Journal of Pharmaceutical and Biomedical Analysis 50 (2009) 987–993

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Validation of a new HPLC method for determination of midazolam and its metabolites: Application to determine its pharmacokinetics in human and measure hepatic CYP3A activity in rabbits

Fawzy Elbarbry[a], Ahmed Attia[b], Ahmed Shoker[b,*]

[a] School of Pharmacy, Pacific University, Hillsboro, OR 97123, USA
[b] Department of Medicine, Royal University Hospital, University of Saskatchewan, Saskatoon, SK, Canada

ARTICLE INFO

Article history:
Received 11 March 2009
Received in revised form 3 July 2009
Accepted 3 July 2009
Available online 6 August 2009

Keywords:
HPLC
CYP3A
Therapeutic drug monitoring
Midazolam
Pharmacokinetics

ABSTRACT

Midazolam (MDZ) is a commonly used benzodiazepine in clinical practice. In addition, its metabolic oxidation is used as a surrogate marker for Cytochrome P450 (CYP) 3A enzyme activity as well. Thus, a new simpler method to measure MDZ and its metabolites is welcomed. Herein we report a new and simple HPLC method with ultraviolet detection for the simultaneous determination of midazolam and its hydroxyl metabolites using lorazepam as an internal standard. A liquid–liquid extraction was used to extract the compounds from rabbit hepatic microsomes and human plasma. The separation was performed on a Zorbax Eclipse XDB C_{18} column using a mobile phase composed of 0.05 M Na_2PO_4 (pH 4.5) and acetonitrile mixture (67:33) pumped at 1.2 mL/min. The calibration curves showed good linearity with correlation coefficient higher than 0.999 for all analytes in the range 10–500 ng/mL. Accuracy in the measurement of quality control (QC) samples was in the range 95–106% of the nominal values. The intra-day and inter-day precisions in the measurement of QC samples were less than 11% coefficient of variation. Although less sensitive than GC–MS, the proposed method was adequately sensitive to measure midazolam hydroxylase activity as a marker for CYP3A activity, and was applied to measure midazolam pharmacokinetics in human plasma.

1. Introduction

Midazolam (MDZ) is a benzodiazepine with a rapid onset and short duration of action [1,2]. Due to its favorable pharmacokinetic properties, MDZ is commonly used in clinical practice to induce anaesthesia and to treat status epilepticus and induce sedation in patients in intensive care units [1,3]. Clearance of MDZ has been used as a marker for the activity of Cytochrome P450 (CYP) 3A, the most abundant class of CYP enzymes in the liver [4,5]. Midazolam is rapidly and extensively metabolized by CYP3A isoforms to 1-hydroxy (1-OH MDZ) and 4-hydroxy (4-OH MDZ) midazolam with the ratio of these metabolites indicative of the CYP3A isoform [5,6]. Although 4-OH MDZ is pharmacologically inactive, 1-OH MDZ is 80% as potent as its parent drug [2].

A significant correlation appears to exist between plasma concentration of MDZ and its active metabolite and the degree of sedation [3]. A routine drug monitoring for MDZ has been recommended in patients with neurologic damage and in patients who have renal failure [3]. In addition, measurement of midazolam and its hydroxyl metabolites in body fluids and tissue is commonly used as a surrogate marker for CYP3A activity. Accordingly, analytical methods should be available to measure MDZ and its metabolites to allow for therapeutic drug monitoring at clinical settings, and to measure CYP3A activity for drug metabolism studies. An ideal analytical method should be sensitive, accurate and precise. The method should also be specific to avoid interference from endogenous chemicals and drugs that may be co-administered with MDZ. A short analysis time is crucial in clinical practice, routine drug analysis, and when large number of samples is processed per day.

Several analytical methods have been published for the determination of MDZ with or without its metabolites in different biological fluids. These techniques utilized high performance liquid chromatography (HPLC) with UV detection [7–13], HPLC–mass spectrometry (MS) [1,14–19], gas chromatography (GC)–MS [20,21]. Although methods utilizing MS detectors are the most sensitive, and usually require small sample volume, these detectors are not always available in clinical settings and traditional research laboratories. Most of the HPLC–UV methods suffer from various limitations, including inadequate sensitivity [8–10,12], long run

* Corresponding author at: Department of Medicine, Division of Nephrology, University of Saskatchewan, 103 Hospital Drive, Saskatoon, SK, Canada, S7N 0W8. Tel.: +1 306 966 2630; fax: +1 306 966 7996.
E-mail address: ass787@mail.usask.ca (A. Shoker).

0731-7085/$ – see front matter © 2009 Published by Elsevier B.V.
doi:10.1016/j.jpba.2009.07.004

times [10,12], and use of expensive solid phase extraction cartridges [9,10,21].

