolam after an overnight fast. Oral fluid samples were collected at 0, 10, 20, 30, 40, 60, 80, 100, 120, 150, 180, 240, 360 and 480 min after MDZ administration. Blood samples were drawn into heparinised tubes at the following time points: 0, 10, 20, 30, 40, 60, 90, 120, 240, 360, 480, 600, 1440 min after drug administration. After centrifugation of both

matrices at 2500 x g for 10 min, plasma and oral fluid samples were separated and stored frozen at -70°C until analysis. The analysis of plasma was carried out by a validated LC-MS/MS method as described in Chapter 3.

4.4 Results and Discussion

4.4.1 Liquid chromatography and mass spectrometry

MDZ and its metabolites could be well separated in less than 8 min by reversed phase liquid chromatography using gradient elution. The retention times for 4-OHMDZ, MDZ-d_6 (IS), MDZ and 1´-OHMDZ were 6.55, 6.99, 7.05 and 7.61 min, respectively. The total run time was 11 min. Figure 1B shows a representative chromatogram (product ion scan) of a blank oral fluid sample spiked with 0.05 ng/ml MDZ and metabolites (LLOQ) and 12 ng of IS under the developed conditions. For mass spectrometry analysis, stably labeled isotopes of the analytes of interest are desirable as internal standard. They are essentially identical regarding their chemical and chromatographic properties to the respective unlabeled compound, while being easily distinguishable by mass spectrometry because of their mass difference. In the present study, MDZ-d_6 was used, which, as expected, elutes in the same time segment as unlabeled MDZ. As shown in Figure 1A, no interfering peaks were observed following the extraction of representative pooled oral fluid samples from seven different human controls who were not receiving any medication.

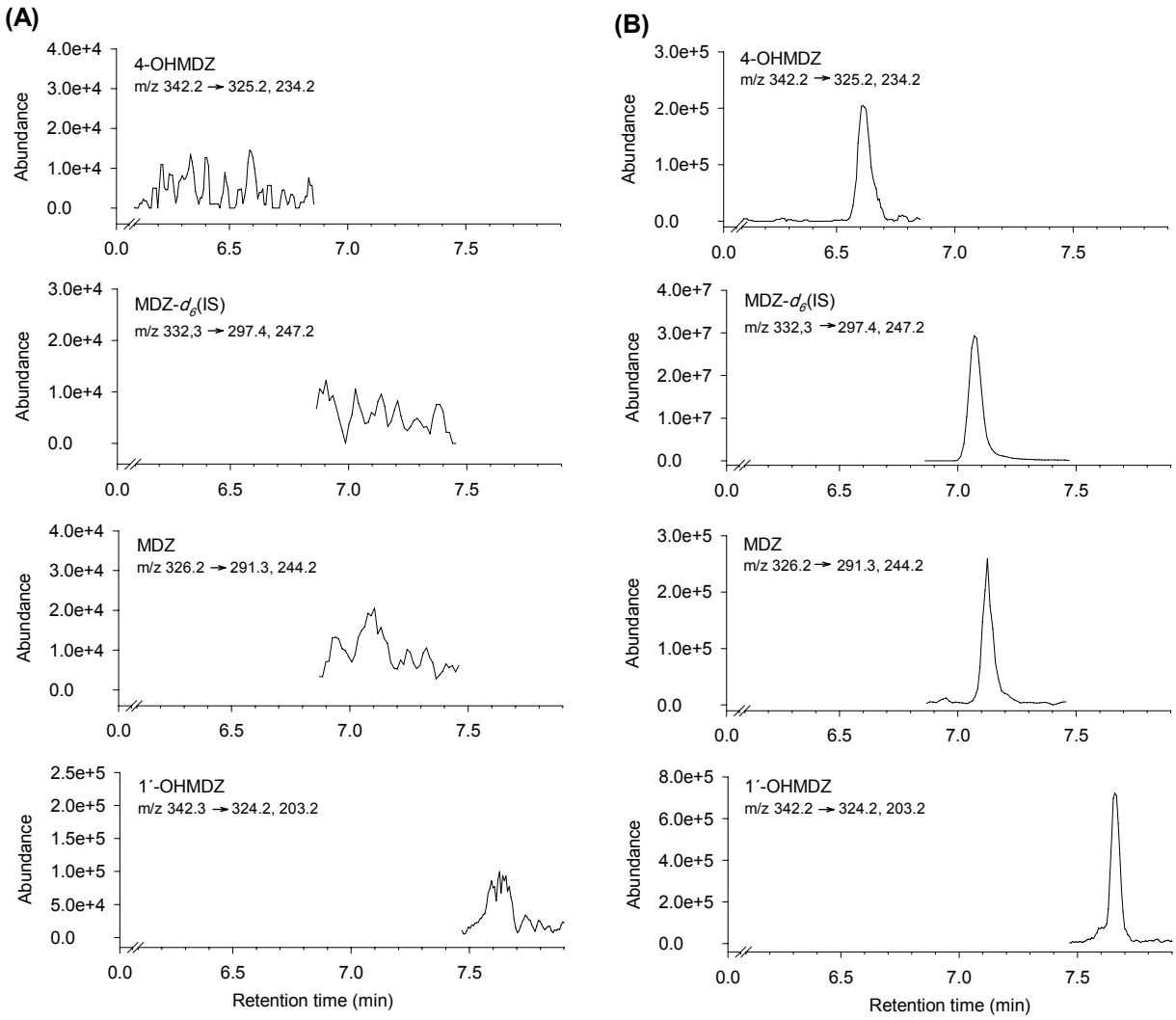

Figure 1. LC-MS/MS chromatograms (product ion scan) of (A) an extract of a blank oral fluid sample and (B) an extract of blank oral fluid spiked with 0.05 ng/ml midazolam (MDZ), 1´-hydroxymidazolam (1´-OHMDZ), 4-hydroxymidazolam (4-OHMDZ) and 12 ng of MDZ-d_6 (IS). The product ions used for quantification are as follows: MDZ *m/z* 326.2 → 291.3, 244.2; 1´-OHMDZ *m/z* 342.2 → 324.2, 203.2; 4-OHMDZ *m/z* 342.2 → 325.2, 234.2; IS *m/z* 332.3 → 297.4, 247.2.

As MDZ and its metabolites are basic compounds, possessing a tertiary amine in their structures, the positive ion mode was chosen for their determination. For all analysed compounds, the electrospray ionisation process showed a predominant protonated molecule $[M+H]^+$ (data not shown). These $[M+H]^+$ ions were selected as precursor ions to undergo collision-induced dissociation in the ion trap. The resultant MS/MS product ion scan spectra and structures are presented in Figure 2. The precursor and product ions selected for each analyte are given in Table 1, together with the optimal collision energy and isolation width.

Formation of the positively charged ions may also be supported using acidic condition in the mobile phase. Among the acids tested, acetic acid provided high production efficiency of $[M+H]^+$ combined with low baseline noise.

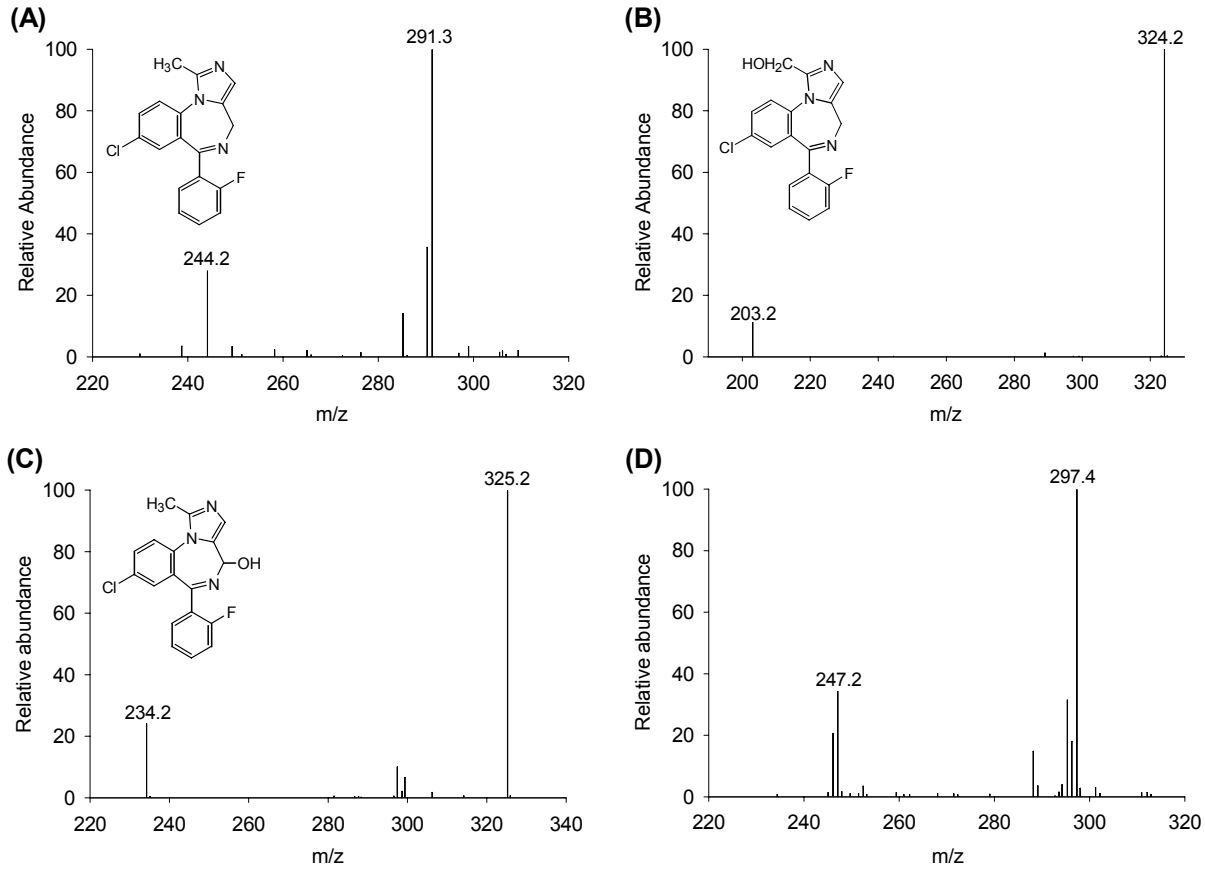

Figure 2. Chemical structures and/or product ion spectra of (A) midazolam, its two metabolites (B) 1´-hydoxymidazolam and (C) 4-hydroxymidazolam, and (D) midazolam-d_6, which was used as internal standard.

For the determination of MDZ and its two metabolites in the pg/ml range in oral fluid, a very sensitive analytical method was required. Therefore, the MS/MS settings were adjusted to maximise the response of each precursor-product ion combination. In addition, the chromatographic run was divided into segments in which only one or two precursor ions were stored for further fragmentation. In this way, the number of cycles of measurement increased and more data points per peak could be gained.

4.4.2 Validation data

The linearity of the method was evaluated for MDZ, 1´-OHMDZ and 4-OHMDZ in oral fluid. Calibration curves were linear from 0.05 to 20 ng/ml for all analytes. Using 1/x weightened linear regressions, coefficients of determination (R^2) of better than 0.999 were obtained in six independent analytical runs (Table 2). Quantification was carried out in the MS/MS product ion scan mode using the two most abundant product ions (see Table 1, Figure 2) and calibration curves were constructed by plotting the analyte/IS peak area ratio against the nominal concentrations of the respective analyte. The recalculated calibrator concentrations (n=6) were used to validate linearity. Deviations of the mean calculated concentrations should not exceed ±15% of the nominal concentration, except for the lower limit of quantification (LLOQ) where a deviation of 20% was permitted. With accuracies of 100.1 ± 4.1% and CV of better than 5.4%, the results for MDZ fulfil the defined validation criteria. Similar data were obtained for the metabolites with accuracies between 95.0 and 105.4% and CV of better than 7%.

Table 2. Calibration and recovery data for midazolam (MDZ), 1´-hydroxymidazolam (1´-OHMDZ) and 4-hydroxymidazolam (4-OHMDZ) in human oral fluid

				Limits (ng/ml)		Recovery (n=6)		
Compound	Linear range (ng/ml)	Mean R^2	Slope (CV %)	LOD	LOQ	Nominal conc. (ng/ml)	Mean ± SD (%)	CV (%)
MDZ	0.05-20	0.9992	0.181	0.025	0.05	0.3	94.8 ± 4.4	4.6
			(2.2)			3.7	95.5 ± 3.2	3.4
						14.9	95.6 ± 4.7	4.7
1´-OHMDZ	0.05-20	0.9994	0.197	0.025	0.05	0.3	97.9 ± 1.3	1.4
			(4.2)			3.7	96.7 ± 2.5	2.6
						14.8	95.1 ± 4.7	4.9
4-OHMDZ	0.05-20	0.9991	0.111	0.025	0.05	0.3	87.2 ± 2.7	3.1
			(3.9)			3.7	85.9 ± 3.5	4.1
						14.7	87.4 ± 4.9	5.6

SD, standard deviation; CV, coefficient of variation; R^2, coefficient of determination; LOD, limit of detection; LOQ, limit of quantification

Limits of quantification were determined as the lowest concentration with a relative deviation of replicate runs of less than 20%. Using 1 ml oral fluid sample, the LLOQ were found to be 0.05 ng/ml for all three compounds (Table 2) with accuracies between 102.6 and 105.4% and CV between 5.4 and 7.4%. The limit of detection was 0.025 ng/ml. This represents a 2-45-fold increase in sensitivity as compared to previously published analytical methods for

the quantitative determination of MDZ and 1´-OHMDZ in oral fluid (Gunnar et al., 2005; Kintz et al., 2005; Quintela et al., 2005; Quintela et al., 2004; Smink et al., 2006).

Using a mixture of hexane:dichlormethane (73:27 v/v), MDZ and two of its metabolites can be simultaneously extracted from oral fluid yielding extraction recoveries between 86 and 98% with CV < 5.6% (see Table 2). As shown in Table 3, intra-day precision and accuracy were determined at three concentrations, covering the concentration range of the calibration standards used, by analysing on the same day six replicates of each QC level. CV ranged from 1.3 to 4.0% and accuracies were between 96.6 and 108.0%. Inter-day variabilities were assessed by the analysis of QC samples at three concentrations each day for six days. The accuracy values ranged from 96.8 to 104.3% and CV were between 1.3 and 7.6%.

An important problem when using ESI is the possible reduction of ionisation. The so-called ion suppression results from the presence of co-eluting compounds (salts, drugs, endogenous compounds, ion-pair agents) that can change the efficiency of droplet formation or droplet evaporation, which affects the amount of charged ions that reaches the detector (Annesley, 2003; Dams et al., 2003; Liang et al., 2003; Muller et al., 2002). Ion suppression could possibly influence assay sensitivity, accuracy and precision of an LC-MS(/MS) method. Due to the susceptibility of ESI to ion suppression, this matrix effect in oral fluid was evaluated by postcolumn continuous infusion, in order to observe the chromatographic profile and the analyte response in the presence of the possible interfering species (Annesley, 2003; Bonfiglio et al., 1999; Muller et al., 2002). Large differences in matrix effect were observed between various sample preparation techniques (data not shown). Liquid-liquid extraction with hexane:dichlormethane (27:73 v/v) yields very clean oral fluid extracts. No suppressive effects could be detected in the regions of interest under assay conditions.

To evaluate the specificity of the method, 500 ng of the following drugs, which are often co-administered with MDZ, were injected under assay conditions: alprazolam, diazepam, flunitrazepam, oxazepam, lorazepam, carbamazepine, rifampicin and ketokonazole. Interferences could be excluded for all the compounds on the basis of different retention times and mass spectra (data not shown).

Table 3. Intra-day and inter-day precision and accuracy for midazolam and metabolites in human oral fluid (n=6 determinations)

	Intra-day			Inter-day		
	Low	Medium	High	Low	Medium	High
Midazolam						
Nominal concentration (ng/ml)	0.3	3.7	14.9	0.3	3.7	14.9
Mean found concentration (ng/ml)	0.3	3.9	14.7	0.3	3.8	14.6
CV (%)	4.0	2.5	2.7	2.9	2.2	1.3
Accuarcy (%)	98.0	103.2	98.3	98.3	101.9	97.8
1´-hydroxymidazolam						
Nominal concentration (ng/ml)	0.3	3.7	14.8	0.3	3.7	14.8
Mean found concentration (ng/ml)	0.3	3.7	14.3	0.3	3.8	14.4
CV (%)	1.3	3.4	2.8	1.5	7.4	3.2
Accuarcy (%)	99.6	99.4	96.6	102.1	101.5	96.8
4-hydroxymidazolam						
Nominal concentration (ng/ml)	0.3	3.7	14.7	0.3	3.7	14.7
Mean found concentration (ng/ml)	0.3	4.0	15.7	0.3	3.8	15.1
CV (%)	3.4	2.8	2.6	5.0	7.6	3.6
Accuarcy (%)	108.0	107.8	106.6	104.3	102.6	103.0

CV, coefficient of variation

All analytes were found to be stable in oral fluid when stored at -70°C for up to 10 months, kept in the autosampler at 15°C for up to 48h or after three freeze-thaw cycles. The mean deviations in the concentration of the analytes were between -17.7 and 7.4%. No significant loss for MDZ and 1´-OHMDZ was observed when samples were stored at ambient temperature for 24h or in the refrigerator for 48h. However, the 4-hydroxy metabolite seems to be less stable in oral fluid at these conditions with deviations in concentrations up to -24.5%. A later eluting degradation product was present in the chromatogram with a base ion m/z 343.2 and a product ion m/z 315.2. Therefore, to overcome this problem, the oral fluid samples were immediately extracted and analysed after thawing.

