Available online at www.sciencedirect.com
ScienceDirect
ELSEVIER
Journal of Chromatography B, 852 (2007) 571–577
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

HPLC determination of midazolam and its three hydroxy metabolites in perfusion medium and plasma from rats

Jan Juřica [a,b], Miroslav Dostálek [b], Jiří Konečný [c], Zdeněk Glatz [c], Eva Hadašová [b], Josef Tomandl [a,*]

[a] *Department of Biochemistry, Faculty of Medicine, Masaryk University, Komenskeho nam. 2, 662 43 Brno, Czech Republic*
[b] *Department of Pharmacology, Faculty of Medicine, Masaryk University, Komenskeho nam. 2, 662 43 Brno, Czech Republic*
[c] *Department of Biochemistry, Faculty of Science, Masaryk University, Kamenice 5, 625 00 Brno, Czech Republic*

Received 5 December 2006; accepted 12 February 2007
Available online 25 February 2007

Abstract

A new, simple, rapid, sensitive, and repeatable isocratic reverse-phase HPLC method was developed and validated for simultaneous determination of midazolam and its main three hydroxylated metabolites, i.e. 1′-hydroxymidazolam, 4-hydroxymidazolam, and 1′,4-dihydroxymidazolam in rat liver perfusate and also plasma. Diazepam was used as an internal standard to ensure precision and accuracy of this method. Analytes were extracted from alkalinized samples into diethyl ether using single-step liquid–liquid extraction. A C18 analytical column and a mobile phase composed of acetonitrile and sodium acetate buffer were used for the chromatographic separation with UV detection. Limits of detection varied between 7.9 and 19.6 μg/L for midazolam and its hydroxy metabolites. The overall recovery for the analytes exceeded 92%, for concentrations twice the limits of detection. The intra- and inter-day precision at three different concentrations never exceeded 8 and 11% variation, respectively. This method is applicable for modeling and description of possible pharmacological interactions on rat (CYP3A1/2) or human (CYP3A4/5) cytochrome P450 enzymes.
© 2007 Elsevier B.V. All rights reserved.

Keywords: Cytochrome P450; Midazolam; 1′-Hydroxymidazolam; 4-Hydroxymidazolam; 1′,4-Dihydroxymidazolam; Rat; Perfusion; Plasma; HPLC

1. Introduction

Midazolam (MDZ, 8-chloro-6-(2-fluorophenyl)-1-methyl-4H-imidazo-[1,5-a][1,4]-benzodiazepine, CAS [59467-70-8]) is a short-acting benzodiazepine, commonly used in intravenous anesthesia induction, short-term sedation and oral hypnotic medication [1]. MDZ is basic and very stable in water solution [2]. The nitrogen in the 2-position provides sufficient basicity (pK_a 6.15). In strong acidic solutions the diazepine ring reversibly opens between the positions 4 and 5, producing a polar water-soluble primary amine derivative [3]. At physiological pH values, approximately 96% of MDZ is bound to plasma proteins [3].

MDZ is relatively quickly metabolized, compared to other benzodiazepines [2]. The major metabolic pathway for MDZ involves hepatic hydroxylation via cytochrome P450 to form 1′-hydroxymidazolam (1′-OH MDZ), 4-hydroxymidazolam (4-OH MDZ) and 1′,4-dihydroxymidazolam (1′,4-diOH MDZ) (Fig. 1) [3,4]. Hydroxy derivatives of MDZ undergo rapid conjugation with glucuronic acid and then are excreted into urine [3,5,6].

Hydroxylation of MDZ has been a valuable tool for the measurement of metabolic activity of the 3A subfamily of cytochrome P450 in rats (CYP3A1/2) [7,8], as well as in humans (CYP3A4/5) [9–11]. Some prediction of the metabolic activity of human CYP3A4/5 can be made by the measurement of rat CYP3A1/2 activity [12].

Numerous methods have been reported for the determination of MDZ and its metabolites in serum [13–30], plasma/serum and urine [9,22,31], or whole blood [32], with the limit of detection mostly around 10 μg/L. Gas chromatographic methods seem to be more sensitive than HPLC methods, but require derivatization. They provide a better limit of detection even at ng/L levels [13] comparing with the HPLC-UV methods with the limit

* Corresponding author.
E-mail address: tomandl@med.muni.cz (J. Tomandl).