Herein, we present a HPLC–UV method for the simultaneous assay of MDZ and its hydroxyl metabolites in human plasma and rabbit hepatic microsomes. The method offers the advantage of simplicity, specificity, sensitivity, and low sample volume to perform pharmacokinetic studies of midazolam and measure CYP3A activity. The simplicity of our proposed method is facilitated by using a single-step liquid extraction procedure rather using the expensive and tedious solid phase extraction cartridges. Endogenous compounds in human plasma and rabbit hepatic microsomes did not show any interference with the analytes of interest during the run time. The method's sensitivity was good enough to monitor MDZ level in human plasma for 6 h after oral administration and capture its pharmacokinetic parameters. Lower sample volume (200 μL) compared to other existing methods [8,11,22,23] provides an advantage when plasma volume or hepatic microsomal fraction is very low or limited. As well, we report an application of the method to determine MDZ pharmacokinetics in human after a single oral administration, and to measure CYP3A activity in hepatic microsomes from rabbits.

2. Materials and experimental protocols

2.1. Chemicals and reagents

Midazolam (MDZ), 1-hydroxymidazolam (1-OH MDZ), 4-hydroxymidazolam (4-OH MDZ) and all chemicals used for microsomal preparation, determination of microsomal protein content, and enzyme assays were purchased from Sigma–Aldrich Canada Ltd. (Oakville, ON, Canada). Lorazepam (LOR, 4 mg/mL) was purchased from Sandoz Canada Inc. HPLC grade acetonitrile and methanol were purchased from EMD chemicals (Darmstadt, Germany). Euthanol (Pentobarbital Sodium) was purchased from Bimeda-MTC Animal Health Inc. (Cambridge, ON, Canada). A Milli Q Synthesis (Millipore, Bedford, MA) water purification system provided purified deionized water. All other chemicals used were analytical grade.

2.2. Subjects

Six healthy adult subjects participated after giving written informed consent all had normal liver enzymes and normal serum albumin. Subjects were instructed to abstain from caffeine or alcohol-containing products for at least 12 h and to be fasting overnight before the day of the study visit.

2.3. Protocol of human sample collection

Upon arrival at 9–10 AM to the research unit, subjects were placed in a supine position and baseline blood pressure, pulse and oxygen saturation were determined. Heparin cannula was placed in an arm vein and a blood sample was collected to measure baseline MDZ level (time zero). Then, an oral midazolam dose (0.075 mg/kg) was mixed in 200 mL apple juice and administered. Subjects were allowed to take breakfast thereafter. Blood samples for further midazolam measurement were collected at 30, 60, 90 min, 2, 4, and 6 h. Plasma were separated from blood cells by centrifugation and stored at −80 °C. The subjects were allowed to move freely 2 h after midazolam administration and to have a standard lunch.

2.4. Animals

This study was conducted on a number of six female New Zealand White rabbits. The animals were obtained from Charles River Laboratories, Inc. (Wilmington, MA, USA). All the rabbits were at the age of 6–8 weeks old weighing between 1.5 and 2.0 kg at the beginning of the study. The animals were housed as per the University of Saskatchewan regulations in the Health Sciences Building and all procedures followed the animal assurance quality guidelines. The rabbits were housed in cages at room temperature of 22–24 °C and a relative humidity of 40–60% under a 12-h-light/12-h-dark cycle to approved standards for laboratory animal care. On arrival, all the rabbits were left for 7 days for acclimatization. The animals received regular rabbit diet and water ad libitum throughout the acclimatization period. After 12 h fasting, animals were anaesthetized by injecting Euthanol in the marginal ear vein. Livers were rapidly removed and rinsed in ice-cold 0.9% NaCl, and were flash frozen in liquid nitrogen and stored at −80 °C until use. All procedures were conducted in accordance with the Canadian Council of Animal Care guidelines for the care and use of laboratory animals and were approved by the Animal Care and Supply Committee of the University of Saskatchewan.

2.5. HPLC instrumentation

The HPLC system consisted of Waters model 2695 Alliance separation module, model 2996 photodiode array detector and Empower data module (Millipore-Waters, Milford, MA, USA). Chromatographic separation was carried out on Zorbax Eclipse XDB C_{18} column (150 × 4.6 mm I.D., 5 μm particle size). The column was kept at 25 °C.

3. Methods

3.1. Preparation of hepatic microsomes

Hepatic microsomes were prepared as described previously [24,25]. Briefly, 0.5 g of liver was homogenized in 2 mL homogenization buffer (50 mM Tris buffer, 150 mM KCl, 0.1 mM dithiothreitol, 1 mM EDTA, 20% glycerol, and 0.1 mM phenylmethyl sulfonylfluoride). The homogenate was centrifuged at 9000 × *g* for 30 min in a Beckman L8-55 Ultracentrifuge (Palo Alto, CA, USA). The supernatant was carefully transferred to clean ultracentrifuge tubes and centrifuged at 100,000 × *g* for 30 min. The pellet was washed in 2 mL of 150 mM KCl and centrifuged again at 100,000 × *g* for 30 min. The pellet was resuspended in 2 mL of 0.25 M sucrose solution and 400 μL aliquots were transferred to cryogenic microcentrifuge tubes (1.5 mL). Microsomal suspensions were stored at −80 °C until use.