4.4.3 Method application

We have successfully applied the described LC-MS/MS method for the determination and quantitative measurement of midazolam and metabolites in oral fluid samples originated from a small pharmacokinetic study. Figure 3 shows an example of a chromatogram (product ion scan) obtained from the analyses of an oral fluid sample drawn 30 min after the administration of a single intravenous dose of 2 mg midazolam to a healthy volunteer. The measured concentration of MDZ and 1´-OHMDZ were 0.9 and 0.2 ng/ml, respectively.

Despite the very low concentrations of parent drug found in oral fluid, the analytical method showed a sufficient sensitivity to detect also the minor metabolite 4-OHMDZ, which has not been described in the literature so far. However, quantification of this metabolite was not possible in this volunteer (concentrations were <0.05 ng/ml).

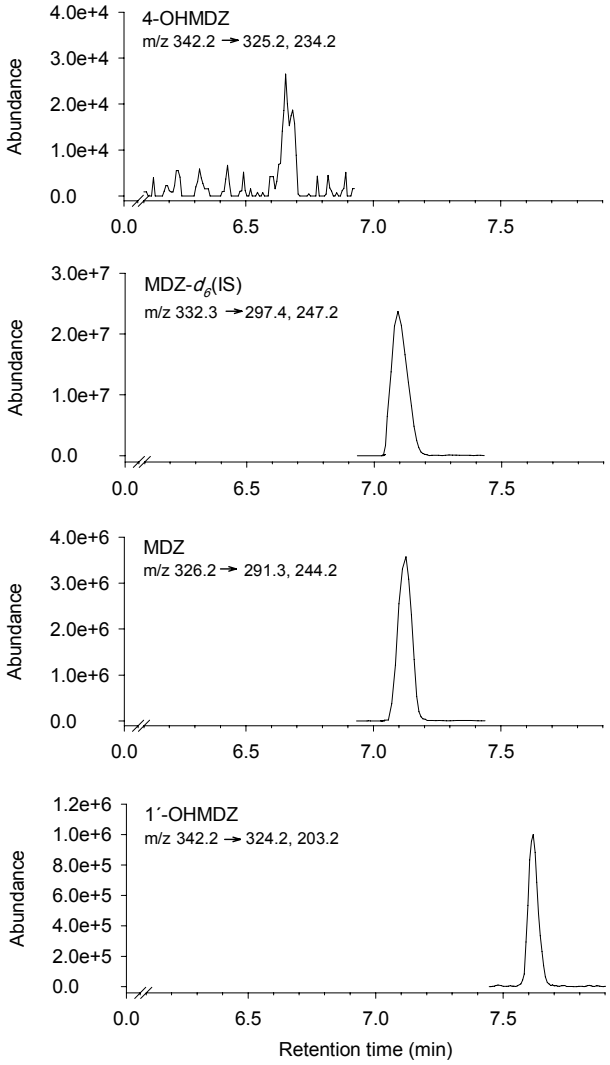

Figure 3. LC-MS/MS chromatograms (product ion scan) obtained from the analysis of an oral fluid sample drawn 30 min after the intravenous administration of a 2 mg single dose of midazolam to a healthy volunteer. The measured concentrations were 0.9 ng/ml for midazolam (MDZ) and 0.2 ng/ml 1´-hydroxymidazolam (1´-OHMDZ). The concentration of 4-OHMDZ was too low for quantification. The product ions used for quantification are the same ones as those shown in Figure 1.

In the present study, concentrations of MDZ, 1´-OHMDZ and 4-OHMDZ have also been determined in plasma, in order to evaluate a possible correlation between the oral fluid and plasma drug concentration. The semi-logarithmic concentration-versus-time curves of MDZ

and its major metabolite in oral fluid and plasma are shown in Figure 4. To our knowledge, this is the first method that has shown a sufficient sensitivity permitting the determination of the pharmacokinetic profiles of MDZ and its major metabolite in oral fluid. Quantification of MDZ was at least possible for 8 h after intravenous administration of a single intravenous dose of 2 mg MDZ. As compared to plasma, the oral fluid MDZ concentrations were roughly two orders of magnitude lower, which was assigned to the extensive binding of MDZ to plasma proteins of about 96%. However, Figure 4 shows that the patterns of MDZ and 1´-OHMDZ pharmacokinetic profiles in oral fluid and plasma agreed well with each other. The comparison of oral fluid and plasma concentrations from 30 to 480 min after administration showed a linear relationship (R^2=0.9717), suggesting the existence of a good correlation (Figure 5). Further studies are necessary to definitely confirm these results and to proof the suitability of oral fluid for CYP3A phenotyping.

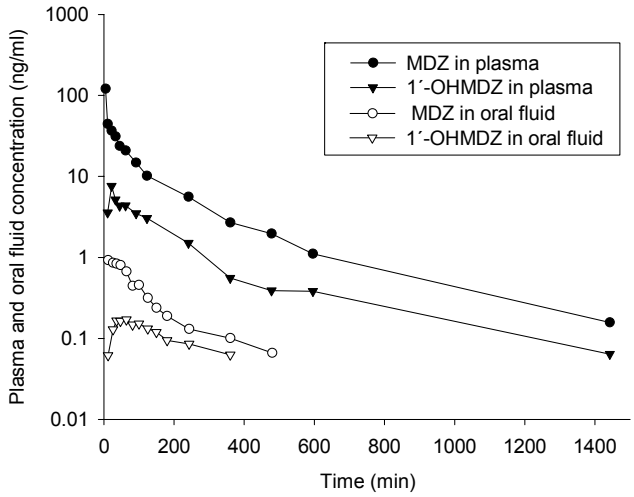

Figure 4. Semi-logarithmic concentration-versus-time profiles of midazolam (MDZ) and 1´-hydroxymidazolam (1´-OHMDZ) in oral fluid and plasma following a single intravenous dose (2 mg) of midazolam in a healthy volunteer.

One of the major disadvantages of using oral fluid as a diagnostic specimen is that subjects may produce insufficient sample volume for analysis. In the study of Quintela *et al.,* an adequate oral fluid volume could be obtained in <50% of cases using a Salivette® device (Quintela et al., 2004) . Stimulation of the oral fluid flow by various methods might increase the sample volume and therefore permit the collection of larger samples in a shorter time, but this method may impact the analytical results, e.g. by altering the oral fluid pH (Choo and Huestis, 2004). In our study, collection of oral fluid by spitting into a polypropylene tube

produced enough oral fluid volume (> 1 ml) without necessitating stimulation of saliva production for the quantitative analysis.

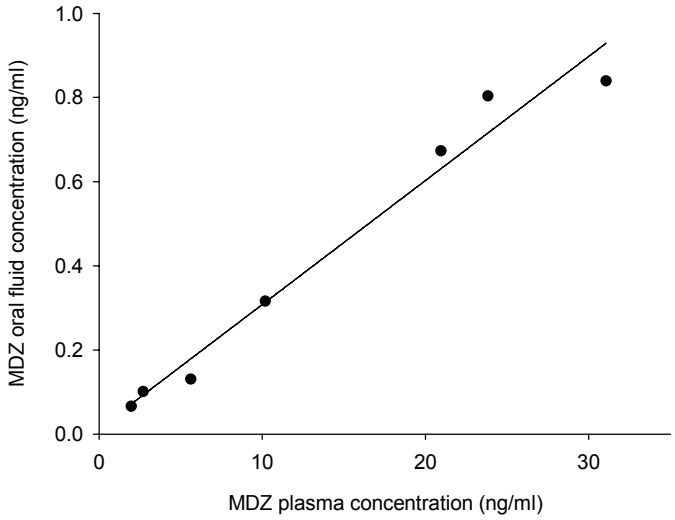

Figure 5. Correlation between MDZ plasma and saliva concentrations at 30-480 min after the administration of a 2 mg single intravenous dose of MDZ. The relationship is characterised by the following equation y=0,0295x + 0,0132 (R^2=0,9717).

4.5 Conclusions

In this paper, we presented the development of a sensitive, selective and reliable LC-MS/MS method which allows for the first time the simultaneous determination of MDZ and two of its metabolites in human oral fluid. It has been completely validated, showing good results for all the parameters studied. Since the method can be used to determine the pharmacokinetics of MDZ and 1´-OHMDZ in oral fluid and since a correlation between plasma and oral fluid MDZ concentrations seems to be very probable, the described method should be suitable for assessing the CYP3A activity in oral fluid. This may offer several advantages as compared to other matrices such as plasma or urine. The possibility of conducting simple and non-invasive CYP3A phenotyping with our method has to be investigated in a larger population under various conditions of CYP3A activity.

Acknowledgements

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5 Pharmacokinetics of intravenous and oral midazolam in plasma and saliva in humans: usefulness of saliva as matrix for CYP3A phenotyping

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5.1 Summary

Aims

To compare midazolam kinetics between plasma and saliva and to find out whether saliva is suitable for CYP3A phenotyping.

Methods

This was a two way cross-over study in eight subjects treated with 2 mg midazolam iv or 7.5 mg orally under basal conditions and after CYP3A induction with rifampicin.

Results

Under basal conditions and iv administration, midazolam and 1′-hydroxymidazolam (plasma, saliva), 4-hydroxymidazolam and 1′-hydroxymidazolam-glucuronide (plasma) were detectable. After rifampicin, the AUC of midazolam [mean differences plasma 53.7 (95% CI 4.6, 102.9) and saliva 0.83 (95% CI 0.52, 1.14) ng ml^{-1} h] and 1′-hydroxymidazolam [mean difference plasma 11.8 (95% CI 7.9, 15.7) ng ml^{-1} h] had decreased significantly. There was a significant correlation between the midazolam concentrations in plasma and saliva (basal conditions: r = 0.864, $P < 0.0001$; after rifampicin: r = 0.842, $P < 0.0001$). After oral administration and basal conditions, midazolam, 1′-hydroxymidazolam and 4-hydroxymidazolam were detectable in plasma and saliva. After treatment with rifampicin, the AUC of midazolam [mean difference plasma 104.5 (95% CI 74.1, 134.9) ng ml^{-1} h] and 1′-hydroxymidazolam [mean differences plasma 51.9 (95% CI 34.8, 69.1) and saliva 2.3 (95% CI 1.9, 2.7) ng ml^{-1} h] had decreased significantly. The parameters separating best between basal conditions and post-rifampicin were: (1′-hydroxymidazolam + 1′-hydroxymidazolam-glucuronide)/midazolam at 20–30 min (plasma) and the AUC of midazolam (saliva) after iv, and the AUC of midazolam (plasma) and of 1′-hydroxymidazolam (plasma and saliva) after oral administration.

Conclusions

Saliva appears to be a suitable matrix for non-invasive CYP3A phenotyping using midazolam as a probe drug, but sensitive analytical methods are required.

5.2 Introduction

Midazolam (MDZ) is a short-acting benzodiazepine with hypnotic, anticonvulsant, muscle-relaxant and anxiolytic properties. In clinical practice, it is used for the induction of anesthesia, sedation and the treatment of generalized seizures or status epilepticus (Nordt and Clark, 1997). Furthermore, MDZ is a widely accepted probe drug for CYP3A phenotyping (Streetman et al., 2000). MDZ is rapidly metabolized by cytochrome P450 3A (CYP3A) to its primary, pharmacologically active metabolite 1´-hydroxymidazolam (1´-OHMDZ) and, to a smaller extent, to 4-hydroxymidazolam (4-OHMDZ) and 1,4-dihydroxymidazolam. These metabolites are conjugated and then excreted as glucuronides in the urine (Bauer et al., 1995; Heizmann and Ziegler, 1981; Kronbach et al., 1989).

Oral fluid, consisting of saliva from the salivary glands and the gingival cervical sulci, mucosal cells, bacteria and food debris, has been increasingly used as an alternative biological matrix for therapeutic drug monitoring, pharmacokinetic studies, and detection of illicit drugs (Aps and Martens, 2005; Choo and Huestis, 2004; Drummer, 2005; Kidwell et al., 1998; Kintz and Samyn, 2002; Maurer, 2005). Oral fluid testing has several advantages over more conventional biological matrices used for these purposes, such as blood or urine. Specimen collection is noninvasive and therefore less objectionable to patients, easy to perform without medical supervision and may represent a cost-effective approach for screening of large populations. Disadvantages include that people are sometimes unable to produce sufficient amounts of oral fluid for quantitative analysis and that substances tend to be detectable for shorter periods in oral fluid than in plasma (Choo and Huestis, 2004).

The most common way of drug molecules to pass from blood to oral fluid is passive diffusion through lipid membranes, which is restricted to molecules that are lipophilic, uncharged, and not bound to plasma proteins (Aps and Martens, 2005; Choo and Huestis, 2004). Therefore, the salivary drug concentration reflects only the free concentration of drugs in plasma. Active transport and ultrafiltration through pores in cell membranes are additional mechanisms for drugs and metabolites to reach oral fluid. Due to the extensive binding of benzodiazepines (MDZ 96%) to plasma proteins (Nordt and Clark, 1997), their concentrations in oral fluid are generally very low compared with plasma. For this reason, highly sensitive analytical methods must be used for their detection and quantification (Link et al., 2007).

CYP3A phenotyping with MDZ is based on the determination of MDZ clearance, necessitating serial blood sampling (Lee et al., 2002; Lee et al., 2006; Li et al., 2006). Although the more recently proposed single point methods in plasma are less invasive (Chaobal and Kharasch, 2005; Lin et al., 2001; Rogers et al., 2002; Zhu et al., 2001),

noninvasive methods would be welcome, both for research and for routine use. If the kinetics of MDZ and its principal metabolites could be assessed in saliva, it might be possible to use saliva as a matrix for CYP3A phenotyping after oral or intravenous administration of MDZ. As shown recently in a single subject, using a sensitive liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) method, it appears to be possible to determine the kinetics of MDZ in saliva (Link et al., 2007). Based on these results, we decided to study the kinetics of MDZ in human subjects after intravenous (iv) or oral (po) administration of the drug both under basal conditions and after CYP3A induction with rifampicin.

5.3 Methods

The clinical protocol was approved by the Ethics Committee of both Cantons of Basel. All subjects gave written informed consent before undergoing any study-related procedures.

5.3.1 Subjects

Ten healthy male volunteers (all non-smokers) were included in the study. The use of any drug, including over the counter medication, as well as vitamins and dietary or herbal supplements known to affect CYP3A activity was prohibited at least 14 days before study start and for the entire duration of the study. Subjects had to abstain from grapefruit-containing foods or juices at least 1 week prior to the study, as well as during and between the study sessions. Subjects with clinically significant abnormalities on pre-study examination, known or suspected history of alcohol or drug abuse or known hypersensitivity to benzodiazepines or to rifampicin were excluded. Caffeine containing food and beverages, and alcohol (24 h before and during study visits) were not permitted.

5.3.2 Study design

This study used an open-label, randomized, two way cross-over design. In phase I, subjects were randomly assigned to receive either an oral dose of 7.5 mg midazolam (Dormicum® F. Hoffmann-LaRoche Ltd., Basel, Switzerland, sequence A, $n = 4$) with 200 ml of water in the morning or an iv infusion of 2 mg midazolam (sequence B, $n = 4$) to assess the pharmacokinetics of midazolam and two of its metabolites under baseline conditions (control). After the 24 h blood sample, subjects started to take 600 mg rifampicin (Rimactan®, 600 mg coated tablet, Medika AG, Aesch, Switzerland) once daily in the

morning for 6 days to induce hepatic as well as intestinal CYP3A activity. One day after the last rifampicin dose, subjects received a second dose of 7.5 mg midazolam orally (sequence A) or 2 mg midazolam iv (sequence B) to assess the kinetics of MDZ and its metabolites after CYP3A induction. Phase I was followed by a wash out phase of at least 5 weeks. During this washout phase, subjects were probed weekly with oral MDZ (7.5 mg, *n* = 4 subjects) or with iv MDZ (2 mg, *n* = 4 subjects) to assess CYP3A induction. On day 43 phase II started: subjects assigned to sequence A received 2 mg midazolam iv and subjects assigned to sequence B received 7.5 mg midazolam orally and again the pharmacokinetics of midazolam and its metabolites were assessed before and 1 day after induction with rifampicin 600 mg orally once daily for 6 days. Rifampicin was provided in blister packages that were returned by the subjects for the assessment of compliance.

All subjects were asked to stay in supine position until the sedative effects of MDZ had resolved. Additionally, subjects were monitored with a pulse oximeter for 2 h after MDZ administration. Standardized meals were served 4 h and 8 h following drug administration. Subjects were permitted to drink water as desired.

5.3.3 Sampling

On each test day, after an overnight fast, an iv catheter was inserted into a forearm vein and blood samples were collected into heparinized tubes. For iv administration of MDZ, a second iv catheter was placed on the opposite forearm.