1570-0232/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2007.02.034

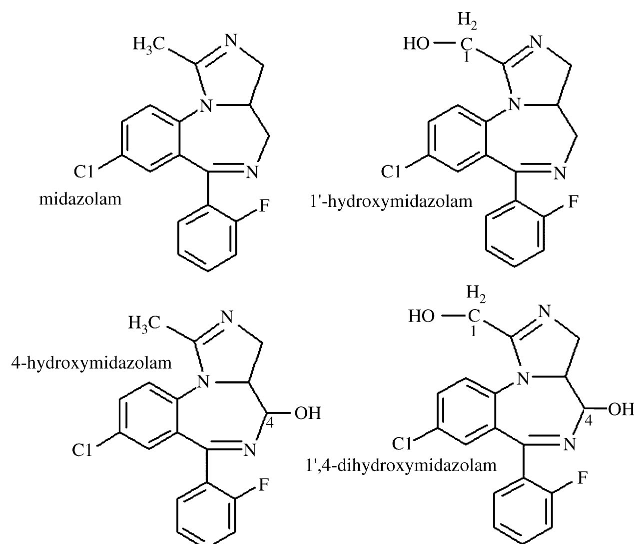

Fig. 1. Structures of midazolam and its hydroxy metabolites.

of detection at μg/L levels [19,22–24,32,34]. The assessment of MDZ, 1′-OH MDZ and 4-OH MDZ in plasma has been successfully accomplished by gas chromatography–negative chemical ionization-mass spectrometry [13,14]. HPLC mass spectrometry-based methods are about 100 times more sensitive and specific than HPLC-UV ones and provide outstanding limits of detection [15–17], but the essential equipment is expensive and often not available in laboratories.

Several HPLC-UV methods have been reported [18–27,32,33]. These methods detect particularly MDZ [20,27] or MDZ with other benzodiazepines [22,32,33]. Some methods allow determination of MDZ and 1′-OH MDZ [18,25], MDZ and 1′-OH MDZ together with 4-OH MDZ [21,23,26] or only 1′-OH MDZ [19]. However, only one of the published methods allows determination of all three main hydroxy metabolites of MDZ [34]. On the other hand, the metabolites 4-OH MDZ and 1′,4-diOH MDZ were not detected in the experimental samples and moreover, the precision and accuracy data for these metabolites were not presented, as well as the extraction recovery data [34]. Moreover, the time for a single analysis varies from about 15 to 25 min in presented methods [18,19,23,26], but only two methods reported an analysis time around 10 min [21,34]. Extraction procedures used both solid phase extraction [15,17,19,26,30,31], and liquid–liquid extraction into organic solvent or mixture of solvents [13,16,18,20–22,24,27,28,32]. The reported methods have extraction recoveries varying from 67 to 101% at different concentrations of analytes.

Here, we describe a new, simple, rapid, and repeatable reversed-phase HPLC method for the simultaneous determination of midazolam and its three main hydroxy metabolites, 1′-OH MDZ, 4-OH MDZ and 1′,4-diOH MDZ from rat liver perfusate and also from plasma. The advantage of the presented method is determination of 1′,4-diOH MDZ (besides the two main hydroxy metabolites) which offers a wide scope of applications.

2. Experimental

2.1. Chemicals

Midazolam and Dormicum® (midazolam 5 mg/mL) was kindly provided by Hoffmann-LaRoche (Basel, Switzerland). The hydroxy derivatives of midazolam (1′-OH MDZ, 4-OH MDZ, and 1′,4-diOH MDZ) were synthesized according to the method of Walser [35].

The internal standard, diazepam, was obtained from Tamda (Olomouc, Czech Republic). Ketamine (Narkamon® 5% inj. ad us. vet.) and xylasine (Rometar® 2% inj. ad us. vet.) were obtained from Spofa (Prague, Czech Republic). The acetic acid and sodium acetate were purchased from Fluka Chemie (Buchs, Switzerland). Acetonitrile, methanol and Williams medium E were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and diethyl ether from Merck (Darmstadt, Germany). All reagents used were of analytical grade except methanol and acetonitrile, which were of HPLC grade.