3.2. Determination of microsomal protein content

Microsomal protein concentrations were determined by the method of Lowry et al. [26] using bovine serum albumin as the standard. Analysis was carried out on an Agilent 8453E UV–visible spectrophotometer using Chemstation software (Palo Alto, CA, USA). Absorbance was measured at 750 nm.

3.3. Chromatographic conditions

The isocratic mobile phase consisted of 0.05 M Na_2HPO_4 (pH 4.5) adjusted with phosphoric acid and acetonitrile mixture (67:33) was run at a flow rate of 1.2 mL/min. Absorbance was monitored at 227 nm. This wavelength was found adequate to monitor MDZ, 1-OH MDZ, 4-OH MDZ, and LOR as indicated by using the PDA detector.

3.4. Preparation of standard solutions and calibration standards

MDZ, 1-OH MDZ, and 4-OH MDZ were dissolved in methanol to prepare stock solutions (1.0 mg/mL) of each. The stock solutions

were stored at −20 °C where analytes were found to be stable for at least 8 weeks. Using these stock solutions, calibration standards were prepared in drug-free human plasma or drug-free and heat-inactivated rabbit hepatic microsomes at concentrations of 0, 10, 25, 50, 100, 250, and 500 ng/mL of each analyte. Three quality control (QC) samples were prepared by spiking drug-free human plasma or rabbit hepatic microsomes with MDZ and its metabolites for method validation studies. These QC samples were prepared to contain 20 ng/mL (QC1); 100 ng/mL (QC2); and 250 ng/mL (QC3) of each analyte. A working internal standard (IS) solution was prepared by diluting lorazepam stock solution (4 mg/mL) in methanol to obtain 20 μg/mL.

3.5. Microsomal incubation

MDZ hydroxylase activity was determined by quantification of 1-OH MDZ and 4-OH MDZ formation rates in rabbit hepatic microsomes. Preliminary experiments were conducted to determine linear metabolite formation kinetics with respect to MDZ concentration, incubation time and microsomal protein concentration. Microsomal incubation mixtures consisted of MDZ (100 μM), 0.4 mg/mL liver microsomal protein, 2 mM $MgCl_2$, 1 mM NADPH, and 50 mM phosphate buffer, pH 7.8, in a final volume of 0.5 mL. After a preincubation period of 1 min at 37 °C, the reaction was started by addition of NADPH and incubated at 37 °C for 30 min in a shaking water bath. The reaction was terminated by addition of 50 μL ice-cold acetonitrile and processed as indicated below. Metabolite formation rate was calculated by dividing the amount of the metabolites formed by the incubation time and microsomal protein content (nmol/min/mg).

3.6. Sample preparation

To 200 μL calibration standards, QC samples, human plasma, or microsomal incubation mixtures, 50 μL of the working internal standard solution, 50 μL of 2.5 M NaOH, and 1 mL of toluene were added. The mixtures were vortex mixed for 2 min. After centrifugation at 10,000 × *g* in an Eppendorf microcentrifuge (Model 5417C, Brinkmann instruments, Westbury, NY, USA) for 15 min, the tubes were kept at −70 °C for 5–10 min to freeze the aqueous layer. The organic layer was separated and evaporated to dryness using a gentle stream of nitrogen at room temperature. The residue was reconstituted in 70 μL of the mobile phase and 20 μL was injected directly onto the analytical column for immediate HPLC analysis.

3.7. Method validation

Method validation procedures were performed according to FDA guidelines and as explained previously [27,28] to evaluate the suitability of the method for the quantitative determination of MDZ and its metabolites in rabbit hepatic microsomes and MDZ alone in human plasma.

Specificity was tested by analysis of five independent drug-free rabbit hepatic microsomes and human plasma samples supplemented only with internal standard to ensure the absence of endogenous compounds with the same retention times at the analytes of interest.

The linearity of the method was evaluated by processing a six-point calibration curves range 10–500 ng/mL on six different days. The peak area ratios between each analyte and the internal standard were plotted against the analyte's nominal concentration. A linear least-squares regression analysis was conducted to determine slope, intercept and coefficient of determination (R^2) to demonstrate linearity of the method. The low limit of detection (LLOD) was defined as the lowest detectable concentration, taking into consideration a signal-to-noise (S/N) ratio of 3 [27,28]. Low limit of quantification (LLOQ) was determined as the lowest concentration at which the precision, expressed as % coefficient of variation (CV), is less than 20% and S/N ratio of 10 [27,28].

The accuracy and precision of the proposed method were determined by analysis of the QC samples. The intra-day accuracy and precision were assessed from the results of five replicate analyses of QC samples on a single assay day. The inter-day accuracy and precision were determined from the same QC samples analyzed on 10 consecutive days. Precision is expressed as CV %, while accuracy (%) is expressed as [(calculated amount/predicted amount) × 100].