Blood samples (5.5 ml each) were taken before and at 10, 20, 30, 40, 60, 90, 120, 180, 240, 360, 480, 600 and 1440 min after MDZ administration. After iv midazolam administration, an additional blood sample was drawn at 5 min and the 180 min sample was skipped. For the assessment of CYP3A induction during the washout phase, blood samples were obtained before drug administration and after 30, 60, 90, 120 and 240 min. Oral fluid samples were collected by spitting into a polypropylene tube without pharmacological stimulation before and at 10, 30, 40, 60, 80, 100, 120, 150, 180, 240, 360 and 480 min after drug intake. An additional sample was drawn 20 min after iv midazolam administration. All blood and oral fluid samples were placed on ice and centrifuged within 30 min at 2500 *g* for 10 min at 5°C after collection. Plasma and saliva (after centrifugation of oral fluid, the supernatant was considered to be saliva) were then separated and stored at -70°C until analysis.

In order to test for MDZ contamination in the oral cavity, subjects were instructed to rinse their mouth carefully with 200 ml of water after having ingested oral MDZ and 200 ml of water. After having rinsed the mouth, a saliva sample was obtained as described above. These saliva samples were randomly analyzed for MDZ. MDZ was not detectable in any of

these saliva samples, indicating that there was no carry over of orally administered MDZ into saliva.

5.3.4 Drug analysis

The concentrations of unconjugated MDZ, 1´-OHMDZ and 4-OHMDZ were determined in plasma and saliva by LC-MS/MS analysis as described previously (Link et al., 2007). In brief, 1 ml plasma or saliva were spiked with 10 µl internal standard (1.2 µg ml^{-1} solution of MDZ-d_6, IS) and 1 ml 0.75 M glycine buffer (pH 9.2). Standards containing known amounts of MDZ and its metabolites were prepared similarly using the respective blank matrix. Liquid-liquid extraction was carried out by using n-hexane : dichlormethane (73 : 27, v : v). After centrifugation, the aqueous layer was frozen in a dry ice/acetone bath and the organic phase was transferred into a silanized glass tube. The organic fraction was evaporated to dryness under nitrogen and the remaining residue was reconstituted in 150 µl acetonitrile : water (23 : 77, v : v). 20 µl of the sample was injected into the LC-MS/MS system. Separation was performed at 40°C using a reversed-phase Luna C18 analytical column, 100 x 2.1 mm i.d., 3 µm (Phenomenex, Torrance, CA, USA) and gradient elution at a flow rate of 0.24 ml min^{-1}. Detection was carried out using an LCQ^{Deca} ion trap mass spectrometer (Thermo Fischer Scientific, Inc., Waltham, MA, USA) equipped with an electrospray ionization (ESI) source operating in the positive ion mode (4 kV). For the quantification of MDZ and its metabolites the MS/MS product ion scan mode was chosen using a mass filter for the two must abundant product ions: MDZ, m/z 326.2→291.3, 244.2; 1´-OHMDZ, m/z 342.2→324.2, 203.2; 4-OHMDZ, 342.2→325.2, 234.2 and MDZ-d_6, 332.3→297.4, 247.2. Intra- and interday coefficients of variation (CV) were determined at three different concentrations by analyzing six replicates of each quality control concentration (0.6, 3.7 and 15 ng ml^{-1} for plasma and 0.3, 3.7 and 15 ng ml^{-1} for saliva). The corresponding CV ranged from 1.2 to 7.3% for MDZ, from 1.3 to 7.4% for 1′-OHMDZ and from 2.6 to 7.6% for 4-OHMDZ, respectively, in plasma and saliva. The limit of quantification (LOQ) was determined based on the criteria that 1) the signal to noise ratio at the LOQ is at least 5 and that 2) the analyte response at LOQ can be determined with a CV of ≤ 20% and an accuracy of 80–120% (n = 6). For MDZ and both metabolites the LOQs were 0.05 ng ml^{-1} in both biological matrices with accuracies between 102.1 and 106.4% and CV between 5.4 and 10.4%. The accuracy for all compounds was between 92.1 and 102.3% in plasma and between 96.6 and 108.0% in saliva. Linear calibration curves were obtained between 0.05 to 20 ng ml^{-1} for midazolam and the hydroxy-metabolites in plasma and saliva (r^2 > 0.998). Plasma samples with high concentrations of MDZ and metabolites were diluted up

to 10-fold with drug free human plasma in order to reach the calibration range. No interference from rifampicin was noted during the analysis.

For the determination of total 1´-OHMDZ in plasma (conjugated and unconjugated) the same analytical method was used after treatment of the sample with β-glucuronidase. To 1 ml of plasma, 1 ml 0.2 M ammonium acetate buffer (pH 4.7) and 40 µl of a solution containing 20 000 U ml^{-1} of β-glucuronidase from *Helix pomatia* (G7770, Sigma, Buchs, Switzerland) were added and the mixture was incubated at 37°C. After 20 h, the reaction was stopped by adding 50 µl of aqueous ammonia (8%). IS (10 µl) and 1 ml 0.75 M glycine buffer (pH 9.2) were added and the sample was then further processed in the same manner as described above. Calibration curves, ranging from 0.05–20 ng ml^{-1} for all three analytes, were constructed in drug-free plasma under the same conditions. 1´-OHMDZ glucuronide concentration was determined by subtracting unconjugated 1´-OHMDZ concentrations from those obtained after treatment with β-glucuronidase. 4-OHMDZ was found to be unstable under hydrolytic conditions, with a later eluting degradation product of this metabolite (specific precursor ion m/z 343.2 and product ion m/z 315.5) being apparent in the chromatograms (Link et al., 2007; Paine et al., 1996). Therefore, 4-OHMDZ glucuronide concentrations were not determined.

Plasma and saliva samples were prepared and measured once. Each subject's samples for assessing the concentration of MDZ and its metabolites at baseline and after induction of CYP3A4 were analyzed on the same day for the intravenous as well as for the oral MDZ administration.

5.3.5 Pharmacokinetic analysis

Plasma and saliva MDZ, 1´-OHMDZ and 4-OHMDZ data were analyzed using either non-compartmental or compartmental methods (WinNonlin, version 5.01, Pharsight Corp., Mountain View, CA, USA), depending on the model fitting the concentration vs. time data the best. The area under the drug concentration–time curves (AUC_{inf}) were calculated using the trapezoidal rule with extrapolation to infinity. The elimination rate constant (k_e) was calculated by log-linear regression analysis after semilogarithmic transformation of the data. The elimination half-life ($t_{1/2}$) was calculated as follows:

$$t_{1/2} = \frac{ln2}{k_e} \qquad (1)$$

Systemic clearances (CL_{iv}) after iv MDZ administration were calculated as follows:

$$CL_{iv} = \frac{dose_{iv}}{AUC_{iv}} \qquad (2)$$

The apparent oral clearances (CL/F) were calculated as the ratio of dose administered over AUC_{po}. Bioavailability (F_{oral}) was calculated as follows:

$$F_{oral} = \frac{AUC_{po} \times dose_{iv}}{AUC_{iv} \times dose_{po}} \qquad (3)$$

The maximum drug concentration in plasma or saliva (C_{max}) and time to reach these peak concentrations (t_{max}) were obtained directly from the concentration–time data. The volume of distribution (V_d) was calculated as follows:

$$V_d = \frac{dose}{AUC * k_e} \qquad (4)$$

5.3.6 Statistics

Results are expressed as median and range. Statistical analysis was performed using the SPSS software package for Windows, version 15.0 (SPSS, Chicago, IL, USA). The nonparametric Wilcoxon signed rank test for paired data was used to analyse changes in the pharmacokinetic parameters before (baseline conditions, control) and after rifampicin administration (CYP3A induction) in both biological matrices. For relevant endpoints mean difference and corresponding 95% confidence intervals (CI) of the difference were calculated. A two-tailed *P* value less than 0.05 was considered to be statistically significant. Linear regression analysis was performed using least squares fitting to assess the relation between the concentrations of MDZ or 1´-OHMDZ in plasma and saliva.

5.4 Results

Ten male subjects were enrolled in the study. Eight subjects completed the study, one subject withdrew during the second induction phase (phase II) because of adverse reactions to rifampicin (headache, flu-like symptoms, abdominal pain and diarrhoea) and one subject discontinued the treatment for personal reasons unrelated to the study medication. The data of the excluded subjects were not used for analysis. Mean ± standard deviation (SD) age was 27.7 ± 9.6 years (range: 21–46 years) and a mean weight 70 ± 6 kg

(range: 60–78 kg). As expected, most subjects experienced light sedation after the administration of midazolam. No other adverse events were detected.

After iv administration of 2 mg MDZ, MDZ, 1´-OHMDZ and 4-OHMDZ could be detected in plasma and the kinetics of MDZ and its metabolites could be assessed. MDZ was rapidly hydroxylated, maximal concentrations of 1´-OHMDZ and 4-OHMDZ were reached after 22 and 42 min, respectively (Figure 1 and Table 1). The half-lives of the two hydroxy-metabolites were similar to MDZ, indicating rapid glucuronidation of the metabolites. MDZ and 1´-OHMDZ could also be detected in saliva, whereas the concentration of 4-OHMDZ was too low for reliable quantification. There was a significant linear correlation between the MDZ concentrations obtained in plasma and in saliva, both under basal conditions (r = 0.864, $P < 0.0001$) and after induction with rifampicin (r = 0.842, $P < 0.0001$, Figure 2). As expected from the extensive protein-binding of MDZ (Bodmer et al., 2008), the concentrations of MDZ were much lower in saliva than in plasma (Figure 1). For the same reason, V_d and clearance of MDZ in saliva were much higher compared with the values obtained in plasma, whereas the elimination half-life was lower (Table 1). The kinetics of 1′-OHMDZ could also be assessed in saliva (Figure 1). Compared with plasma, the maximal concentrations in saliva appeared clearly later (22 vs. 52 min). While the concentrations of 1′-OHMDZ were also lower in saliva than in plasma, the difference was not as large as for MDZ, compatible with lower binding to serum proteins (Bodmer et al., 2008).

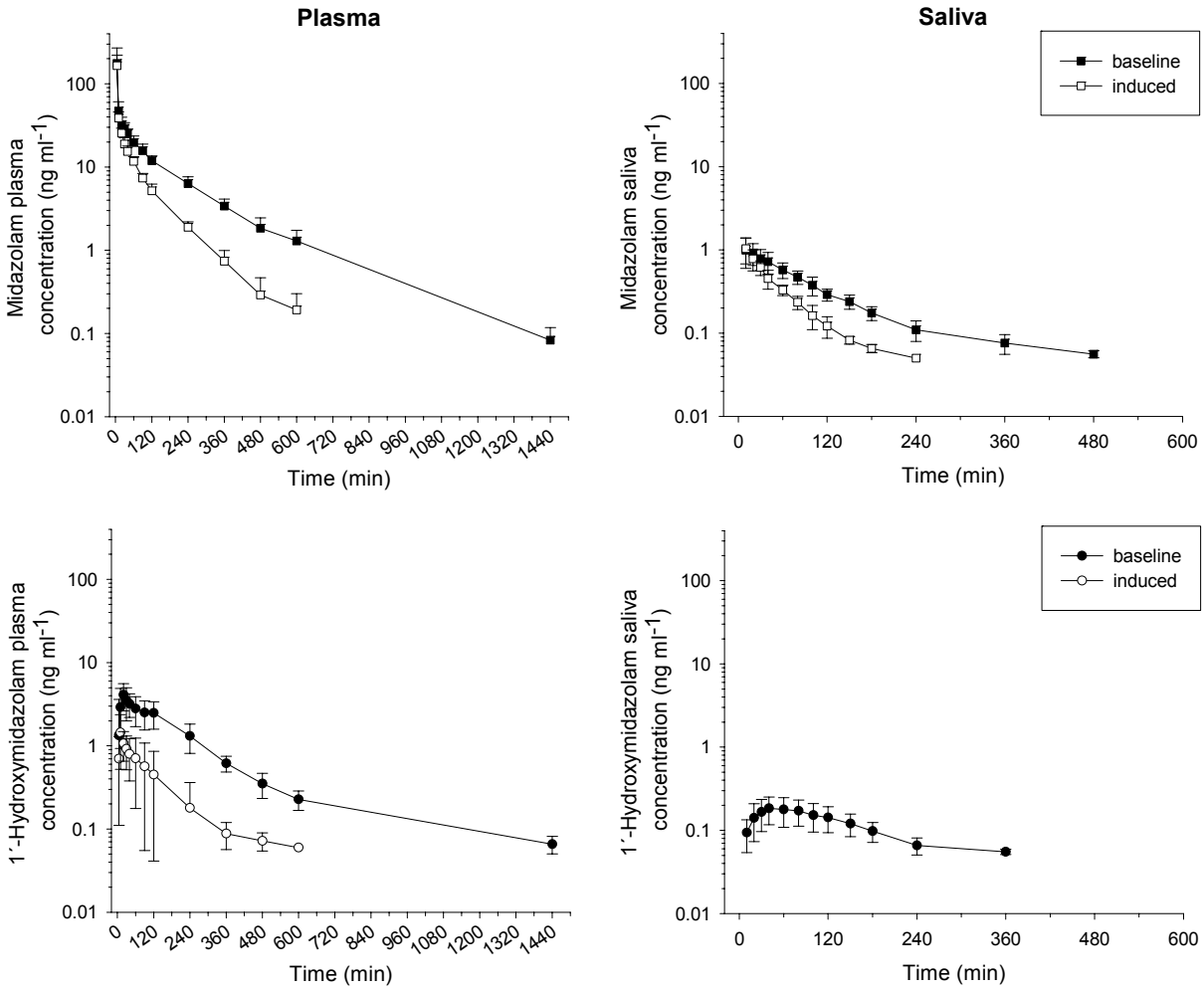

Figure 1. Kinetics of MDZ and 1´-OHMDZ in plasma (left panel) and saliva (right panel) after iv administration of 2 mg MDZ in eight healthy volunteers. CYP3A induction by rifampicin is associated with a decrease in the concentrations MDZ (plasma and saliva) and 1´-OHMDZ (plasma). The data are presented as mean ± SD. The results of the kinetic calculations are presented in Table 1.

Table 1. Pharmacokinetic parameters of midazolam and its metabolites after intravenous administration of 2 mg midazolam before (control) and after treatment with rifampicin in eight healthy subjects

	Plasma		**Saliva**	
	Control	Rifampicin	Control	Rifampicin
Midazolam				
V_d (l)	3.53 (0.35-26.97)	2.6 (1.2-16.8)	1918 (943-2963)	1793 (836-2146)
V_d/kg (l kg^{-1})	0.05 (0.01-0.39)	0.04 (0.02-0.23)	28.2 (15.7-43.6)	26.2 (13.9-29.0)
AUC_{inf} (ng ml^{-1} h)	125.8 (83.7-268.7)	82.4 (58.5-101.9)**	1.7 (1.4-2.4)	0.96 (0.77-1.37)**
$t_{1/2}$ (h)	2.9 (1.8-3.4)	1.4 (1.1-1.8)**	1.3 (0.7-1.6)	0.58 (0.40-0.73)**
CL (l h^{-1})	15.9 (7.4-23.9)	24.4 (19.6-34.2)*	1185 (841-1448)	2081 (1457-2590)**
C_{max} (ng ml^{-1})	151 (89-388)	167 (83-253)	1.05 (0.67-2.12)	1.12 (0.93-2.39)
1´-hydroxymidazolam				
AUC_{inf} (ng ml^{-1} h)	14.7 (8.9-21.6)	2.0 (1.4-6.4)**	0.68 (0.45-1.12)	na[1]
$t_{1/2}$ (h)	2.2 (1.8-4.2)	1.4 (1.2-2.7)*	2.1 (1.7-3.5)	na
C_{max} (ng ml^{-1})	3.5 (1.7-6.5)	1.3 (0.59-3.54)**	0.18 (0.11-0.31)	na
t_{max} (min)	22.0 (20.0-47.0)	10.5 (10.0-61.0)	52.0 (35.0-80.0)	na
4-hydroxymidazolam				
AUC_{inf} (ng ml^{-1} h)	1.9 (1.5-2.5)	na	na	na
$t_{1/2}$ (h)	2.9 (2.7-3.6)	na	na	na
C_{max} (ng ml^{-1})	0.39 (0.26-0.52)	na	na	na
t_{max} (min)	44.4 (28.05-61.30)	na	na	na

Data are presented as median and range. V_d, volume of distribution; AUC_{inf}, area under the concentration-time curve extrapolated to infinity; $t_{1/2}$, elimination half-life; CL, clearance; C_{max}, maximum plasma or saliva concentration; t_{max}, time to maximum plasma or saliva concentration. * $P<0.05$ rifampicin versus control in the same matrix. ** $P=0.012$ rifampicin versus control in the same matrix. na[1]: not assessable.

After induction of CYP3A by rifampicin, the AUC and the half-life of MDZ in plasma dropped significantly [mean difference for AUC 53.7 ng ml^{-1} h (95% CI 4.6, 102.9), P = 0.012, mean difference for half-life 1.2 h (95% CI 0.7, 1.7)], whereas the clearance increased [mean difference 8.9 l h^{-1} (95% CI 4.2, 13.7), P < 0.05, Figure 1, Table 1]. Unexpectedly, AUC and half-life dropped also for 1′-OHMDZ, whereas 4-OHMDZ could not be quantified reliably. In saliva, only MDZ could be quantified, but not 1′-OHMDZ or 4-OHMDZ. Similar to plasma, AUC and half-life of MDZ dropped significantly [mean difference AUC 0.83 ng ml^{-1} h (95% CI 0.52, 1.14), mean difference half-life 0.62 h (95% CI 0.38, 0.85), P = 0.012], whereas the clearance of MDZ increased.