All water solutions were prepared with Ultrapur water (Premier MFG Systems, Phoenix, AZ, USA) filtered through a 0.45 μm membrane filter.

2.2. Standard solutions and calibration standards

Diazepam (internal standard), MDZ, 1′-OH MDZ, 4-OH MDZ, and 1′,4-diOH MDZ were dissolved in methanol to make stock solutions (1.0 mg/mL). The stock solutions were stored at −18 °C. Working standard solutions were made by dilution of the stock solutions with water-methanol (1:1, v/v). The calibration standards were prepared by spiking blank medium/plasma samples with the working standard solutions to obtain final concentrations of 0.05, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, and 10.0 mg/L. The internal standard solution was prepared at a final concentration of 40 mg/L. For liver perfusions, midazolam was dissolved in ethanol (1.0 mg/mL) before its addition into the circulating perfuse medium.

2.3. Equipment

An HPLC system consisted of a gradient HPLC pump Knauer 64 (Berlin, Germany) equipped with an LCD 2084 UV–VIS detector (ECOM, Prague, Czech Republic). Data from the detector were collected and analyzed with CSW software (DataApex Ltd., Prague, Czech Republic).

2.4. Animals

Experiments were done with male Wistar SPF rats (weighing 220 ± 20 g, BioTest, Konarovice, Czech Republic) with free access to food and water under controlled environmental conditions (lights from 6:00 a.m. to 6:00 p.m., temperature 21–22 °C, relative humidity 50–60%). The study and its experimental protocol were approved and monitored by the Ethical Committee of the Faculty of Medicine of Masaryk University.

2.5. Plasma collection

Dormicum® was applied intravenously to male Wistar albino SPF rats at the dose of 5 mg/kg of body weight, and 2 mL blood samples were collected from *retrobulbal plexus* in short ether anesthesia. The blood samples were collected into the EDTA-treated test tubes at times from 10 to 240 min after administration. The blood samples were centrifuged immediately after their collection, and 0.5 mL of plasma was then stored at −40 °C until analysis.

2.6. Liver perfusion

In the experiment with isolated rat liver, the Wistar albino male rats were anesthetized with combined ketamine (Narkamon® 5%, 2 mL/kg, i.p.) and xylasine (Rometar® 2%, 0.8 mL/kg, i.p.). As glass canula was introduced into the portal vein and liver was shortly perfused with tempered (38 °C) saline, which was then changed with the perfusion medium (120 mL of Williams medium E, equilibrated with 95% O_2 and 5% CO_2). The recirculating perfusion apparatus was manufactured according to the principle developed by Miller et al. [36]. After 20 min of pre-perfusion, midazolam (in ethanolic solution) was added as a bolus into the circulating perfusion solution to a final concentration of 12.5 mg/L. The perfusion flow was maintained at 25 mL/min. Samples of liver perfusate (2 mL) were collected at the 30, 60, and 120 min after perfusion began and stored at −40 °C until analysis.

2.7. Sample preparation

Aliquots (450 μL) of plasma or liver perfusate sample or spiked plasma/perfuse medium were transferred into a screw-capped glass tube and alkalinized with 200 μL of 0.1 M sodium hydroxide. Then 50 μL of the internal standard solution and 4 mL of diethyl ether were added, and the tubes were mixed using vortex device for 10 min at 2000 rpm and centrifuged for 5 min at 3000 × *g* at room temperature. The screw-capped tubes, containing the samples, were frozen at −40 °C for about 35 min. The organic layer was decanted into conical glass centrifuge tubes and concentrated (at 40 °C) under gentle stream of nitrogen. The residues, after evaporation, were redissolved in 150 μL of mobile phase; 50 μL was injected in the HPLC system.

Quality control samples for precision, accuracy, extraction recovery, and sample stability assessment were prepared by spiking blank plasma or perfuse medium with the working standard solutions and extracted as described above.