The extraction recovery of MDZ and its metabolites was determined by comparison of peak areas obtained from injection of 20 μL aliquots of standards prepared in methanol with samples containing the same amount after spiking in blank rabbit hepatic microsomes or human plasma and processed as indicated above. The recovery of the IS was calculated at a single concentration of 20 μg/mL. Recovery was calculated using the following equation:

$$\text{Recovery}(\%) = \frac{\text{Peak area after extraction of hepatic microsomes or human plasma}}{\text{Peak area after direct injection of a methanolic solution}} \times 100$$

3.8. Pharmacokinetic analysis

The maximum plasma concentration (C_{max}) and time to reach C_{max} (T_{max}) following MDZ administration were obtained directly from the individual plasma–concentration time data for MDZ. The area under concentration–time curve from time zero to infinity ($AUC_{0-\infty}$) was measured using linear trapezoidal summation with extrapolation. The terminal elimination rate constant (β) was estimated by linear least-square regression analysis of the terminal log-linear portion of plasma–concentration time curve. The terminal elimination half-life ($t_{1/2}$) was determined as $\ln 2/\beta$.

4. Results and discussion

Midazolam and its hydroxyl metabolites (4-OH MDZ and 1-OH MDZ) are basic compounds with a tertiary amine group in their structure. These analytes were rendered non-polar in the biological samples (plasma or hepatic microsomes) through using a strong alkaline solution (2.5N NaOH), then extracted with a non-polar organic solvent (Toluene). After injection into a reversed phase column, MDZ and its metabolites were eluted from the column by using an acidic (pH 4.5) mobile phase. Lorazepam is structurally similar to MDZ and its metabolites, and was found to be a good internal standard in this method. Chromatographic conditions (stationary phase, mobile phase composition, isocratic versus gradient elution) were optimized towards achieving symmetric peaks, good resolution, as well as acceptable accuracy and precision.

Under the chromatographic conditions explained in Section 3, 4-OH MDZ, LOR, 1-OH MDZ, and MDZ were eluted and well separated at retention times 3.1, 3.7, 4.8, and 6.2 min, respectively, in both human plasma and rabbit hepatic microsomes (Fig. 1a).

4.1. Method validation

4.1.1. Specificity

Specificity of the proposed analytical method was indicated by the absence of any endogenous interference at retention times of