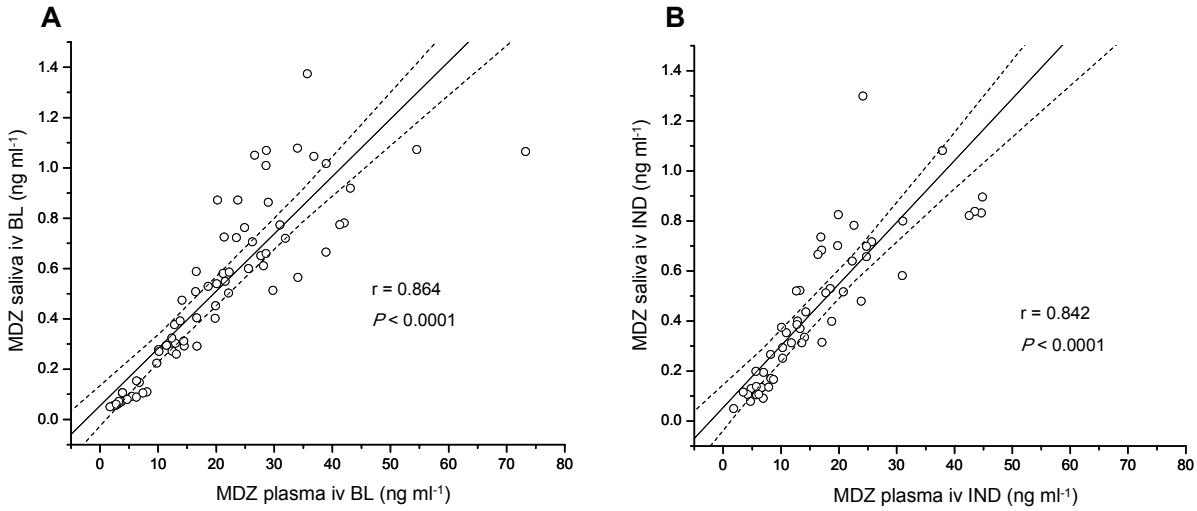

Figure 2. Correlation between MDZ concentrations in plasma and saliva. Single values and the 95% confidence interval are shown. After iv administration of 2 mg MDZ, there is a linear correlation between the MDZ concentrations determined in plasma and saliva both under basal conditions (left panel, r = 0.864) and after treatment with rifampicin (right panel, r = 0.842).

In order to find the reason for the unexpected drop of the 1′-OHMDZ concentrations in plasma and in saliva after induction with rifampicin, 1′-OHMDZ-glucuronide was analyzed in both matrices. As shown in an individual subject, the reason for the fall of the plasma concentration of 1′-OHMDZ was the rapid conversion into the corresponding glucuronide (Figure 3). Accordingly, 20 and 30 min after iv administration of MDZ, the 1′-OHMDZ glucuronide plasma concentrations were approximately doubled in subjects treated with rifampicin compared with basal conditions (Table 2). In contrast to plasma, 1′-OHMDZ-glucuronide could not be detected in saliva (data not shown).

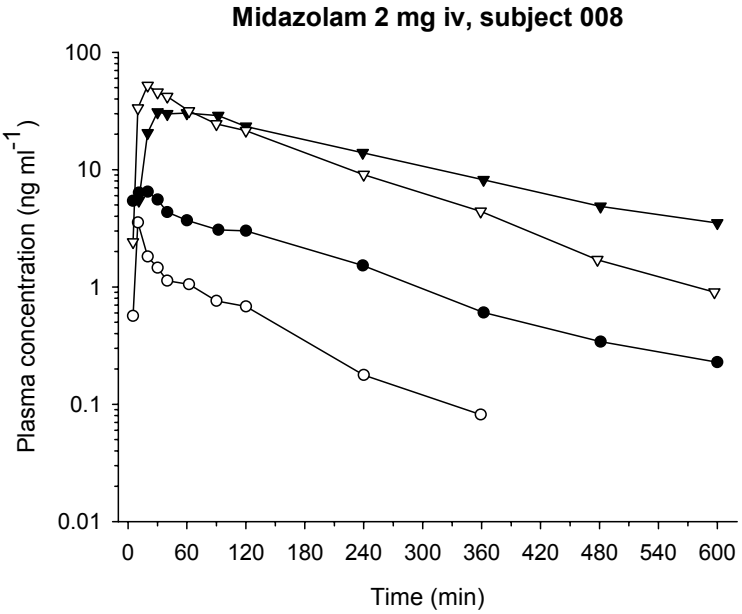

Figure 3. Plasma concentrations of 1´-OHMDZ and 1´-OHMDZ-glucuronide under basal conditions and after treatment with rifampicin in one subject. Surprisingly, CYP3A induction by rifampicin is associated with a decrease in the plasma concentrations of 1´-OHMDZ. The explanation for this finding is an increase in the plasma concentrations of 1'-OHMDZ-glucuronide. The 1´-OHMDZ concentrations at 20 and 30 min are presented in Table 2. 1´-OHMDZ baseline (●); 1´-OHMDZ-glucuronide baseline (▼);1´-OHMDZ induced (○); 1´-OHMDZ-glucuronide induced (▽).

Table 2. Plasma concentrations of midazolam (MDZ), 1´-hydroxymidazolam (1´-OHMDZ), 1´-hydroxymidazolam-glucuronide (1´-OHMDZ-gluc) and 1´-OHMDZ + 1´-OHMDZ-gluc (1´-$OHMDZ_{total}$) after intravenous administration of 2 mg midazolam before (control) and after pre-treatment with rifampicin in eight healthy subjects

	20 minutes		**30 minutes**	
	Control	Rifampicin	Control	Rifampicin
MDZ (ng ml^{-1})	32.4 (22.2-43.1)	24.8 (19.8-31.1)	29.2 (20.2-34.1)	19.2 (13.2-23.9)*
1´-OHMDZ (ng ml^{-1})	3.52 (2.06-6.46)	0.99 (0.59-1.81)*	3.10 (1.47-5.54)	0.73 (0.53-1.58)*
1´-OHMDZ-gluc (ng ml^{-1})	18.0 (11.9-25.2)	40.9 (29.3-61.6)*	22.7 (16.4-38.1)	40.5 (31.0-55.7)*
1´-$OHMDZ_{total}$ (ng ml^{-1})	20.9 (15.2-30.5)	41.8 (30.0-62.6)*	21.5 (16.4-38.1)	39.6 (31.0-55.7)*
1´-OHMDZ/MDZ	0.136 (0.074-0.150)	0.040 (0.024-0.073)*	0.104 (0.069-0.169)	0.040 (0.031-0.093)*
1´-OHMDZ-gluc/MDZ	0.497 (0.305-0.920)	1.56 (0.94-2.55)*	0.733 (0.446-1.16)	2.39 (1.27-2.87)*
1´-$OHMDZ_{total}$/MDZ	0.627 (0.391-1.07)	1.52 (0.968-2.59)*	0.840 (0.551-1.33)	2.34 (1.30-2.91)*

*P<0.008 rifampicin versus control at the same time point.

Good markers of induction with rifampicin (no overlap of the basal values with the values obtained after treatment with rifampicin) after iv administration of MDZ were in plasma the drop in the AUC of 1′-OHMDZ, the drop in the 1′-OHMDZ concentration (20 min), the increase in the 1′-OHMDZ-glucuronide concentration (20 min), the drop in the ratio 1′-OHMDZ : MDZ (20 min) and the increases in the ratios 1′-OHMDZ-glucuronide : MDZ and 1′-OHMDZ$_{total}$: MDZ (20 and 30 min) (Tables 1 and 2). Interestingly, while the AUC of MDZ in plasma showed some overlap between basal conditions and after treatment with rifampicin, there was no overlap of this ratio in saliva (Figure 4).

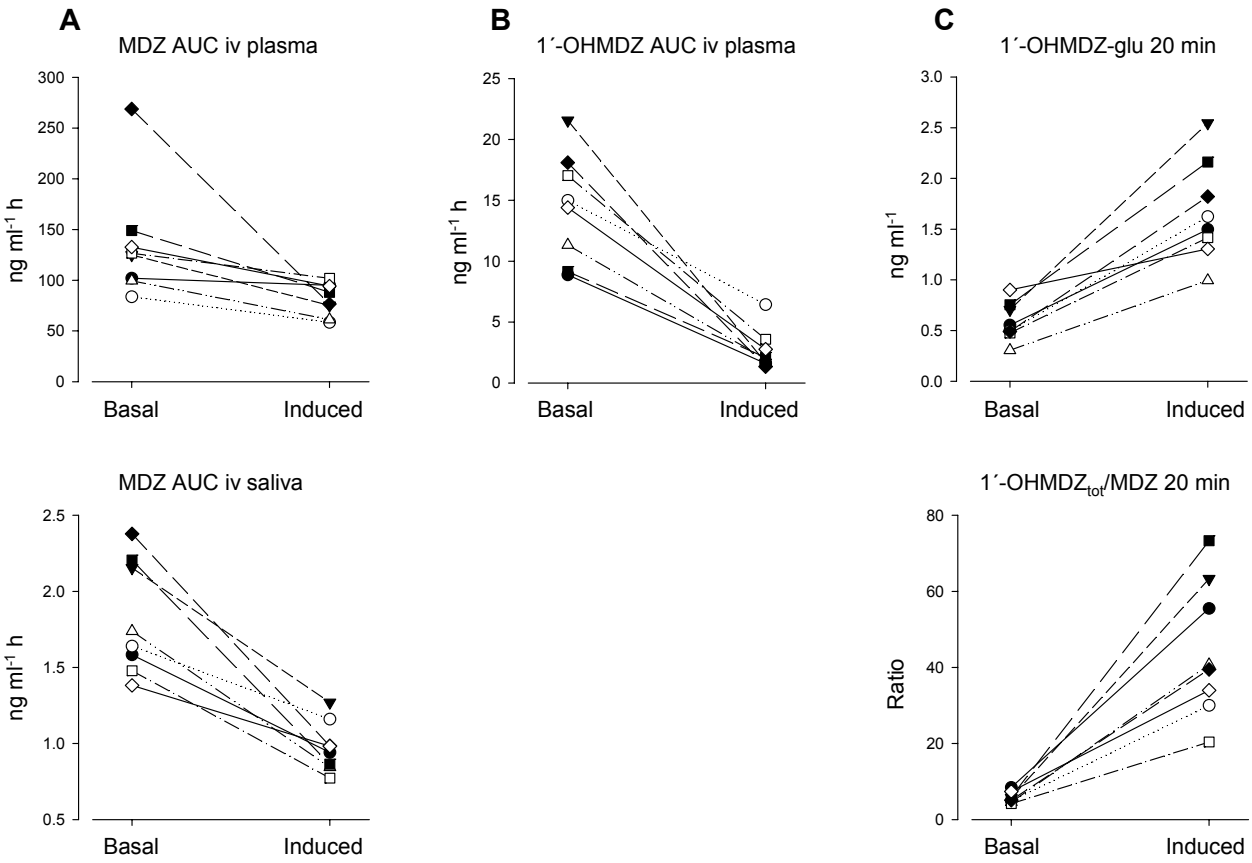

Figure 4. Markers of CYP3A induction after iv administration of MDZ. A) The AUC of MDZ under basal conditions and after treatment with rifampicin partially overlap in plasma, but not in saliva. B) The AUC of 1´-OHMDZ separates well between basal conditions and CYP3A induction in plasma, but could not be determined in saliva after treatment with rifampicin. C) A good separation between basal conditions and CYP3A induction in plasma was also obtained by the concentration of 1´-OHMDZ-glucuronide or the ratio (1´-OHMDZ + 1´-OHMDZ-glucuronide) : MDZ 20 or 30 minutes after administration of MDZ. (1´-OHMDZ + 1´-OHMDZ-glucuronide) = 1´-OHMDZ$_{tot}$

During the washout phase, the decrease in CYP3A induction was assessed to ascertain that the probands had reached basal conditions before starting phase II of the study. After stopping the treatment with rifampicin, it lasted 3 weeks until the AUC(0,4 h) (oral and iv administration of MDZ) had reached the values before ingestion of rifampicin in every subject (data not shown).

After oral ingestion of 7.5 mg MDZ, MDZ and its hydroxy-metabolites could be detected in plasma and in saliva (Figure 5) and the respective kinetics could be assessed (Table 3). Similar to iv administration, there was a significant linear correlation between MDZ plasma and saliva concentrations ($r = 0.913$, $P < 0.0001$; data not shown). Under basal conditions, the bioavailability calculated from the plasma data was in the range of 20%, whereas the half-life was 3.2 h, a value close to that obtained after iv administration. The ratios between the AUC of 1′-OHMDZ or 4-OHMDZ to MDZ were much higher after oral compared with iv administration of MDZ, most probably reflecting intestinal metabolism of MDZ. Accordingly, the concentrations of 1′-OHMDZ and 4-OHMDZ were higher in saliva after oral compared with iv administration, whereas the MDZ concentrations in saliva were comparable with the values after iv administration. After administration of rifampicin, the AUC of MDZ in plasma decreased dramatically compared with basal conditions [mean difference 104.5 ng ml^{-1} h (95% CI 74.1, 134.9), $P = 0.012$, Figure 5]. Similar to iv administration of MDZ, the concentrations of 1′-OHMDZ and 4-OHMDZ in plasma were lower after rifampicin compared with basal conditions. Accordingly, in saliva, MDZ and 4-OHMDZ could not be detected after treatment with rifampicin, whereas the concentration of 1′-OHMDZ was lower compared with basal conditions.

Good markers of induction with rifampicin (no overlap of the basal values with the values obtained after treatment with rifampicin) after oral administration of MDZ were the drop in AUC and C_{max} of MDZ and the drop in the AUC of 1′-OHMDZ in plasma as well as the drop in AUC and C_{max} of 1′-OHMDZ in saliva (Figure 6).

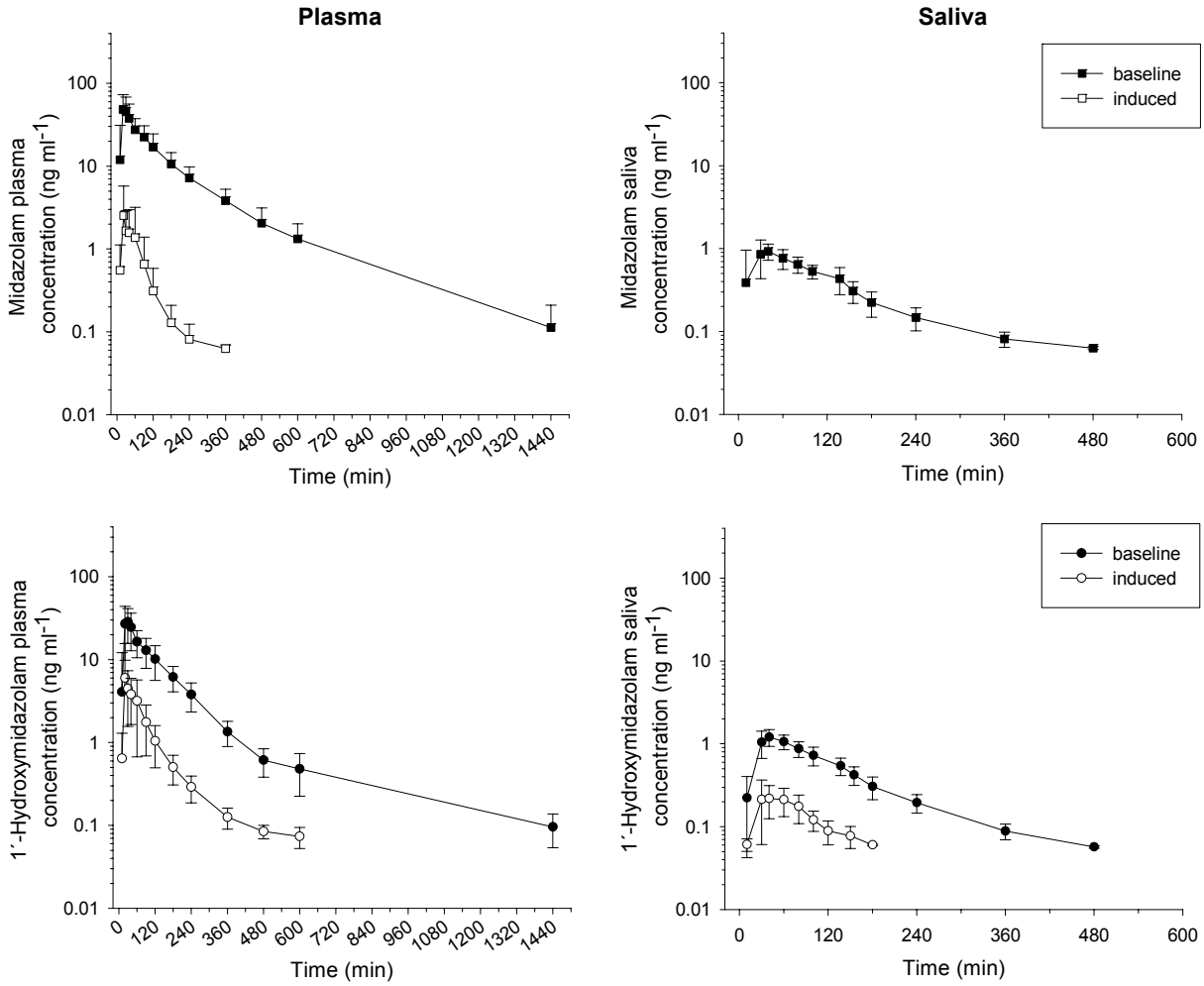

Figure 5. Kinetics of MDZ and 1´-OHMDZ in plasma (left panel) and saliva (right panel) after oral administration of 7.5 mg MDZ in eight healthy volunteers. Similar to iv administration, CYP3A induction is associated with a decrease in the plasma concentrations of MDZ (plasma) and 1´-OHMDZ (plasma and saliva). In saliva, MDZ can only be detected under basal conditions, but not after CYP3A induction. The data are presented as mean ± SD. The results of the kinetic calculations are presented in Table 3.