2.8. Chromatography

HPLC separations were carried out on the Luna C18(2) column (150 mm × 4.6 mm i.d.) protected with a SecurityGuard column (8.0 mm × 3.0 mm i.d.) both packed with 5 μm particles (Phenomenex, Torrance, CA, USA). An isocratic mode was used for the separation of the analytes. The mobile phase consisted of 10 mM sodium acetate buffer (pH 4.7) and acetonitrile (55:45, v/v) and was filtered through a 0.45 μm filter (Supelco, Bellefonte, PA, USA) before use. The flow rate was 1 mL/min, at ambient temperature, and the absorbance of the eluent was monitored at 220 nm. All compounds, including internal standard, were eluted within 10 min.

3. Results and Discussion

3.1. Method evaluation

Analysis was done with rat liver perfusates and plasma samples. In comparison with other HPLC methods [18,26], we achieved good resolution within acceptable analysis time despite using isocratic elution to separate four analytes and an internal standard. Resolution was at least 2.6 for all analytes in experimental or spiked blank samples. Using isocratic elution, the retention times were 2.7, 3.6, 4.3, 6.3, and 9.8 min for 1′,4-diOH MDZ, 4-OH MDZ, 1′-OH MDZ, MDZ and the internal standard diazepam, respectively. Fig. 2 shows chromatograms of blank perfusion medium spiked only with the internal standard and perfusion medium spiked with standards of MDZ, 1′-OH MDZ, 4-OH MDZ, and 1′,4-diOH MDZ, respectively.

Typical chromatograms of experimental samples are shown in Fig. 3: (a) rat liver perfusate collected in the 30 min of perfusion after an addition of a bolus of MDZ into the perfusion medium (to final concentration 12.5 mg/L) and b) a plasma sample from the 4th min after the i.v. administration of midazolam (5 mg/kg body weight), respectively. In all of the chromatograms presented, the samples were extracted using the same liquid–liquid procedure, employing diazepam as an internal standard.

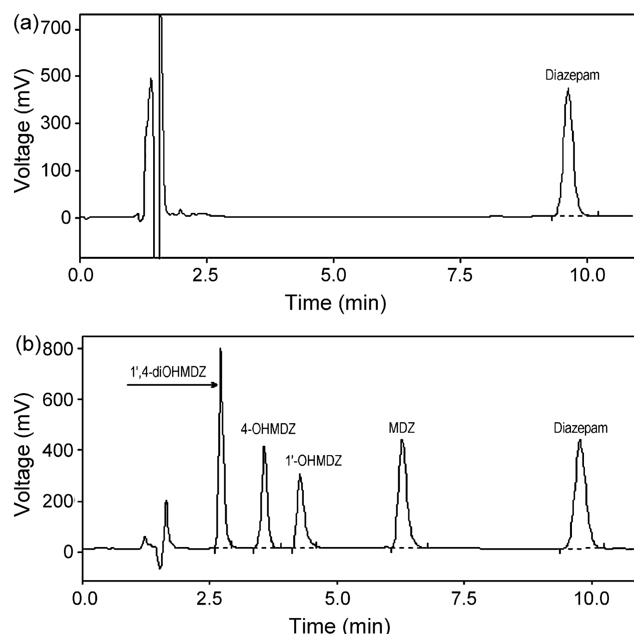

Fig. 2. Typical chromatograms of blank perfusion medium and medium spiked with standards. (a) Blank perfusion medium spiked only with diazepam (internal standard, 4 mg/L); (b) blank perfusion medium spiked with 1′,4-diOH MDZ (2.78 mg/L), 4-OH MDZ (2.35 mg/L), 1′-OH MDZ (2.45 mg/L), MDZ (2.52 mg/L), and diazepam (internal standard, 4 mg/L).