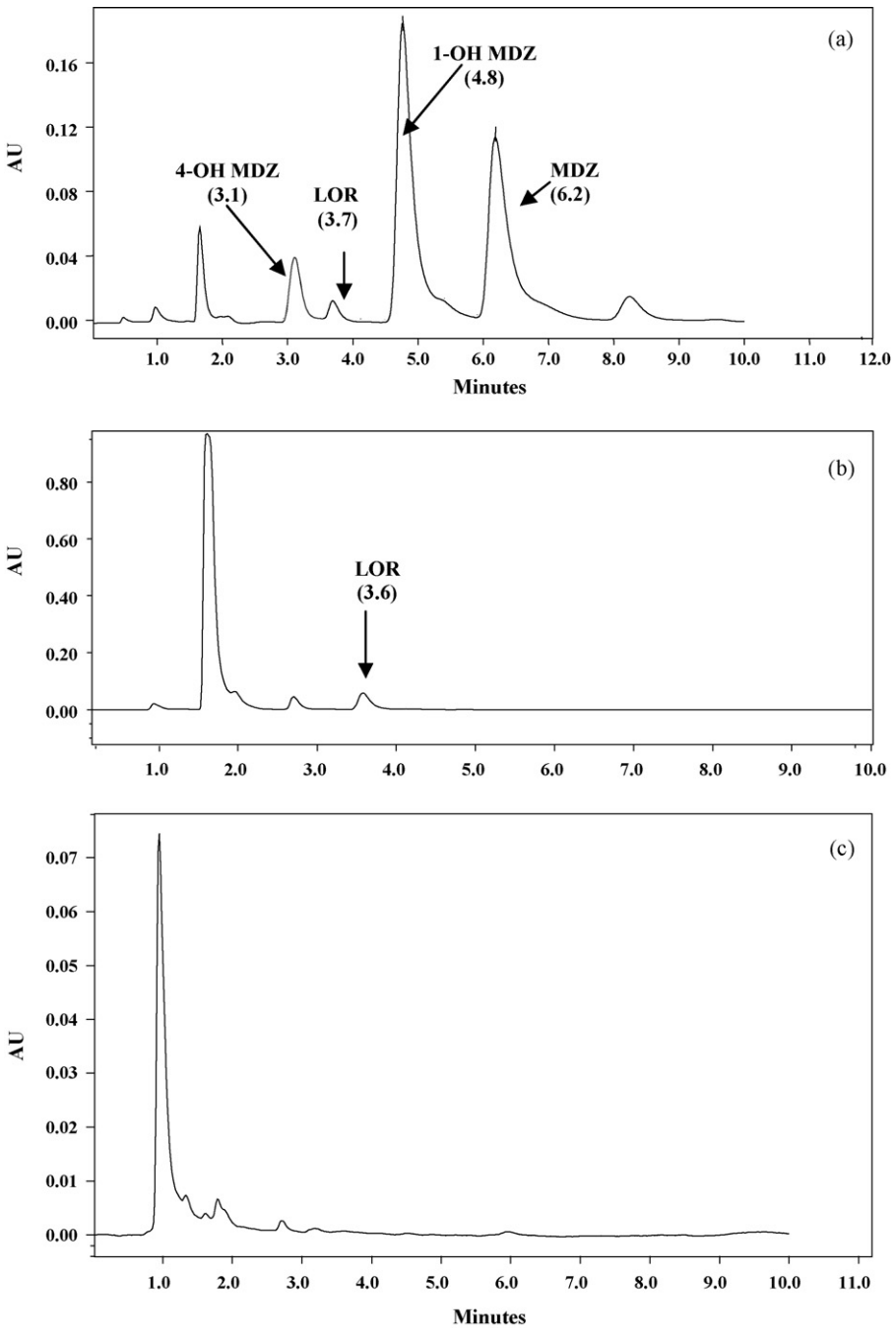

Fig. 1. Typical HPLC chromatograms of MDZ and its metabolites in human plasma or rabbit hepatic microsomes samples. (a) Blank rabbit hepatic microsomes sample spiked with internal standard (LOR), 4-OH MDZ (200 ng/mL), 1-OH MDZ (200 ng/mL), and MDZ (200 ng/mL). (b) Blank human plasma sample spiked only with the internal standard (20 μg/mL) shows no interfering peaks at retention times of MDZ or its metabolites. (c) Blank human plasma sample without any spiking shows no interfering peaks at retention times of MDZ or its metabolites.

peaks of interest as evaluated by chromatograms of blank human plasma or rabbit hepatic microsomes spiked only with the internal standard (Fig. 1b).

4.1.2. Extraction efficiency

The extraction recovery of MDZ and its metabolites was measured using three levels of concentration in rabbit hepatic microsomes and human plasma, while the recovery of the internal standard was determined only at one concentration level (Table 1). Five replicates of these samples were extracted and analyzed as described in Section 3. Despite the simple sample preparation method, recovery of all analytes from spiked human plasma and rabbit hepatic microsomes was in acceptable range (85–94%) with CV less than 7%. Recovery of the internal standard was more than 89% with a CV less than 13%. Compared with other methods utilizing liquid–liquid extraction [7,8,16,19], our method shows better or at least similar results in addition it includes the simultaneous determination of MDZ and its metabolites.

4.1.3. Linearity

The calibration curves of MDZ in blank human plasma, or MDZ and its metabolites in rabbit hepatic microsomes were linear over concentration ranges of 10–500 ng/mL. The coefficient of determination (R^2) was greater than 0.999 in all calibration curves ($n = 6$).

Table 1
Extraction recovery of MDZ, its metabolites and the internal standard (LOR) from spiked rabbit hepatic microsomes ($n = 5$), and extraction recovery of MDZ and LOR in spiked human plasma samples ($n = 5$).

Analyte	Concentration (ng/mL)	Recovery (%)	
		Average*	SD
Rabbit hepatic microsomes			
4-OH MDZ	20	85.3	5.2
	100	90.8	2.4
	250	90.8	2.6
1-OH MDZ	20	89.7	2.8
	100	89.1	3.1
	250	90	2.7
MDZ	20	88.7	1.9
	100	90.5	1.2
	250	88.5	5.7
LOR	20000	89.4	3.7
Human plasma			
MDZ	20	89.8	4
	100	92.9	2.3
	250	88.6	6.3
LOR	20000	94.2	12.2

* $n = 5$.

Low limit of detection (LLOD) and Low limit of quantitation (LLOQ) were determined based upon signal-to-noise (S/N) ratio of 3 and 10, respectively [28]. Spiked human plasma samples (for MDZ alone) or spiked rabbit hepatic microsomes (for MDZ and its metabolites) were assayed for all analytes in decreasing concentration. Chromatograms from the assay of blank plasma or hepatic microsomal samples were used for the establishment of the background noise. LLOD of MDZ in human plasma was 5 ng/mL, however it was 2.5 ng/mL for MDZ and its metabolites in rabbit hepatic microsomes (Table 2). The obtained LLOQ (10 ng/mL) in our method was sufficient for our preliminary pharmacokinetic study of MDZ in human plasma and to measure MDZ hydroxylase activity in rabbit hepatic microsomes.