5.5 Discussion

With our study, we primarily wanted to answer the following questions: i) Can the kinetics of MDZ be assessed reliably in saliva? and ii) Can saliva be used as a matrix to determine CYP3A activity with MDZ as a probe drug? If yes, is the ability of the values obtained in saliva to separate CYP3A-induced from non-induced individuals comparable to plasma values?

Regarding the good correlation between the MDZ concentrations in plasma and saliva (Figure 2), it is evident that the kinetics of MDZ can be reliably assessed also in saliva both

Table 3. Pharmacokinetic parameters of midazolam and its metabolites after oral administration of 7.5 mg midazolam before (control) and after pre-treatment with oral rifampicin to eight healthy subjects

	Plasma		**Saliva**	
	Control	Rifampicin	Control	Rifampicin
Midazolam				
AUC_{inf} (ng ml^{-1} h)	102.9 (64.0-163.7)	1.6 (1.0-7.2)**	2.5 (1.1-3.3)*	na[1]
$t_{1/2}$ (h)	3.2 (2.1-4.8)	1.8 (0.98-8.21)	6.9 (1.3-13.4)	na
C_{max} (ng ml^{-1})	63.1 (25.9-80.2)	2.2 (0.64-10.4)**	0.94 (0.61-1.58)	na
t_{max} (min)	20.6 (12.9-72.4)	29.9 (13.2-39.6)	39.2 (12.7-86.8)	na
F	0.19 (0.15-0.33)	0.005 (0.004-0.02)	na	na
1´-hydroxymidazolam				
AUC_{inf} (ng ml^{-1} h)	56.6 (30.7-91.9)	5.4 (3.9-13.1)**	2.8 (1.9-3.2)	0.44 (0.31-0.62)**
$t_{1/2}$ (h)	5.8 (1.8-21.8)	3.2 (1.8-6.3)	1.6 (1.2-2.0)	0.91 (0.55-1.26)*
C_{max} (ng ml^{-1})	36.9 (15.0-52.6)	4.4 (2.0-11.7)**	1.3 (0.75-1.65)	0.21 (0.14-0.58)**
t_{max} (min)	24.5 (13.7-70.1)	31.2 (18.2-39.4)	43.5 (30.0-80.0)	43.5 (32.0-80.0)
4-hydroxymidazolam				
AUC_{inf} (ng ml^{-1} h)	6.3 (5.4-9.8)	na	0.37 (0.29-0.70)	na
$t_{1/2}$ (h)	1.8 (1.4-2.0)	na	2.3 (1.5-3.3)	na
C_{max} (ng ml^{-1})	3.1 (1.5-3.9)	na	0.10 (0.09-0.12)	na
t_{max} (min)	31.5 (20.0-93.0)	na	61.0 (42.0-100.0)	na

Data are presented as median and range. V_d, volume of distribution; AUC_{inf}, area under the concentration-time curve extrapolated to infinity; $t_{1/2}$, elimination half-life; CL, clearance; C_{max}, maximum plasma or saliva concentration; t_{max}, time to maximum plasma or saliva concentration. * $P<0.05$ rifampicin versus control in the same matrix. ** $P=0.012$ rifampicin versus control in the same matrix. na[1]: not assessable.

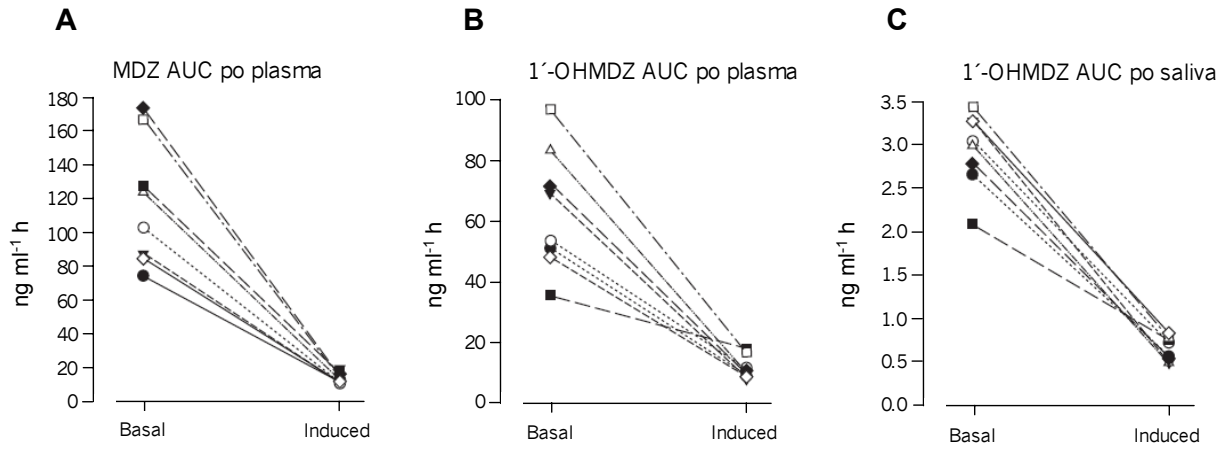

Figure 6. Markers of CYP3A induction after oral administration of MDZ. In plasma, the drop in the AUC of MDZ (A) and in the AUC of 1´-OHMDZ (B) separate well between basal conditions and CYP3A induction. In saliva, the drop in the AUC of 1´-OHMDZ (C) shows an excellent separation between basal conditions and CYP3A induction.

after iv and oral administration of MDZ. However, several points have to be taken into account. Because MDZ is highly plasma protein-bound and only free MDZ distributes into saliva, the MDZ concentration is much lower in saliva than in plasma. In contrast to plasma, where we determined the kinetics of total MDZ, the kinetics of free MDZ was therefore assessed in saliva. Accordingly, the AUC of MDZ was much lower and the clearance much higher in saliva compared with plasma. Similar to MDZ, 1´-OHMDZ kinetics could be calculated in plasma as well as in saliva. Interestingly, 1´-OHMDZ reached its maximum concentration in saliva much later than in plasma, indicating a slow transport into saliva. The exact mechanism of how 1´-OHMDZ gains access to saliva is not known but may be by diffusion, depending on protein binding and lipophilicity of the compound (Loscher and Frey, 1984). Most probably due to the lower binding to plasma proteins compared with MDZ (Bodmer et al., 2008), the ratio of 1´-OHMDZ to MDZ was higher in saliva than in plasma. 1´-OHMDZ-glucuronide could not be detected in saliva, most probably reflecting low or even lacking transport into this compartment due to its hydrophilic properties. Similar findings have been reported for the glucuronides of morphine (Friedrich et al., 1992).

After induction with rifampicin, the clearance of MDZ was increased compared with basal values, and, accordingly, the AUC decreased both after iv and oral administration of MDZ. However, the effects of rifampicin differed in their extent between iv and oral administration, most probably reflecting both hepatic and intestinal induction of the first pass metabolism of oral MDZ. While the AUC of MDZ in plasma dropped only by 34.5% after iv administration, this drop was 98.5% after oral ingestion of MDZ. These effects of rifampicin are consistent

with other reports in the literature (Backman et al., 1996a; Gorski et al., 2003). After oral administration, MDZ could therefore not be detected in saliva, precluding its use as a marker of CYP3A induction with the quantification limits of current analytical methods.

Interestingly, the plasma concentrations and AUCs of the hydroxy-metabolites of MDZ did not show the expected increase, but a decrease, after treatment with rifampicin. As evidenced by the current study for 1´-OHMDZ, the hydroxy-metabolites of MDZ undergo extensive glucuronidation, explaining our findings. Although it is well known that intestinal and hepatic metabolism of MDZ is altered significantly by induction (Gorski et al., 2003) or inhibition (Kupferschmidt et al., 1995) of CYP3A activity, it was less clear that induction of UDP-glucuronyltransferases exhibits such dramatic effects on the kinetics of the hydroxy-MDZ metabolites. Different authors have reported an up to three-fold increase in glucuronidation activity in humans (Ebert et al., 2000), human hepatocytes (Soars et al., 2004) or rats (Oesch et al., 1996) after treatment with rifampicin (Ebert et al., 2000; Oesch et al., 1996; Soars et al., 2004). In contrast, Reinach *et al*. did not find a significant effect of rifampicin on AZT glucuronidation in human hepatocytes (Reinach et al., 1999). Until very recently, glucuronidation of 1´-OHMDZ was not well characterized. Recently, Zhu *et al*. (Zhu et al., 2008) demonstrated *in vitro* that glucuronidation of 1´-OHMDZ was catalyzed by UGT2B4 and UGT2B7. Nakajima *et al*. (Nakajima et al., 1999) studied the effects of glucocorticoids (moderate inducers of CYP3A activity) on MDZ kinetics in humans. Although not statistically significant, a trend towards a lower AUC of MDZ and 1´-OHMDZ in plasma, and a higher cumulative excretion of 1´-OHMDZ-glucuronide in the urine compared with baseline could be observed. In addition, Eap *et al*. (Eap et al., 2004) studied single dose MDZ kinetics before and after induction with rifampicin in human subjects. After 4 days of treatment with 450 mg rifampicin per day, they could demonstrate a significant decrease of MDZ and 1´-OHMDZ plasma concentrations. Interestingly, in their study, the ratio 1´-$OHMDZ_{tot}$: MDZ (1´-$OHMDZ_{tot}$ represents the concentration of 1´-OHMDZ plus 1´-OHMDZ-glucuronide) obtained after 30 min discriminated well ($P < 0.0001$) between constitutive and induced CYP3A activity. Unfortunately, they did not report specifically the pharmacokinetics of the glucuronide metabolites. In our work, the ratio 1´-$OHMDZ_{tot}$: MDZ showed also a significant increase after administration of rifampicin ($P < 0.05$) but there was a slight overlap of values obtained at baseline and after treatment with rifampicin both after 20 and 30 min (Table 2).

MDZ is a well established probe drug to assess *in vivo* CYP3A activity (Streetman et al., 2000). Various authors demonstrated that the AUC of MDZ, in most cases obtained by limited sampling models (Chainuvati et al., 2003; Lee et al., 2006), or even single point sampling strategies in plasma (Chaobal and Kharasch, 2005; Lin et al., 2001) are able to

discriminate between constitutive, induced, and inhibited CYP3A activity. Various authors investigated the ratio of 1´-OHMDZ : MDZ in plasma as an index for CYP3A activity, but with inconsistent results (Eap et al., 2004; Lee et al., 2006; Rogers et al., 2002; Zhu et al., 2001). As nicely demonstrated in our work, glucuronidation plays a major role in the kinetics of the hydroxy-MDZ metabolites, especially after induction with rifampicin. Therefore, the ratio (1´-OHMDZ + 1´-OHMDZ-glucuronide) : MDZ rather than 1´-OHMDZ : MDZ may be used to assess induction of CYP3A activity in plasma.

Whereas various limited sampling strategies to assess CYP3A activity using MDZ are simpler, less invasive and cost saving compared with the determination of the entire AUC, all techniques so far reported require invasive blood sampling. In our work, we were able to show that MDZ kinetics in saliva, an easily assessable body fluid, may be a useful tool to differentiate between constitutive and induced CYP3A activity. After iv administration of MDZ, there was no overlap in the AUCs of MDZ under basal conditions and after treatment with rifampicin in saliva, showing a better separation between these two conditions compared with the AUC in plasma. Similarly, after oral ingestion of MDZ, there was no overlap in the AUC for 1´-OHMDZ in saliva, a finding obtained also for the corresponding AUC in plasma. Saliva may therefore be suited at least as well as plasma for the determination of the CYP3A phenotype using MDZ as a probe drug. However, more work is needed regarding the use of saliva as a matrix for this purpose, for example for evaluating the most suitable sampling strategy. Furthermore, it has to be shown that inhibition of CYP3A can be assessed in saliva as well.

In conclusion, we provide evidence that MDZ and 1´-OHMDZ can be determined reliably in saliva and that the concentrations in saliva correlate well with those in plasma. Saliva may be a suitable matrix for the non-invasive determination of the CYP3A phenotype using MDZ as a probe drug.

Acknowledegments

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6 Pharmacokinetics of midazolam and metabolites in a patient with refractory status epilepticus treated with extraordinary doses of midazolam

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6.1 Abstract

The authors present a patient with refractory epilepsy who was treated with very high doses (up to 4 mg/min) of intravenous midazolam, phenytoin, carbamazepine, and other antiepileptics. Because it was known from the literature that the half-life of midazolam can increase at high dosage, the kinetics of midazolam (MDZ), 1´-hydroxymidazolam, and 4-hydroxymidazolam were assessed at steady state (dosage 1 mg/min) and after stopping treatment. Total body clearance of MDZ (33 l/kg) and intrinsic hepatic clearance (19 ml/min/kg) at steady state were both 5 to 10 times higher than after normal therapeutic doses, demonstrating hepatic cytochrome (CYP) 3A induction. Despite the high body clearance, the half-life of MDZ was in the range of 24 hours, approximately 10 times higher than after normal therapeutic doses. The volume of distribution at steady state was 33 l/kg, approximately 50 times higher than after normal therapeutic doses. The free fraction of MDZ was 58% at steady state, much higher than the 3-6% at normal therapeutic doses. The kinetics of intravenous MDZ is strongly dependent on its dose and on hepatic CYP3A activity. Even in patients with hepatic CYP3A induction, the half-life of MDZ increases with high doses as a result of a rise in its volume of distribution, which is a consequence of an increase in the free fraction of MDZ.

6.2 Introduction

Midazolam is often used for long-term sedation and treatment of status epilepticus in patients in intensive care units. The short elimination half-life (1.8-3.5 h) in healthy persons after intravenous administration (Gorski et al., 2003; Klotz and Ziegler, 1982; Tsunoda et al., 1999) is an advantage compared with longer-acting benzodiazepines. Midazolam is highly protein-bound (94-97%), primarily to albumin (Fragen, 1997). It is metabolized by cytochrome P450 (CYP) 3A to 1´-hydroxymidazolam and 4-hydroxymidazolam, which are subsequently glucuronidated and eliminated mainly in urine. Midazolam is an intermediate-to-high clearance drug with a hepatic extraction ratio (E_{hep}) in the range of 0.27-0.44 (Masica et al., 2004; Thummel et al., 1996) rising to 0.6 after CYP3A induction (Gorski et al., 2003).

In patients in intensive care units, the half-life ($t_{½}$) of midazolam shows greater variation and tends to be longer compared with healthy individuals (Fragen, 1997; Malacrida et al., 1992; Vree et al., 1989). Total body clearance of midazolam, in healthy subjects in the range of 240 to 620 ml/min (Gorski et al., 2003; Klotz and Ziegler, 1982; Lee et al., 2002; Tsunoda et

al., 1999), is similar in patients in intensive care units but with a higher variation (Dirksen et al., 1987; Oldenhof et al., 1988). The volume of distribution of midazolam ranges between 1.0 and 3.3 l/kg in healthy subjects treated with common therapeutic doses (Gorski et al., 2003; Kupferschmidt et al., 1995; Tsunoda et al., 1999) and increases with decreasing albumin plasma concentrations (Vree et al., 1989).

In this report, we describe the pharmacokinetics of midazolam administered at extraordinarily high doses in a patient with prolonged nonconvulsive status epilepticus (NCSE) who was treated concomitantly with phenytoin and carbamazepine. The report illustrates the value of plasma level monitoring for understanding the kinetics of midazolam in patients treated with very high doses of this drug.

6.3 Methods

Midazolam and its metabolites were analyzed by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) as described previously (Link et al., 2007). The metabolite 1´-hydroxymidazolam glucuronide was measured as 1´-hydroxymidazolam after deglucuronidation using the same LC-MS/MS method. Free concentrations of midazolam and 1´-hydroxymidazolam were determined after ultrafiltration of the plasma samples.