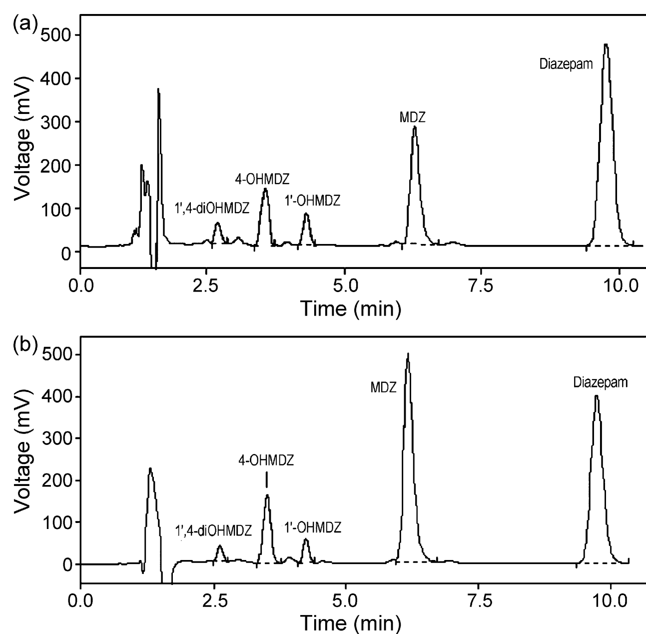

Fig. 3. Typical chromatograms of rat liver perfusate and plasma samples. (a) Liver perfusate (30 min of perfusion, after an addition of a bolus of MDZ into perfusion medium to a final concentration 12.5 mg/L); (b) plasma sample (4 min after the i.v. administration of midazolam, 5 mg/kg).

3.2. Extraction recovery

The extraction recoveries for MDZ and its hydroxy metabolites were measured with three levels of concentration in spiked perfuse medium (data are not presented) and rat plasma (Table 1). The recovery for the internal standard diazepam (I.S.) was measured only at one concentration level. Ten replicates of these samples were extracted and analyzed according to the method described above. Recovery was calculated using following equation: Recovery = (peak area after extraction/peak area of direct injection) × 100 (%). Despite the simple one step extraction, the recoveries of all analytes from spiked plasma and perfuse medium were in acceptable range of 72.0–94.2% and 89.6–98.9%, respectively. The extraction recovery of the analytes in spiked perfuse medium were similar or slightly better than in plasma (due to the voluminosity and similarity, data are not presented). Comparing with other methods utilizing liquid–liquid extraction [18,21], the recoveries of analytes were similar or better. The recoveries were, in general, slightly better in perfusate, which may be due to the presence of proteins in plasma.

3.3. Linearity, precision and accuracy

Linearity was investigated in the range of 0.05–10 mg/L for MDZ and its hydroxy metabolites and in the range of 0.2–40 mg/L for the internal standard. The calibration curves for the standards and the internal standard were done at eight levels of concentration: 0.05–0.1–0.25–0.5–1.0–2.5–5.0–10.0 and 0.2–0.4–1.0–2.0–4.0–10.0–20.0–40.0 mg/L, respectively. The linear regression equations of the detector response (mV) at 220 nm for the concentration (mg/L) of MDZ, 1′-OHMDZ, 4-OH MDZ, 1′,4-diOHMDZ and diazepam were $y = 4002(\pm 61)x - 63(\pm 6)$; $y = 2068(\pm 47)x - 79(\pm 6)$; $y = 2794(\pm 42)x - 44(\pm 5)$; $y = 3661(\pm 82)x - 44(\pm 7)$; $y = 4064(\pm 121)x - 235(\pm 20)$, respectively ($n = 8$). The determination coefficients (r^2) of the curves exceeded 0.999. To determine intra- and inter-day precision and accuracy of the assay, replicate sets ($n = 10$ and $n = 6$, respectively) of three concentrations of each analyte in perfusion medium and rat plasma were analyzed. Precision was calculated as intra- and inter-day R.S.D. values and accuracy was expressed as percent relative error [(measured concentration − spiked concentration) × 100/(spiked concentration)]. The intra- and inter-day precisions for measured analytes were <9.0 and 9.2%, respectively (Tables 2 and 3). The intra- and inter-day precision and accuracy data of the analytes in spiked perfuse medium were similar or slightly better than in plasma (data are not presented).