4.1.4. Accuracy and precision

To determine intra-day and inter-day accuracy and precision of the assay, replicate set ($n = 5$ and 10, respectively) of three concentrations (low, medium and high) of each analyte in rabbit hepatic microsomes, or MDZ only in human plasma were analyzed. Accuracy and precision were calculated as described in Section 3 and results are summarized in Table 3. The intra-assay and inter-assay precisions for MDZ and its metabolites were less than 11% in all cases. Blindly assayed spiked samples of MDZ alone (human plasma) or MDZ and its hydroxyl metabolites (rabbit hepatic microsomes) showed average accuracy in the range of 95–106% (Table 3). These data show that the proposed method is both accurate and precise for the determination of MDZ and its metabolites in rabbit hepatic microsomes and MDZ alone in human plasma.

4.2. Application of the proposed method

Midazolam is rapidly and extensively metabolized by CYP3A isozyme to 1-OH MDZ and 4-OH MDZ [4,5]. Accordingly, MDZ hydroxylase is a commonly used marker for CYP3A activity. Our validated HPLC–UV method is adequately sensitive, accurate, and precise to evaluate MDZ hydroxylase activity in rabbit hepatic microsomes. Fig. 2a indicated that MDZ hydroxylation is exclusively mediated by microsomal CYP enzymes and requires NADPH for electron transfer. Maximum enzyme activity (V_{max}) for metabolite formation was 11.5 ± 6.2 and 32.1 ± 14.9 nmol/min/mg for 4-OH MDZ and 1-OH MDZ, respectively. To our knowledge, this is the first study to measure hepatic CYP3A activity in rabbit microsomes. Weber et al. determined CYP3A activity in rabbit microsomes using triazolam as a substrate for CYP3A, however the activity was at least 3 times lower than our reported results [22,29].

The method was also successfully applied for monitoring of plasma levels of midazolam in six healthy individuals. Fig. 3 represents concentration–time profile for MDZ in plasma samples collected over a period of 6 h after oral administration of

Table 2
Linearity data for the quantification of MDZ in human plasma and MDZ and its metabolites in rabbit hepatic microsomes by the proposed HPLC method.

Analyte	Intercept		Slope		R^2		LLOQ (ng/mL)	LLOD (ng/mL)	Linearity range (ng/mL)
	Average*	SD	Average*	SD	Average*	SD			
Rabbit hepatic microsomes									
4-OH MDZ	0.0408	0.006	0.005	0.0004	0.9993	0.00036	10	2.5	10–500
1-OH MDZ	0.0739	0.001	0.011	0.0009	0.9995	0.0003	10	2.5	10–500
MDZ	0.0812	0.005	0.018	0.0002	0.9994	0.0002	10	2.5	10–500
Human plasma									
MDZ	0.0029	0.01	0.019	0.0017	0.9991	0.0009	10	5	10–500

* $n = 6$.

Table 3
Intraday ($n = 5$) and inter-day (10 consecutive days) accuracy and precision data for the quantitation of MDZ in human plasma, and MDZ and its metabolites in rabbit hepatic microsomes by the proposed HPLC method.

Analyte	Nominal concentration (ng/mL)	Intra-day ($n = 5$)				Inter-day ($n = 10$)			
		Average	SD	% Accuracy	CV%	Average	SD	% Accuracy	CV%
Rabbit hepatic microsomes									
4-OH MDZ	20	19.1	1.4	95.5	7.2	19.5	1.96	97.5	10.1
	100	105	6.8	105.0	6.4	106	5.7	106	5.3
	250	265	20	106.0	7.6	259	19.1	103.6	7.4
1-OH MDZ	20	20.1	2.1	100.5	10.4	20.4	1.97	102	9.6
	100	103	6.1	103.0	5.9	102	5.9	102	5.8
	250	265	20	106.0	7.6	255	23	102	9
MDZ	20	19.5	1.5	97.5	7.5	20.8	2.1	104	10.2
	100	103	6.7	103.0	6.5	100	6.2	100	6.2
	250	253	15	101.2	6	256	18.4	102.4	7.2
Human plasma									
MDZ	20	20.3	1.9	101.5	9.4	20.2	1.84	101	9.1
	100	95.3	2.9	95.3	3.1	96.5	3.1	96.5	3.2
	250	248	17	99.2	6.8	252	16.6	100.8	6.7