The steady-state plasma concentration (C_{ss}) of midazolam was determined during a period of continuous infusion of midazolam (1 mg/min), which had been administered at this dosage for more than 4 days. CL_{ss} was then calculated as follows (IR = infusion rate of midazolam = 1 mg/min):

$$CL_{ss} = \frac{IR}{C_{ss}} \qquad (1)$$

The elimination rate constant (k_e) was calculated by least square regression of the log concentration-time curve assuming first order terminal elimination kinetics. The terminal half-life ($t_{½}$) was then calculated using equation (2):

$$t_{1/2} = \frac{ln2}{k_e} \qquad (2)$$

The volume of distribution (V_{ss}) of midazolam was calculated according to equation (3)

$$V_{ss} = \frac{CL_{ss}}{k_e} \qquad (3)$$

According to the venous equilibrium model (Shand et al., 1975), hepatic clearance (CL_{hep}) of midazolam is given by equation (4). The blood flow across the liver (Q) was assumed to be 25.5 ml/min/kg (Wynne et al., 1989). Because the renal clearance of midazolam is negligible (Dirksen et al., 1987; Gorski et al., 2003), CL_{hep} equals CL_{ss}:

$$CL_{hep} = \frac{Q \cdot (f_u \cdot CL_{int})}{Q + (f_u \cdot CL_{int})} \approx CL_{ss} \qquad (4)$$

CL_{int} represents the intrinsic hepatic clearance and f_u the unbound fraction of midazolam. From equation (4), the intrinsic hepatic clearance (CL_{int}) can be derived as follows:

$$CL_{int} = \frac{Q \cdot CL_{ss}}{f_u (Q - CL_{ss})} \qquad (5)$$

Finally, taking into account that $CL_{hep} = CL_{ss}$, the hepatic extraction ratio (E_{hep}) of midazolam can be calculated according to equation (6):

$$E_{hep} = \frac{CL_{ss}}{Q} \qquad (6)$$

The formation clearances of 1´-hydroxymidazolam and 1´-hydroxymidazolam glucuronide (CL_m) were calculated according to equation (7):

$$CL_m = f_m \cdot CL_{ss} \qquad (7)$$

f_m represents the fraction of midazolam which is converted to 1´-hydroxymidazolam or 1´-hydroxymidazolam glucuronide, respectively.

6.4 Case report

A 52-year-old woman (body weight 60 kg, calculated creatinine clearance 77 ml/min/1.72m^2) with a history of temporal lobe epilepsy, chronic schizophrenia and benzodiazepine abuse for decades was admitted to the hospital with an acute confusional state. A few weeks previously, she had deliberately stopped her antiepileptic treatment consisting of carbamazepine and lamotrigine. A focal NCSE was diagnosed and antiepileptic treatment with lorazepam and valproic acid was started. Because of refractory NCSE, lorazepam was stopped and oral topiramate and levetiracetam as well as intravenous midazolam and propofol were started on day 3. However, focal epileptic activity persisted, midazolam and propofol were replaced by pentobarbital and oxcarbazepine on day 9, and phenytoin was added on day 13. This treatment led to a complete suppression of convulsive activity on the electroencephalogram. As a result of toxicity associated with this

treatment, on day 27, valproate, oxcarbazepine, pentobarbital, levetiracetam and topiramate were stopped and intravenous midazolam was added to phenytoin, which was continued. Epileptic activity reappeared, however, and pulse therapy with methylprednisolone was initiated from day 35 to day 40. On day 41, oral carbamazepine was added to midazolam and phenytoin. Intravenous midazolam was tapered from day 54 to 57 and replaced by oral lorazepam. The patient was discharged on day 61 without electroencephalographic evidence of epileptic activity. Her treatment at that time consisted of oral lorazepam, phenytoin and carbamazepine.

It is noteworthy that the patient experienced variable degrees of sedation, but she could always be aroused during this treatment, even when the highest midazolam doses (4 mg/min) were infused. Bonati et al. already described the same patient in a report focussing on the EEG findings (Bonati et al., 2006).

6.5 Results

Continuous infusion of midazolam at a rate of 1 mg/min led to high steady-state plasma concentrations of 860 ng/ml for midazolam, 433 ng/ml for 1´-hydroxymidazolam and 3608 ng/ml for 1´-hydroxymidazolam glucuronide (Figure 1). In addition, minor amounts of 4-hydroxymidazolam could be detected. After dose reduction, plasma concentrations of midazolam and metabolites decreased accordingly.

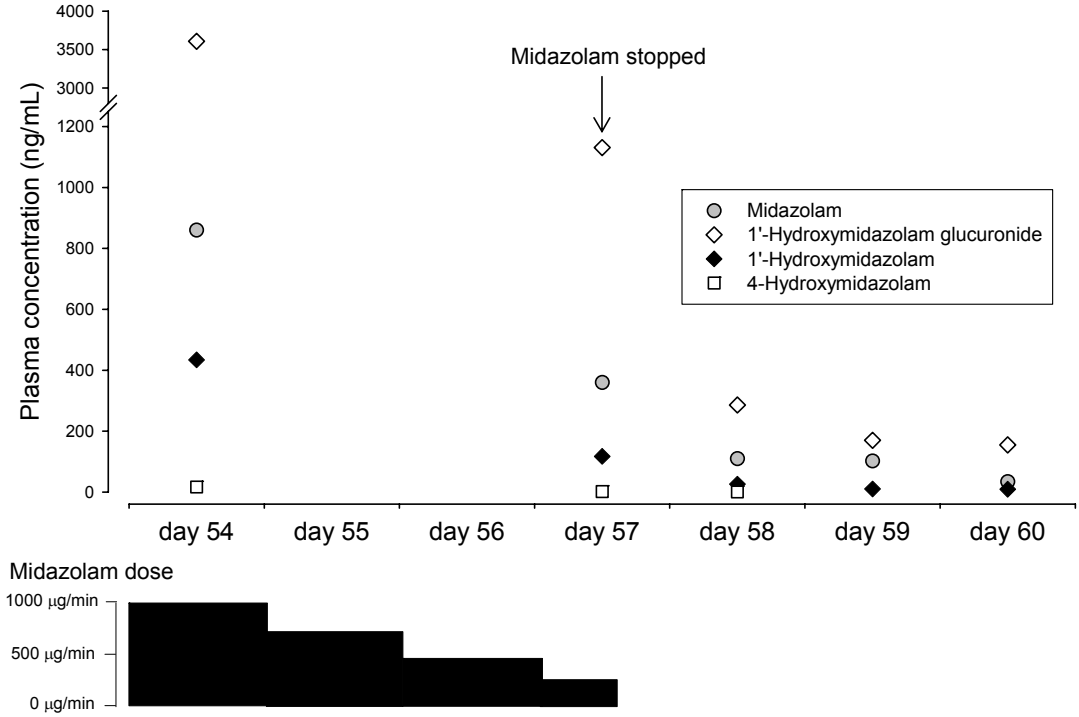

Figure 1. Plasma concentrations of midazolam, its hydroxymetabolites and 1´-hydroxymidazolam glucuronide. The determinations were performed using liquid chromatography-mass spectrometry/mass spectometry.

After stopping the continuous infusion on day 57, the half-life ($t_{½}$) of midazolam was found to be in the range of 24 h (Figure 1; Table 1), which is much longer than the $t_{½}$ of 1.8 to 3.5 h after common therapeutic doses of midazolam (Gorski et al., 2003; Klotz and Ziegler, 1982; Tsunoda et al., 1999). In accordance with, and as a possible explanation of the long $t_{½}$, the volume of distribution (V_{ss}) of midazolam was found to be 33 l/kg, a value up to 30 times higher than after common therapeutic doses (Table 1) (Gorski et al., 2003; Kupferschmidt et al., 1995; Tsunoda et al., 1999; Vree et al., 1989). As a possible explanation for this increase in V_{ss}, the unbound fraction (f_u) of midazolam was very high (58% versus 3-6% at a therapeutic dosage), compatible with saturation of the protein-binding sites. This statement is further substantiated by the drop in the free fraction of midazolam with decreasing midazolam dose rate (Figure 2). For 1´-hydroxymidazolam, the protein-binding was generally lower than for midazolam, a finding most probably explained by its lower lipophilicity, and showed no saturation at high concentrations (Figure 2). 4-Hydroxymidazolam showed protein-binding properties similar to 1´-hydroxymidazolam (data not shown).

Table 1. Pharmacokinetic parameters of Midazolam*

Parameter	Patient	Comparative values
C_{ss} (ng ml^{-1})	860	116-2817 (Dirksen et al., 1987; Vree et al., 1989)
f_u (%)	58	3-6 (Fragen, 1997)
CL_{ss} (ml min^{-1})	1160	240-620 (Gorski et al., 2003; Klotz and Ziegler, 1982; Lee et al., 2002; Tsunoda et al., 1999)
CL_{int} (ml min^{-1} kg^{-1})	138	13.3 (Tsunoda et al., 1999)
V_{ss} (l kg^{-1})	33	1.0-3.3 (Gorski et al., 2003; Kupferschmidt et al., 1995; Tsunoda et al., 1999)
E_{hep}	0.76	0.25-0.44 (Lee et al., 2002; Masica et al., 2004; Thummel et al., 1996)
k_e (h^{-1})	0.03	0.09-0.19 (Vree et al., 1989); 0.34 (Kupferschmidt et al., 1995)
$t_{½}$ (h)	24	1.8-3.5 (Gorski et al., 2003; Klotz and Ziegler, 1982; Tsunoda et al., 1999); 3.7-9.4 (Vree et al., 1989)
CL_m (1´-hydroxymidazolam) (ml min^{-1} kg^{-1})	2.9	na
CL_m (1´-hydroxymidazolam glucuronide) (ml min^{-1} kg^{-1})	16.4	na

* Steady-state values were determined at a midazolam dose rate of 1000 µg/min on November 9, 2006.

C_{ss}, steady state plasma concentration; f_u, free fraction; CL_{ss}, total blood clearance in steady-state; CL_{int}, intrinsic hepatic clearance; V_{ss}, volume of distribution in steady-state; E_{hep}, hepatic extraction ratio; k_e, terminal elimination constant; $t_{½}$, terminal elimination half-life; CL_m, formation clearance. Comparative values are values published from studies with healthy volunteers or with patients as indicated by the references; na, not available.

Despite the prolonged half-life, total body clearance (CL_{ss}) of midazolam was 19 ml/min/kg, which is much higher than usual clearance values at therapeutic doses (Gorski et al., 2003; Klotz and Ziegler, 1982; Lee et al., 2002; Tsunoda et al., 1999) and which was approaching the blood flow across the liver (Table 1). The intrinsic hepatic clearance (CL_{int}), calculated according to equation (5), reached a value of 138 ml/min/kg. This value is approximately 10 times higher than after common therapeutic doses (Table 1), demonstrating hepatic CYP3A induction. The product of the free fraction of midazolam and the intrinsic hepatic clearance of midazolam (f_u x CL_{int}) was found to be substantially larger than the blood flow across the liver:

$$f_u \times CL_{int} = 0.58 \times 138 \frac{ml}{min \cdot kg} = 80.0 \frac{ml}{min} >> 25.5 \frac{ml}{min}$$

According to equation (6), the hepatic extraction ratio (E_{hep}) was calculated to be 0.76. This value is approximately 100% higher than the values observed after common therapeutic doses of midazolam in normal subjects (see Table 1) (Lee et al., 2002; Masica et al., 2004;

Thummel et al., 1996) again demonstrating hepatic CYP3A induction. The formation clearance of 1´-hydroxymidazolam was 2.9 ml/min/kg (f_m =0.15) and for the main metabolite 1´-hydroxymidazolam glucuronide 16.4 ml/min/kg (f_m=0.85).

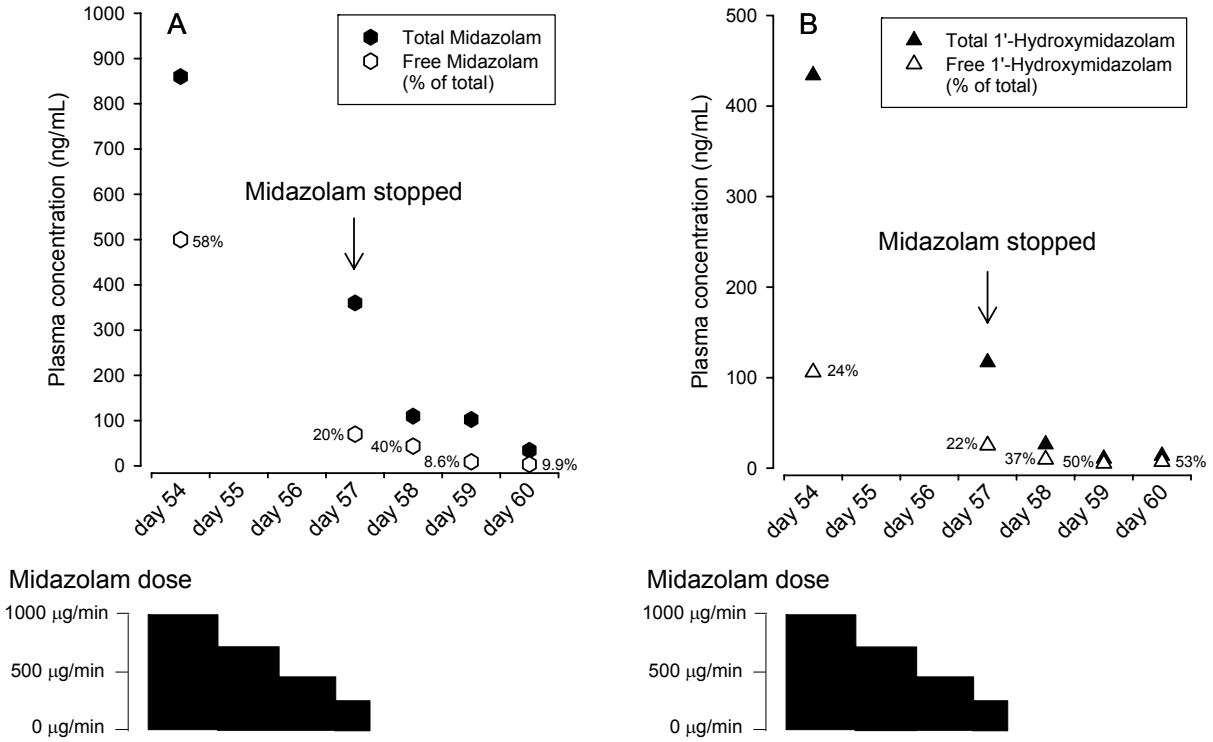

Figure 2. Plasma concentrations of total and free midazolam (A) and total and free 1´-hydroxymidazolam (B). The respective free fractions are given as a percentage of the total concentration. The determinations were performed using liquid chromatography-mass spectrometry/mass spectometry after ultrafiltration of the plasma.

6.6 Discussion

We describe a patient with refractory focal NCSE for weeks, who was treated with multiple antiepileptic drugs. In addition to carbamazepine, phenytoin and methylprednisolone, all including CYP3A, and thereby increasing the clearance of midazolam (Backman et al., 1996b; Nakajima et al., 1999), midazolam was administered at very large doses (up to 4 mg/min) as a continuous infusion for approximately 30 days. To our knowledge, there are no other reports in the literature describing the administration of similarly high cumulative doses of midazolam in critically ill patients. Taking into account the possible development of

tolerance with long-term use of high doses of midazolam (Bateson, 2002), such high doses of midazolam may have been necessary to control seizure activity in this patient.

As mentioned earlier, midazolam is highly bound to plasma protein (94-97%), primarily to albumin (Fragen, 1997). Before the continuous midazolam infusion of 1 mg/min (administered for the last 4.5 days before November 9, 2006) was tapered, an unbound midazolam fraction of 58% could be measured in our patient, which is much higher than the 3 to 6% observed after therapeutic dosages. Possible explanations were displacement by other drugs or endogenous substances, a very low serum albumin concentration or saturation of the protein binding resulting from the high midazolam concentration. The slightly decreased serum albumin concentration (26 g/l) was considered to contribute, but not to offer a full explanation for this finding (Vree et al., 1989). Competition with another drug could also not be ruled out completely, because phenytoin is also highly bound to albumin. After stopping the administration of midazolam, however, the free fraction of midazolam (f_u) significantly dropped (from 58% to approximately 9%), although the serum albumin concentration remained low and phenytoin continued to be administered, indicating that saturation of the protein binding of midazolam contributed most to this finding.

As expected regarding the high free fraction of midazolam and hepatic CYP3A induction, the calculated total body clearance (CL_{ss}) of 1160 ml/min was much higher than reported after therapeutic doses in healthy volunteers (Table 1) and in most patients in intensive care units who were described in the literature. Gorski *et al.* administered intravenous midazolam to healthy subjects before and after pre-treatment with the CYP3A inducer rifampicin and could demonstrate an increase in total body clearance of midazolam from 470 ml/min at baseline to 920 ml/min in men and from 430 ml/min to 1120 ml/min in women (Gorski et al., 2003). They calculated a hepatic extraction ratio (E_{hep}) of 0.6 and concluded that the hepatic clearance remained sensitive to increases in the intrinsic hepatic clearance and is not limited by hepatic blood flow. Our patient was treated concomitantly with carbamazepine and phenytoin, most probably leading to maximal CYP3A induction. Accordingly, the total body clearance in our patient had reached a value close to the estimated hepatic blood flow, and the product of the free fraction of midazolam and the intrinsic hepatic clearance CL_{int} of midazolam (f_u x CL_{int}) of 80 ml/min/kg exceeded hepatic blood flow (estimated to be 25.5 ml/min/kg (Wynne et al., 1989)) by far. The interpretation of this finding is that the hepatic clearance of midazolam can become limited by hepatic blood flow in patients treated with very high midazolam doses and induction of CYP3A. In accordance with this interpretation, a very high hepatic extraction ratio (E_{hep}) of 0.76 could be calculated (Delco et al., 2005).

The determined intrinsic hepatic clearance (CL_{int}) of 138 ml/min/kg in our patient is much higher than reported in healthy volunteers receiving a single dose of midazolam. Tsunoda *et al.* reported a mean value for CL_{int} of 13.3 ml/min/kg (Tsunoda et al., 1999) after a single intravenous midazolam dose in healthy volunteers. Therefore, we believe that the CYP3A isozymes of our patient were induced substantially. At this point, it has to be mentioned that we applied a conservative estimation of the hepatic blood flow; other authors (Thummel et al., 1996) suggested 21.6 ml/min/kg (1296 ml/min in our patient), which would result in a higher estimate of CL_{int}.