Table 1
Extraction recoveries of midazolam and its hydroxy metabolites and diazepam (internal standard) from spiked rat plasma ($n = 10$)

Compound	Nominal concentration (mg/L)	Estimated concentration[a] (mg/L)	Recovery (%)	R.S.D. (%)
MDZ	0.52	0.46 ± 0.04	88.5	8.7
	2.60	2.45 ± 0.11	94.2	4.5
	5.20	4.83 ± 0.14	92.9	2.9
1′-OH MDZ	0.50	0.43 ± 0.03	86.0	7.0
	2.48	2.19 ± 0.13	88.3	5.9
	4.95	4.38 ± 0.17	87.6	3.9
4-OH MDZ	0.50	0.36 ± 0.03	72.0	8.3
	2.50	1.98 ± 0.13	79.2	6.6
	5.00	4.03 ± 0.14	80.6	3.5
1′,4-diOH MDZ	0.59	0.43 ± 0.04	72.9	9.3
	2.93	2.19 ± 0.13	74.7	5.9
	5.85	4.42 ± 0.20	75.6	4.5
Diazepam (internal standard)	4.00	3.58 ± 0.09	89.5	2.5

[a] Expressed as mean ± S.D.

Table 2
Intra-day precision and accuracy of the determination of midazolam and its hydroxy metabolites in spiked rat plasma ($n = 10$)

Compound	Concentration added (mg/L)	Concentration found[a] (mg/L)	R.S.D. (%)	Accuracy[b] (%)
MDZ	0.55	0.56 ± 0.01	1.3	+1.8
	2.75	2.61 ± 0.14	5.4	−5.1
	5.55	5.24 ± 0.18	3.3	−5.6
1′-OH MDZ	0.50	0.48 ± 0.04	9.0	−4.0
	2.50	2.51 ± 0.11	4.2	+0.4
	5.00	4.93 ± 0.13	2.7	−1.4
4-OH MDZ	0.50	0.48 ± 0.01	2.7	−4.0
	2.50	2.43 ± 0.11	4.4	−2.8
	5.00	5.01 ± 0.10	2.0	+0.2
1′,4-diOH MDZ	0.60	0.56 ± 0.02	3.2	−6.7
	3.00	2.88 ± 0.19	6.5	−4.0
	6.00	5.84 ± 0.24	4.1	−2.7

[a] Expressed as mean ± S.D.
[b] Expressed as relative error = (measured conc. − spiked conc.) × 100/(spiked conc.).

Table 3
Inter-day precision and accuracy of the determination of midazolam and its hydroxy metabolites in rat plasma ($n = 6$)

Compound	Concentration added (mg/L)	Concentration found[a] (mg/L)	R.S.D. (%)	Accuracy[b] (%)
MDZ	0.55	0.53 ± 0.04	7.0	−3.6
	2.75	2.58 ± 0.17	6.5	−6.2
	5.55	5.38 ± 0.18	3.4	−3.1
1′-OH MDZ	0.50	0.55 ± 0.04	6.4	+10.0
	2.50	2.57 ± 0.23	9.1	+2.8
	5.00	4.97 ± 0.19	3.8	−0.6
4-OH MDZ	0.50	0.52 ± 0.04	6.9	+4.0
	2.50	2.51 ± 0.14	5.4	+0.4
	5.00	4.99 ± 0.23	4.6	−0.2
1′,4-diOH MDZ	0.60	0.60 ± 0.05	9.2	−1.7
	3.00	3.02 ± 0.20	6.5	+0.7
	6.00	5.93 ± 0.24	4.1	−1.2

[a] Expressed as mean ± S.D.
[b] Expressed as relative error = (measured conc. − spiked conc.) × 100 /(spiked conc.).

3.4. Limit of detection

For determination of the limit of detection (LOD, $S/N > 3:1$), spiked perfusion medium was assayed medium for all analytes in decreasing concentrations. The LOD of midazolam and its hydroxy metabolites ranged from 7.9 to 19.6 μg/L (Table 4). Chromatograms from the assay of blank samples were used for the establishment of the “background noise” in the assay. These limits of detection were sufficient for our preliminary pharmacokinetic study in rats, although gas chromatography–negative chemical-ionization mass spectrometry-based methods provide about hundred fold lower LOD [13]. However, these methods involve time-consuming sample preparation and derivatization procedures. The limits of detection of presented method are, in general, comparable with the formerly published HPLC-UV methods [19,22–24,32,34].