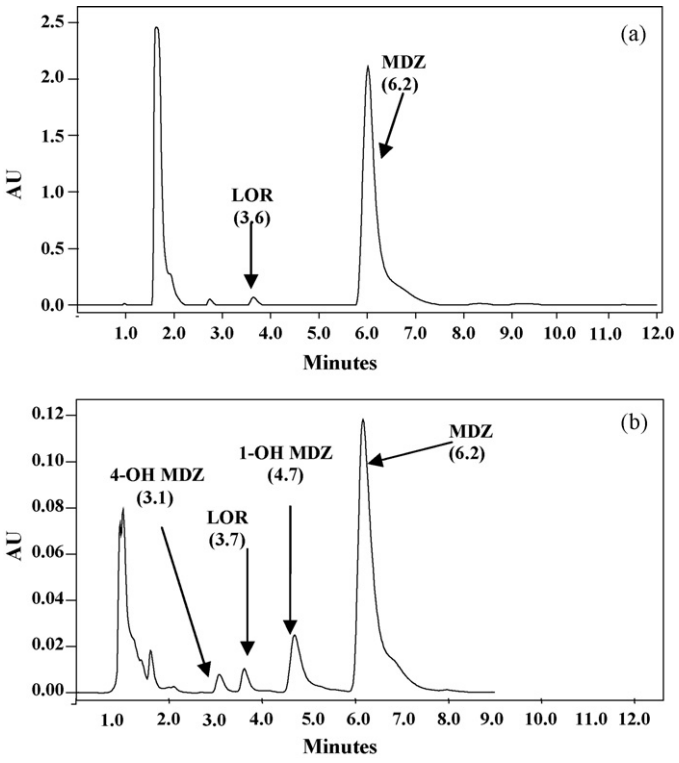

Fig. 2. Representative HPLC chromatograms of rabbit hepatic microsomes after incubation with MDZ (100 μM) in the absence (a) and presence (b) of NADPH. Incubation conditions are explained in Section 3.

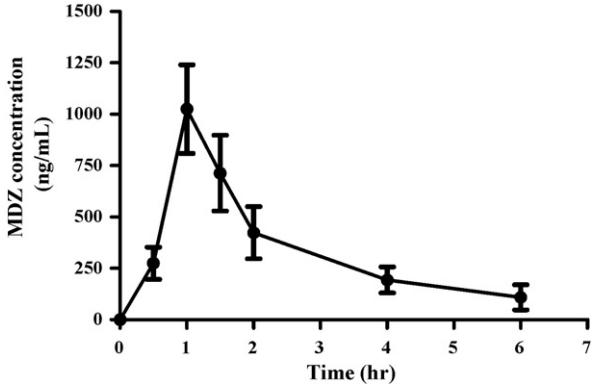

Fig. 3. Midazolam plasma concentration-time profile following a single oral administration of 0.075 mg/kg midazolam to six healthy individuals.

0.075 mg/kg midazolam. A summary of midazolam pharmacokinetic data is presented in Table 4. Our results are in agreement with previously published human data [23,30,31]. There is an increasing interest to study CYP3A activity. A recent plethora of data suggests a pivotal role for its function in a vast array of biological function such as renal function [32], cardiovascular functions [33], and its contribution in drug–drug interactions [34]. Taken together, we reason that this new technique is particularly helpful where mass spectrometry is not readily available. In addition, this new method is suitable for both liquid as well as tissue sample examination.

Table 4
Mean pharmacokinetic parameters of midazolam following a single-dose (0.75 mg/kg) administration of midazolam to six healthy individuals.

	C_{max} (ng/mL)	T_{max} (h)	$AUC_{(0-\infty)}$ (ng h/mL)	β (h^{-1})	$t_{1/2}$ (h)
Average	1025	0.9167	2032	0.3	2.35
SD (±)	215.0	0.2	26.0	0.02	0.19

$AUC_{0-\infty}$: area under the concentration–time curve; C_{max}: maximum plasma concentration; T_{max}: time to maximum plasma concentration, β: the terminal elimination rate constant, $t_{1/2}$: the terminal elimination half-life.

5. Study limitation

The method was applied to measure CYP3A activity in rabbit hepatic microsomes. Although rabbit is not commonly used as a model for this kind of study, we use it in our lab as a model for hypercholesterolemic atherosclerosis. An ongoing project is studying the correlation between hypercholesterolemia and activity of different CYP enzymes, e.g. CYP3A. Accordingly having a validated HPLC assay to measure CYP enzymes in rabbit hepatic microsomes is critical to accomplish our studies.

Pharmacokinetics of MDZ was performed on healthy human volunteers. Therefore it was not possible to study inference of drugs commonly administered with MDZ in this project. However, our results show no interference from endogenous compounds in both human plasma and rabbit hepatic microsomes.

6. Conclusion

In this study, we describe a simple HPLC-UV method for the simultaneous determination of MDZ and its metabolites for their drug therapy monitoring and as a surrogate marker for CYP3A enzyme activity as well. This new method offers the advantages of simplicity, precision, adequate sensitivity, and low sample volume. We successfully applied the method to determine CYP3A activity in rabbit hepatic microsomes and to study the pharmacokinetics of MDZ in healthy human subjects.