Despite of the high total body clearance of midazolam, we observed a markedly prolonged $t_{1/2}$ of 24 h. This value can be explained by the large volume of distribution V_{ss} of midazolam in our patient. An increase in the volume of distribution after high doses of midazolam has been described in other reports (Malacrida et al., 1992; Oldenhof et al., 1988; Vree et al., 1989). Such an increase can be explained by the high free concentration of midazolam resulting from saturation of its protein binding, possibly leading to widespread distribution of midazolam in deep body compartments. Accordingly, prolonged terminal half-lifes, varying from 2 h to 53 h, have been reported in patients in intensive care units treated with intravenous midazolam (Fragen, 1997; Naritoku and Sinha, 2000; Oldenhof et al., 1988; Vree et al., 1989). However, to the best of our knowledge, such a large V_{ss} as observed in our patient has so far not been reported.

As could be demonstrated by our case report, dose-dependent pharmacokinetics and therefore prolongation of the half-life of midazolam can occur in patients in intensive care units even if they have normal renal and hepatic function. The dose-dependent prolongation of the half-life of midazolam may lead to extended sedation in individuals susceptible to the benzodiazepine effects (Spina and Ensom, 2007). Patients with low serum albumin levels may be at a particularly high risk because of their low binding capacity for midazolam. The determination of a pharmacokinetic profile of midazolam in an individual patient may therefore be helpful to rule out or to substantiate the suspicion of midazolam-associated prolonged sedation. On the other hand, single plasma concentrations appear not to be very helpful for guiding the midazolam administration in such patients as a result of the lack of appropriate reference values and a large interindividual variability towards the central effects of this drug.

In conclusion, we presented a patient with refractory focal NCSE who was treated with carbamazepine, phenytoin, methylprednisolone, and very high doses of midazolam over weeks. We observed a substantially prolonged terminal half-life of midazolam, mainly due to an extraordinarily large volume of distribution caused by saturation of protein binding. The

hepatic clearance of midazolam appeared to be limited by liver blood flow and the high intrinsic hepatic clearance suggested a substantial induction of CYP3A.

Acknowledgements

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7 Conclusions and Outlook

The aim of an ideal drug therapy is to provide the patient with the most efficient and safest treatment possible. In this context, pharmacotherapy should also be as price effective as possible to avoid cost explosion in health care. The most efficient treatment considers mainly pharmacodynamic parameters, whereas the safest outlines mostly the pharmacokinetic aspects of an administered drug.

Currently, most medications are dosed based upon the assumption that each individual metabolizes drugs at approximately the same rate. However, patients are often showing pronounced interindividual differences of both the intensity and duration of drug action and side effects, when receiving the same ("standard") dose of a drug. One mechanism leading to those inter-patient differences is the variability in drug metabolizing enzyme activity, and/or the alteration of these enzymes, which may lead to unwanted therapeutic outcomes such as lack of efficacy or harmful response due to sub-therapeutic or toxic drug levels. Both extremes may incur in relevant health care costs.

Among the drug metabolizing enzymes, Cytochrome P450 enzymes - and especially CYP3A, which is the most important subfamily of these enzymes - play a pivotal role in the elimination process. Of the currently marketed drugs, more than 50% are metabolized by CYP3A. Furthermore, it is well known that the enzyme is prone to be involved in clinically significant interactions. Many different factors (such as genetic polymorphism, age, gender, disease status, concomitant drug therapy and dietary interactions) may influence the hepatic and intestinal CYP3A activity which makes the dosing and therapeutic use of many CYP3A substrates difficult, especially those with a narrow therapeutic range. Therefore, the direct assessment of the functional CYP3A activity ("Phenotyping") might be helpful to predict enzyme induction or inhibition, to improve the therapeutic outcome, by minimizing side effects and maximizing therapeutic efficiency, and might be also useful for guiding therapeutic dosing.

Among several possible CYP3A phenotyping probes, midazolam clearance represents one of the most reliable of the available methods, however, necessitating serial intravenous blood samples and mainly the administration of therapeutic doses. This thesis wants to provide improved CYP3A phenotyping procedures, which are easy to perform, safe for the patient, less/non-invasive and suitable for the screening of a larger patient population. To develop such alternatives, very sensitive analytical methods are required to detect and quantify the analytes of interest in different biological matrices. The validated LC-MS/MS methods described in this work, enables to measure midazolam, and to our knowledge for

the first time both of its major metabolites, 1´-hydroxymidazolam and 4-hydroxymidazolam in plasma and oral fluid at pg/ml levels. This allows on one hand for low-dose phenotyping, which is less objectionable to the subjects as compared to using therapeutic doses and which might be of special importance when phenotyping special populations such as elderly patients. On the other hand this opens the possibility to investigate the usefulness of saliva as matrix for non-invasive CYP3A phenotyping. In recent years, saliva has gained more and more interest as an alternative biological matrix for therapeutic drug monitoring and detection of illicit drugs since in principle, salivary drug concentration correlates with the free, pharmacologically active concentration of drugs in plasma, which may reflect for certain drugs, the drug effect better than the total plasma concentration.
Applying the herein described analytical methods to a randomized, two-way cross-over study, provided evidence that MDZ and 1´-OHMDZ can be determined reliably in saliva and that the concentrations in saliva correlate well with those in plasma both after intravenous and oral administration of MDZ. In this work we could show, that MDZ kinetics in saliva may be a useful tool to differentiate between constitutive and induced CYP3A activity. Consequently, saliva appears to be a suitable matrix for non-invasive CYP3A phenotyping using midazolam as a probe drug. Further studies in this field should be conducted to evaluate the most suitable sampling strategy and to investigate whether inhibition of the CYP3A activity could be assessed in saliva as well.

In conclusion, this thesis offers improved CYP3A phenotyping procedures, thus opening new ways to further investigate CYP3A mediated drug interactions and to better predict individual drug responses. In the future, the procedures might also be helpful to guide individual therapeutic dosing.

Appendix: Study Protocol

Untersuchung einer vereinfachten, nichtinvasiven Methode zur *in vivo* Phänotypisierung von Cytochrom P450 3A4 in Speichel mittels Midazolam

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INHALTSVERZEICHNIS

Untersuchung einer vereinfachten, nichtinvasiven Methode zur *in vivo* Phänotypisierung von Cytochrom P450 3A4 in Speichel mittels Midazolam

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Synopsis

Cytochrom P450 (CYP) 3A4 spielt eine entscheidende Rolle im oxidativen Fremdstoffmetabolsimus. Eine gleichzeitige Gabe mehrerer Arzneimittel, welche über CYP3A4 metabolisiert werden, kann zur Induktion oder Inhibition des Enzyms und damit zu potentiellen Interaktionen führen. Weiterhin charakteristisch ist die große interindividuelle Variabilität von CYP3A4. Da mehr als 50% aller therapeutisch eingesetzten Medikamente über CYP3A4 abgebaut werden, sind Kenntnisse über potentielle Interaktionen, und damit verbundenen unzureichenden Arzneimittelwirkungen oder Toxizität, von enormer klinischer Relevanz.

Eine der bisher verlässlichsten CYP3A4 Phänotypisierungsmethoden stellt die Bestimmung der Midazolam Plasmaclearance dar. Leider ist diese Methode invasiv und zeitaufwändig, was den Einsatz als Screeningmethode limitiert. Könnte eine Korrelation zwischen der Midazolam Plasmaclearance und den Konzentrationen an Midazolam im Speichel festgestellt werden, könnte der Nachweis von Midazolam im Speichel eine einfache, nichtinvasive Alternative zur Bestimmung der CYP3A4 Aktivität *in vivo* darstellen. Hierzu wurden bisher noch keine Daten publiziert.

Ziele der Studie

Primäre Fragestellung

Als Hauptziel der Studie soll geklärt werden, ob sich die Bestimmung der Midazolamkonzentration im Speichel zur nichtinvasiven Phänotypisierung von CYP3A4 *in vivo* eignet.

Weitere Fragestellungen

Von Interesse ist außerdem:

- Wie die Kinetik von Midazolam im Speichel unter induzierten und nichtinduzierten Bedingungen, sowie nach p.o. und i.v. Applikation verläuft.

- Welche Metaboliten von Midazolam im Speichel identifiziert werden können und ob es einen Unterschied zwischen i.v. und p.o. Applikation gibt.
- Ob es einen Zusammenhang zwischen dem Verhältnis von 6-β-Hydroxycortisol/Cortisol und der Midazolam Plasmaclearance bzw. der Clearance in Speichel unter induzierten und nichtinduzierten Bedingungen gibt.

Studiendesign

In einer randomisierten cross-over Studie mit 8 gesunden männlichen freiwilligen Probanden soll geklärt werden, ob sich die Messung von Midazolam im Speichel zur nichtinvasiven Phänotypisierung von CYP3A4 *in vivo* eignet. Hierzu erhalten die Probanden 6 Tage lang einmal täglich 600 mg Rifampicin (hepatische und intestinale CYP3A Induktion). Vor und nach Induktion mit Rifampicin wird die Aktivität von CYP3A4 mittels eines Midazolamtests in Plasma und Speichel untersucht. Ausserdem soll zwischen intestinalem (p.o. Midazolam Applikation) und hepatischem Metabolismus (i.v. Midazolam Applikation) unterschieden werden. Zum Vergleich verschiedener Phänotypisierungsmethoden wird die Aktivität des Enzyms zudem über die renale Elimination von 6-β-Hydroxycortisol bestimmt.

Einleitung

Die Cytochrom P450 (CYP) 3A Subfamilie spielt eine zentrale Rolle im oxidativen Fremdstoffmetabolismus. Etwa 30% aller P450 Enzyme in der Leber und 70% im menschlichen Dünndarm entfallen auf CYP3A (Thummel and Wilkinson, 1998). Sie besitzen eine breite Substratspezifität und metabolisieren mehr als 50% aller therapeutisch verabreichten Medikamente (Guengerich, 1999; Thummel and Wilkinson, 1998). Das wichtigste Isoenzym dieser Familie stellt CYP3A4 dar. Charakteristisch für CYP3A4 ist die stark ausgeprägte interindividuelle Enzymaktivität, die in der Leber bis zu 100-fach, im Darm bis zu 30-fach schwanken kann (Lee et al., 2002). Weiterhin hat sich gezeigt, dass eine gleichzeitige Gabe mehrerer Arzneimittel, welche über CYP3A4 abgebaut werden, häufig zur Induktion oder Inhibition des Enzyms und damit zu Arzneimittelinteraktionen führen kann (Dresser et al., 2000; Gorski et al., 2003). Daher ist es von großer klinischer Relevanz, die Aktivität des Enzyms zu bestimmen, um potentielle Drug-Drug Interaktionen und damit verbundene unzureichende Arzneimittelwirkung oder Toxizität verhindern zu können.

Neben verschiedenen anderen Phänotypisierungsmethoden (z.B. Erythromycin, Cortisol oder Nifedipin als CYP3A4 Substrat) stellt die Midazolam (MDZ) Plasmaclearance bzw. das Verhältnis Midazolam/1'-Hydroxymidazolam eine der verlässlichsten Methoden zur quantitativen Bestimmung der *in vivo* Enzymaktivität dar (Streetman et al., 2000; Thummel et al., 1994a; Thummel et al., 1994b). Jedoch erfordert diese Methode eine Vielzahl an Blutproben über den Zeitraum der Studie, was den Einsatz als Screeningmethode limitiert.

Es konnte gezeigt werden, dass das Verhältnis von Paraxanthin/Coffein im Speichel eine hohe Korrelation zum Verhältnis Paraxanthin/Coffein im Plasma und zur totalen Coffein Clearance aufweist und sich daher gut für die Bestimmung der *in vivo* CYP1A2 Aktivität eignet (Carrillo et al., 2000; Fuhr and Rost, 1994). Bisherige Untersuchungen in unserem Labor haben gezeigt, dass Midazolam, sowie dessen Hauptmetabolite 1'-Hydroxymidazolam und 4-Hydroxymidazolam mittels LC-MS/MS auch im Speichel nachgewiesen werden können. Eine erste, kürzlich veröffentlichte Publikation hat dies bestätigt, jedoch konnte dort aufgrund zu weniger Probeentnahmen, sowie ungeeigneter Speichel-kollektionsgefässen, keine Korrelation zwischen der Konzentration an MDZ im Speichel und der MDZ Plasmaclearance festgestellt werden (Quintela et al., 2004). Ergebnisse aus einer bereits von uns durchgeführten Pilotstudie deuten darauf hin, dass eine Korrelation der Pharmakokinetik in beiden Matrices bestehen könnte. Falls sich diese Vermutung bestätigen würde, könnte der Nachweis von MDZ bzw. dessen Metaboliten im Speichel eine einfache, nichtinvasive und kostensparende Screeningmethode zur *in vivo*

Phänotypisierung von CYP3A4 darstellen, welche einfach auf eine große Anzahl an Probanden angewendet werden könnte.

Ziele der Studie

Primäre Fragestellung

Als Hauptziel der Studie soll geklärt werden, ob sich die Bestimmung der Midazolamkonzentration im Speichel zur nichtinvasiven Phänotypisierung von CYP3A4 *in vivo* eignet.

Weitere Fragestellungen

Von Interesse ist außerdem:

- Wie die Kinetik von Midazolam im Speichel unter induzierten und nichtinduzierten Bedingungen, sowie nach p.o. und i.v. Applikation verläuft.
- Welche Metaboliten von Midazolam im Speichel identifiziert werden können und ob es einen Unterschied zwischen i.v. und p.o. Applikation gibt.
- Ob es einen Zusammenhang zwischen dem Verhältnis von 6-β-Hydroxycortisol/Cortisol und der Midazolam Plasmaclearance bzw. der Clearance in Speichel unter induzierten und nichtinduzierten Bedingungen gibt.

Studiendesign

Geplant sind folgende 5 Studienphasen mit 8 gesunden männlichen Freiwilligen:

Session 1:	Baselinemessung (Midazolam p.o.)
	Induktionsphase, Messung nach Induktion (Midazolam p.o.)
Wash out:	Prüfen auf Andauer der CYP3A4 Induktion
Session 2:	Baselinemessung (Midazolam i.v.)
	Induktionsphase, Messung nach Induktion (Midazolam i.v.)

Am Besprechungstag wird dem Probanden die Studie erklärt, die Ein- und Ausschlusskriterien geprüft und die Einwilligungserklärung eingeholt. Danach werden die Testsubstanzen, die Probenbehälter und Protokollblätter verteilt.

Die Studie beginnt mit einer Basislinienmessung, bei der die Aktivität von CYP3A4 ohne Modulation gemessen wird, gefolgt von einer Messung der Enzymaktivität nach Induktion mit Rifampicin. Das Design der Studie (randomized, cross-over) sieht vor, dass die Hälfte der Freiwilligen zuerst mit der p.o. Applikation beginnen (Session 1 gefolgt von einer wash out Phase), während die andere Hälfte zunächst i.v. (Session 2 gefolgt von einer wash out Phase) behandelt wird. Nach der wash out Phase wird der Proband mit der jeweils noch verbleibenden Applikationsart behandelt. Die Probanden werden mittels Los in die entsprechenden Gruppen eingeteilt.
Abbildung 1 und Tabelle 1 veranschaulichen den genauen Studienverlauf für Probanden, die mit p.o. Applikation beginnen.

Basislinienmessungen

Der Proband erhält am Morgen des Versuchstages 7,5 mg Midazolam (Dormicum®) **p.o.** auf nüchternen Magen. Zu folgenden Zeitpunkten werden Blutproben (jeweils 5,5 ml) zur Bestimmung der Midazolamkonzentration über einen Venenkatheter entnommen: 0, 10, 20, 30, 40, 60, 90, 120, 180, 240, 480, 600 min nach Applikation. Eine zusätzliche Blutprobe wird 24 Stunden nach Applikation von Midazolam entnommen.
Speichel (jeweils 2-5 ml) zur Bestimmung der Midazolamkonzentration wird in Speichelröhrchen 0, 15, 30, 40, 60, 80, 100, 120, 150, 180, 240, 360 und 480 min nach Applikation gesammelt.
Zur Bestimmung des Verhältnisses 6-β-Hydroxycortisol/Cortisol wird der Morgenurin, für das Verhältnis 1'-Hydroxymidazolam/Midazolam der Urin zwischen 0-8 und 8-24 Stunden nach Midazolamapplikation gesammelt.
Eine zweite Basislinienmessung wird nach einer **i.v.** Dosis von 2 mg Midazolam durchgeführt. Blutproben werden am contra-lateralen Arm zu folgenden Zeitpunkten entnommen: 0, 5,10, 20, 30, 40, 60, 90, 120, 240, 480 und 600 min. Eine zusätzliche Blutprobe wird 24 Stunden nach Applikation von Midazolam entnommen. Alle weiteren Konditionen folgen dem p.o. Protokoll.

Induktionsphase

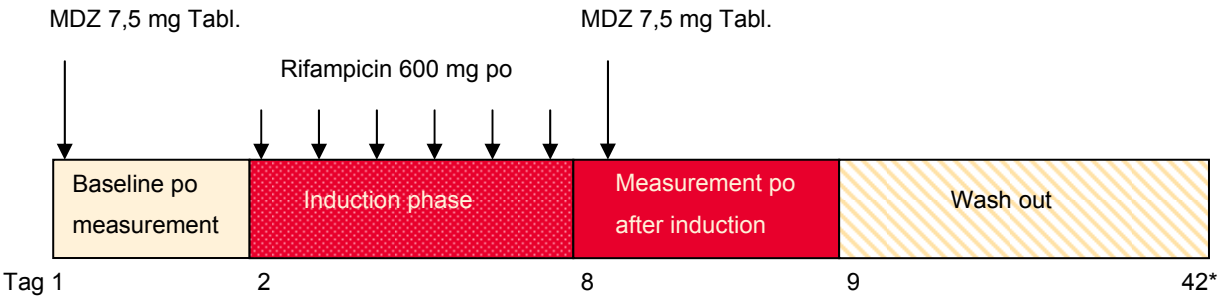
Zur Induktion von CYP3A4 erhalten die Probanden an 6 aufeinanderfolgenden Tagen 600 mg Rifampicin (Backman et al., 1998; Eeckhoudt et al., 2001; Kharasch et al., 2004; Niemi et al., 2003) einmal täglich morgens 30 min vor der Mahlzeit, beginnend am Studientag 2

(bzw. 44). Am Studientag 8 (bzw. 50) wird der gleiche Vorgang analog zur Basislinienmessung wiederholt (jeweils für i.v. und p.o.).

Wash out Phase

Zur Wiederherstellung der ursprünglichen CYP3A4 Aktivität (vor der Induktion) schließt sich eine wash out Phase an (Backman et al., 1998; Fromm et al., 1996; Niemi et al., 2003; Villikka et al., 1999). Da keine konkreten Daten vorhanden sind, wie lange die Induktion von CYP3A4 *in vivo* anhält, wird bei allen Probanden nach 7, 14, 21 und 28 Tagen nach Absetzen der Rifampicintherapie nochmals 7,5 mg MDZ p.o bzw. 2 mg MDZ i.v. verabreicht (je nachdem in welcher Gruppe sich der Proband befindet), und aus Plasma eine reduzierte Kinetik aufgenommen. Blut- und Speichelproben werden zu den Zeitpunkten t=30, 60, 90 und 120 min entnommen. Zudem wird zur Bestimmung des Verhältnisses 6-β-Hydroxycortisol/Cortisol der Morgenurin gesammelt.
Die verbleibende Applikation erfolgt frühestens 4 Wochen nach Absetzen der Rifampicintherapie abhängig von den gewonnenen Daten.

Applikation po



Applikation iv

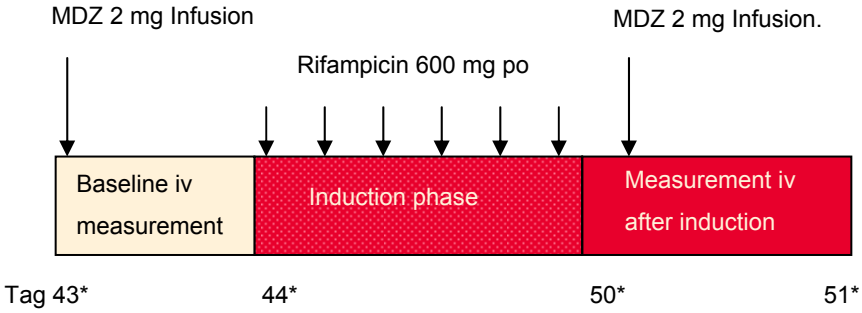


Abbildung 1. Schematische Darstellung des geplanten Studienablaufs Studiendesigns für die Gruppe, welche mit der p.o. Applikation beginnt (* abhängig von Andauer der Induktion).

Tabelle 1. Zusammenfassung des Studiendesigns für die Gruppe, welche mit der p.o. Applikation beginnt (* abhängig von Andauer der Induktion)

Studienphase	**Tag**	**Medikation**	**Maßnahmen**
Besprechungstag			Information
Basislinienmessung nach p.o. Applikation	1		Sammeln des Morgenurins für Cortisolmessungen
			Entnahme von Blankspeichel, -plasma und -urin
Kinetik p.o.		7.5 mg p.o. Midazolam	Blutentnahme für MDZbestimmung zu t=10, 20, 30, 40, 60, 90, 120, 180, 240, 360, 480, 600 min
			Speichelentnahme für MDZbestimmung zu t= 10, 30, 40, 60, 80, 100, 120, 150, 180, 240, 360, 480 min
			Sammeln des Urins zwischen 0-8h, 8-24h
	2		MDZ Plasmakonzentration nach 24h
Induktion	2-7	600 mg p.o. für 6 Tage	
	8		Sammeln des Morgenurins für Cortisolmessungen Entnahme von Blankspeichel, -plasma und –urin,
Kinetik p.o. nach Induktion		7.5 mg p.o. Midazolam	Blut-, Speichel- und Urinentnahmezeitpunkte für MDZbestimmung analog p.o. Basislinienmessung
	9		MDZ Plasmakonzentration nach 24h
Wash out Überprüfen der Andauer der CYP3A4 Induktion im Abstand von je einer Woche	8-42*	7.5 mg MDZ p.o. oder 2 mg i.v. (abhängig von der Proban-dengruppe)	Sammeln des Morgenurins für Cortisolmessungen Blut- und Speichelentnahme für MDZbestimmung zu t=30, 60, 90, 120 min
Basislinienmessung nach i.v. Applikation	43*		Sammeln des Morgenurins für Cortisolmessungen
			Entnahme von Blankspeichel, -plasma und -urin
Kinetik i.v.		2 mg i.v. Midazolam	Blutentnahme für MDZbestimmung zu den Zeitpunkten: 5, 10, 20, 30, 40, 60, 90, 120, 240, 360, 480, 600 min
			Speichelentnahme für MDZbestimmung zu den Zeitpunkten: 10, 20, 30, 40, 60, 80, 100, 120, 150, 180, 240, 360, 480 min
			Sammeln des Urins zwischen 0-8h, 8-24h
	44*		MDZ Plasmakonzentration nach 24h
Induktion	44-49*	600 mg p.o. für 6 Tage	
			Sammeln des Morgenurins für Cortisolmessungen Entnahme von Blankspeichel, -plasma und –urin
Kinetik i.v. nach Induktion	50*	7.5 mg i.v.. Midazolam	Blut-, Speichel- und Urinentnahmezeitpunkte für MDZbestimmung analog i.v. Basislinienmessung
			MDZ Plasmakonzentration nach 24h

Der Proband erhält während des Studientages ein standardisiertes Frühstück (2 Stück Brot, 20 g Butter, Schinken, Marmelade, Früchtetee) 2,5 Stunden nach Midazolam-applikation und ein standardisiertes Mittagessens (mageres Stück Fleisch, Salat, Brot, Wasser oder Früchtetee) 5 Stunden nach Medikamentengabe.

Probanden

Einschlusskriterien

- Vorgesehen sind 8 gesunde männliche Freiwillige
- Unterzeichnete Einverständniserklärung

Ausschlusskriterien

- Überempfindlichkeit gegenüber den Wirkstoffen Midazolam oder Rifampicin
- Einnahme von Arzneistoffen, welche zu einer Induktion und/oder Inhibition von CYP3A führen könnten 14 Tage vor Beginn und während Andauer der Studie
- Einnahme von grapefruithaltigem Essen oder Getränken 5 Tage vor und während des Studientages
- Bekannte Leber- oder Nierenfunktionsstörungen, sowie respiratorische Insuffizienz
- Patienten, welche in der Vergangenheit und aktuell alkohol- oder drogenabhängig waren oder sind
- Überdurchschnittlicher Alkoholkonsum (> 2 Gläser Wein/d)
- Rauchen
- Alter über 50

Eingangsuntersuchung

Die Eingangsuntersuchung wird folgende Maßnahmen umfassen:

- Aufnahme der Krankengeschichte, aktuelle Medikation, Erfassung des Gewichts
- Laboranalyse: Hb, Albumin, Serumglucose, Quick, Bilirubin, ALAT, alkalische Phosphatase, GGT, Kreatinin

Probenahme und Aufbewahrung der Proben

Blutproben werden in heparinisierten Monovetten (je 5,5 ml) gesammelt, zentrifugiert und das separierte Plasma bei –70°C in Polypropylen(PP)gefässen bis zur Analyse gelagert.
Speichelproben (je 2-5 ml) werden in Speichelröhrchen gesammelt, zentrifugiert und der klare Überstand bei –70°C in PPgefässen bis zur Analyse gelagert.
Urinproben (10ml) werden bei –70°C in PPgefässen bis zur Analyse gelagert.

Eigenschaften der Wirkstoffe

Dormicum® ist ein rasch wirksames Einschlafmittel, das zur Kurzzeittherapie von Schlafstörungen eingesetzt wird. Daneben besitzt das Medikament auch angstlösende und muskelentspannende Eigenschaften.
Rimactan® ist ein Antibiotikum, das zur Therapie von Tuberkulose sowie Lepra eingesetzt wird.
Beide Medikamente werden seit Jahren verwendet und deren erwünschte, sowie unerwünschte Wirkungen sind gut bekannt (s.a. Patientensicherheit).

Pharmakokinetik

Zur Berechnung der Fläche unter der Konzentrations-Zeit-Kurve (’area under the curve, AUC 0-24h für Plasma und 0-8h für Speichel) wird die Trapezregel angewendet. Die maximale Konzentration (c_{max}) im Plasma und im Speichel, sowie deren Zeitpunkt (t_{max}) wird direkt aus der Konzentrations-Zeit-Kurve abgelesen. Ebenso von Interesse ist die Eliminationshalbwertszeit ($t_{1/2}$) von Midazolam, die hepatische und intestinale Midazolam Plasmaclearance, sowie die systemische und orale Bioverfügbarkeit. Ausserdem wird das Verhältnis Midazolam/1’-Hydroxymidazolam bestimmt.

Analytische Methoden

Midazolam und Metabolite

Midazolam und dessen Metabolite werden aus Plasma und Speichel (und evt. Urin) mittels flüssig-flüssig Extraktion extrahiert und gemäß eines in unserem Labor erstellten und validierten Protokolls mit HPLC-MS/MS analysiert.

Cortisol

Cortisol und 6-β-Hydroxycortisol im Urin werden nach Festphasenextraktion mittels HPLC-UV gemessen (Vrtic et al., 2003).

Statistik

Die Daten werden vom Investigator selbst ausgewertet. Die Statistik wird mittels SPSS-Software für PC's durchgeführt. Untersucht wird primär die Aktivität von CYP3A4 mittels metabolischer (MDZ) und endogener Testsubstanz (Cortisol) sowohl nach p.o. als auch nach i.v. Applikation jeweils vor und nach Induktion mit Rifampicin. Die Änderungen werden mittels paired Tests untersucht mit einer Signifikanzschwelle von 5%.

Ethische Überlegungen

Patientensicherheit

Die in der Studie verwendeten Wirkstoffe Midazolam (Dormicum®) und Rifampicin (Rimactan®) werden seit vielen Jahren verwendet. Die erwünschten und unerwünschten Wirkungen sind gut bekannt.

Hohe Blutkonzentrationen von **Midazolam** können zu Müdigkeit und Schläfrigkeit führen. Selten wurde bei Patienten Amnesie beobachtet, die jedoch rasch reversibel verläuft. Bei besonders empfindlichen Menschen oder bei Überdosierung kann es zu einer verminderten Atemtätigkeit kommen, welche jedoch während der Studie überwacht wird. Falls sie unter einen bestimmten Wert fallen sollte, wird als Gegenmassnahme ein zweites Medikament (Flumazenil, Anexate®) verabreicht, welches den Effekt von Dormicum® aufhebt.

Unter der Gabe von **Rifampicin** können sich Körperflüssigkeiten wie Speichel, Urin oder Tränenflüssigkeit rötlich anfärben. Dieser Effekt ist völlig ungefährlich und verschwindet nach Absetzen des Medikaments vollständig. Weiterhin können Beschwerden wie Appetitlosigkeit, Übelkeit, Müdigkeit, asymptomatische Veränderung der Leberenzyme und Kopfschmerzen sowie Juckreiz und vorübergehende Veränderungen des Blutbildes auftreten. In seltenen Fällen werden auch grippeähnliche Symptome beobachtet, die dem Studienarzt gemeldet werden sollten. Besondere Vorsicht ist geboten bei Polymedikation,

da Rifampicin aufgrund seiner induzierenden Wirkung, die Wirksamkeit vieler Arzneimittel herabsetzen kann.
Das Legen der Venenkanüle(n), sowie die Blut-, Speichel- und Urinentnahmen sind mit keinem nennenswerten Risiko verbunden.

Versicherung

Die Probanden sind über eine Haftpflichtversicherung des Universitätsspitals Basel versichert.

Entschädigung und Nutzen

Der Proband hat von der Studie keinen direkten Nutzen, abgesehen von einer Entschädigung in Höhe von Fr 500. Im Falle eines vorzeitigen Studienabbruchs, wird diese Entschädigung anteilsmäßig ausbezahlt.

Teilnahme und Gründe für einen vorzeitigen Studienabbruch

Die Teilnahme an der Studie ist freiwillig. Der Proband hat jederzeit das Recht, die Studienteilnahme ohne Angabe von Gründen abzubrechen, ohne dass ihm dadurch ein Nachteil für seine weitere medizinische Betreuung entsteht. Jeder Teilnehmer bekommt sowohl eine mündliche als auch schriftliche Erklärung zur Studie. Außerdem wird ihm eine schriftliche und unterschriebene Einverständniserklärung ausgehändigt.
Der Prüfarzt kann, falls er es für nötig erachtet, den Probanden im Interesse seiner Gesundheit aus dieser Studie auschliessen.

Vertraulichkeit und Schutz der Daten

Alle in dieser Studie gewonnenen Informationen und Aufzeichnungen über den Probanden werden vertraulich behandelt. Die Resultate, die aus der Studie gewonnen werden, werden nur ohne Namensnennung in einer wissenschaftlichen Publikation veröffentlich werden.

Finanzierung

Die Studie wird finanziert über die Klinik für Pharmakologie und Toxikologie des Universitätsspitals Basel.

Literatur

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Curriculum Vitae

Personal data

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24.10.1976 in Karlsruhe, Germany
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Single

Education

2003 –2009	**PhD thesis**, Division of Clinical Pharmacology and Toxicology, University Hospital of Basel, Switzerland (directed by Prof. Dr. Stephan Krähenbühl) Thesis topic: "*In-vivo* phenotyping of CYP3A using midazolam as probe drug: Development of novel approaches based on highly sensitive LC-MS/MS methods"
2001	**Diploma in food chemistry**, University of Karlsruhe (TH), Germany
2001	**Diploma thesis**, Institute of Food Process Engineering, University of Karlsruhe and Federal Research Centre for Nutrition and Food, Karlsruhe, Germany Thesis topic: "Stability of carotenoids in emulsions"
1996-2001	**Study of food chemistry**, University of Karlsruhe (TH), Germany
1993 - 1996	**Grammar school of nutritional sciences**, Karlsruhe, Germany

Professional Experience

Since 09/2008	**Clinical Supply Manager**, Novartis Pharma AG, Basel, Switzerland
12/2006 – 08/2008	**Associate Clinical Supply Manager**, Novartis Pharma AG, Basel, Switzerland
01/2003 – 12/2006	**PhD student,** Division of Clinical Pharmacology and Toxicology, University Hospital of Basel, Switzerland
05/2005 - 10/2005	**Supervisor of a diploma student**, Division of Clinical Pharmacology and Toxicology, University Hospital of Basel, Switzerland
2004 - 2005	**Assistant at the practical course** "**Pharmacology and Toxicology**", University of Basel, Switzerland

08/2002 - 11/2002	**Research associate**, Institute of Process Engineering, Federal Research Centre for Nutrition and Food (BfEL), Karlsruhe, Germany
02/2002 - 08/2002	**Employee at the pharmaanalytical Laboratory** Dr. Heusler, Stutensee, Germany
2000	**Assistant** at the basic practical course in chemistry, Fachhochschule Karlsruhe, Germany
1997 -1999	**Part time employee at a pediatric practice**, Dr. med Schaub, Karlsruhe, Germany

Internship and additional courses

2010	Basics of Supply Chain Management, APICS
2008	Fundamental Project Management (Boston University), Novartis Pharma AG, Basel, Switzerland
2007	Negotiation and Influence, Novartis Pharma AG, Basel, Switzerland
2005 - 2006	Mentoring Program WIN, Novartis Pharma AG, Basel, Switzerland
2004	Advanced training course "Quality and GMP" including case studies, Pharmacenter Basel-Zürich, University Basel, Switzerland
2003/2004	Several training courses in instrumental analytics
2000	One month internship at Heidelberger Naturfarben, Heidelberg, Germany

Languages

German:	mother tongue
English:	good speech and writing abilities
French:	intermediate speech and writing abilities

Informatic skills

MS-Office, Endnote, Internet Explorer, ChemDraw, several software for analytical instruments, SPSS, SigmaPlot, WinNonlin, etc.

Memberships in Academic societies

Since 01/2005	Member of the GDCh section medical chemistry
Since 02/2002	Member of the German Chemical Society (GDCh)

Poster Presentation

Link B, Wenk M and Krähenbühl S. 21st (Montreux) LC/MS Symposium. Poster presentation "Development of a LC-MS/MS method for low-dose phenotyping of cytochrome P450 3A4 with midazolam in human plasma & its comparison towards a validated HPLC method", Nov. 2004

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