References

[1] M. Bodmer, B. Link, N. Grignaschi, O. Kummer, S. Ruegg, M. Haschke, S. Krahenbuhl, Ther. Drug Monit. 30 (2008) 120–124.
[2] M. Pecking, F. Montestruc, P. Marquet, E. Wodey, M.C. Homery, P. Dostert, Br. J. Clin. Pharmacol. 54 (2002) 357–362.
[3] S.P. Spina, M.H. Ensom, Pharmacotherapy 27 (2007) 389–398.
[4] P. He, M.H. Court, D.J. Greenblatt, L.L. von Moltke, Drug Metab. Dispos. 34 (2006) 1198–1207.
[5] J.C. Gorski, S.D. Hall, D.R. Jones, M. VandenBranden, S.A. Wrighton, Biochem. Pharmacol. 47 (1994) 1643–1653.
[6] L.L. von Moltke, D.J. Greenblatt, J. Schmider, J.S. Harmatz, R.I. Shader, Clin. Pharmacokinet. 29 (Suppl. 1) (1995) 33–43.
[7] J. Jurica, M. Dostalek, J. Konecny, Z. Glatz, E. Hadasova, J. Tomandl, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 852 (2007) 571–577.
[8] P.G. ter Horst, N.A. Foudraine, G. Cuypers, E.A. van Dijk, N.J. Oldenhof, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 791 (2003) 389–398.
[9] V. Sautou, J. Chopineau, M.P. Terrisse, P. Bastide, J. Chromatogr. 571 (1991) 298–304.
[10] V. Mastey, A.C. Panneton, F. Donati, F. Varin, J. Chromatogr. B: Biomed. Appl. 655 (1994) 305–310.
[11] R. Lauber, M. Mosimann, M. Buhrer, A.M. Zbinden, J. Chromatogr. B: Biomed. Appl. 654 (1994) 69–75.
[12] F. Ma, C.E. Lau, J. Chromatogr. B: Biomed. Appl. 682 (1996) 109–113.
[13] S.L. Eeckhoudt, J.P. Desager, Y. Horsmans, A.J. De Winne, R.K. Verbeeck, J. Chromatogr. B: Biomed. Sci. Appl. 710 (1998) 165–171.
[14] A. Petsalo, M. Turpeinen, O. Pelkonen, A. Tolonen, J. Chromatogr. A 1215 (2008) 107–115.
[15] S.J. Marin, R. Coles, M. Merrell, G.A. McMillin, J. Anal. Toxicol. 32 (2008) 491–498.
[16] B. Link, M. Haschke, M. Wenk, S. Krahenbuhl, Rapid Commun. Mass Spectrom. 21 (2007) 1531–1540.
[17] F.E. Dussy, C. Hamberg, T.A. Briellmann, Int. J. Legal Med. 120 (2006) 323–330.
[18] H. Kanazawa, A. Okada, E. Igarashi, M. Higaki, T. Miyabe, T. Sano, R. Nishimura, J. Chromatogr. A 1031 (2004) 213–218.
[19] S.N. Muchohi, S.A. Ward, L. Preston, C.R. Newton, G. Edwards, G.O. Kokwaro, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 821 (2005) 1–7.
[20] J. Martens, P. Banditt, J. Chromatogr. B: Biomed. Sci. Appl. 692 (1997) 95–100.
[21] G. Frison, L. Tedeschi, S. Maietti, S.D. Ferrara, Rapid Commun. Mass Spectrom. 15 (2001) 2497–2501.
[22] K. Iwakiri, Y. Oda, Y. Kaneshiro, H. Iwaki, T. Masada, A. Kobayashi, A. Asada, K. Takaoka, J. Orthop. Sci. 13 (2008) 463–468.
[23] M.D. Reed, A. Rodarte, J.L. Blumer, K.C. Khoo, B. Akbari, S. Pou, Pharmd, G.L. Kearns, J. Clin. Pharmacol. 41 (2001) 1359–1369.

[24] F.A. Elbarbry, P.J. McNamara, J. Alcorn, J. Biochem. Mol. Toxicol. 21 (2007) 41–50.
[25] J. Alcorn, F.A. Elbarbry, M.Z. Allouh, P.J. McNamara, Drug Metab. Dispos. 35 (2007) 2225–2231.
[26] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, J. Biol. Chem. 193 (1951) 265–275.
[27] F.A. Elbarbry, A.S. Shoker, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 859 (2007) 276–281.
[28] F.A. Elbarbry, A. Shoker, J. Pharm. Biomed. Anal. 43 (2007) 788–792.
[29] A. Weber, M. Kaplan, S.A. Chughtai, L.A. Cohn, A.L. Smith, J.D. Unadkat, Biopharm. Drug Dispos. 22 (2001) 157–168.
[30] I.H. Patel, P.P. Soni, E.K. Fukuda, D.F. Smith, C.V. Leier, H. Boudoulas, Br. J. Clin. Pharmacol. 29 (1990) 565–569.
[31] P.S. Shih, J.D. Huang, Drug Metab. Dispos. 30 (2002) 1491–1496.
[32] C.J. Ying, T. Noguchi, H. Aso, K. Ikeda, Y. Yamori, Y. Nara, Hypertens. Res. 31 (2008) 1821–1827.
[33] T. Simon, C. Verstuyft, M. Mary-Krause, L. Quteineh, E. Drouet, N. Meneveau, P.G. Steg, J. Ferrieres, N. Danchin, L. Becquemont, N. Engl. J. Med. 360 (2009) 363–375.
[34] S.F. Zhou, Curr. Drug Metab. 9 (2008) 310–322.