Table 4
Limits of detection of midazolam and its hydroxy metabolites

Compound	Limit of detection (μg/L)
MDZ	13.3
1′-OH MDZ	19.6
4-OH MDZ	13.4
1′,4-diOH MDZ	7.9

3.5. Stability

The extracts of spiked Williams medium were used for the stability determination. The nominal concentrations of MDZ, 1′-OH MDZ, 4-OH MDZ, and 1′,4-diOH MDZ, were 2.60, 2.48, 2.50, and 2.93 mg/L, respectively. Recoveries of added standards were measured after extraction and storage. After 5, 10, and 30-day storage at 4 °C in the dark, the sample extracts were dissolved in the mobile phase and measured as described above. The estimated zero-day concentrations (immediately after extraction) of MDZ, 1′-OH MDZ, 4-OH MDZ, and 1′,4-diOH MDZ were 2.51, 2.45, 2.34, and 2.78 mg/L, respectively, and were considered as 100%. The amount of analytes after 30 days of storage slowly fell to ∼80% of the original values (Fig. 4).

The tests for the stability of evaporated extract show that there is no dramatic degradation of MDZ or its hydroxy metabolites

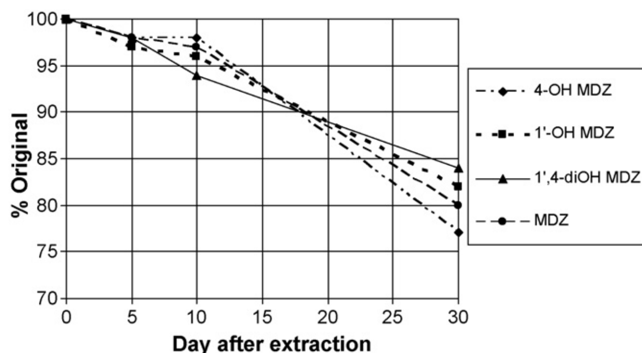

Fig. 4. Stability of midazolam and its metabolites, in evaporated extracts at 4 °C ($n = 3$).

during the storage at 4 °C in the first 10 days. This result allows for immediate extraction (after sample collection) and determination later. Therefore, samples do not need to be frozen because of risk of analytes degradation. We did not find any critical differences in stability of the extracts from plasma samples and extracts from perfusate medium samples.

3.6. Interferences

All of the drugs tested did not interfere with the determination in our study (Table 5). These compounds are substrates or metabolites of parent drugs, which are metabolized via CYP1A, 2C, and 3A enzymes or inhibitors of these subfamilies. Some of them (e.g. ibuprofen) may be co-administered with MDZ in common medication. Their retention times were different than were found for MDZ and compounds of interest.

3.7. Experimental data obtained from the experimental samples

Quantitative analyses were done with experimentation samples of perfusion medium after rat liver perfusion, as well as the plasma samples after Dormicum® administration (i.v., 5 mg/kg). The results suggest very high metabolic turnover of MDZ by rat CYP3A1/2 (Tables 6a and 6b). The metabolites of MDZ may be further biotransformed or conjugated to account for the decreasing concentration in the samples with time.

Table 5
Retention times of compounds of interest and tested drugs

Compound	Retention time (min)
Paracetamol	1.7
1′,4-diOH MDZ	2.7
Phenacetin	3.0
Dexamethasone	3.1
Sulfaphenazole	3.4
4-OH MDZ	3.6
Chlorpropamide	3.9
Alprazolam	4.0
1′-OH MDZ	4.3
MDZ	6.3
Diclofenac	6.8
Ketoconazole	7.0
Nifedipine	8.0
Diazepam (internal standard)	9.8
Ibuprofen	11.8

Table 6a
Estimated concentrations of analytes in experimental samples of perfusion medium after 30, 60, and 120 min of perfusion ($n = 8$)

Analyte	Concentration (μg/L) in timed perfusate samples		
	30 min	60 min	120 min
MDZ	465 ± 23	108 ± 9	47 ± 5
1′-OH MDZ	224 ± 14	76 ± 3	15 ± 1
4-OH MDZ	336 ± 19	116 ± 5	79 ± 6
1′,4-diOH MDZ	200 ± 13	102 ± 10	38 ± 4

Each value is expressed as mean ± S.D.

Table 6b
Estimated concentrations of analytes in experimental samples of plasma (10–240 min after Dormicum® administration, $n = 8$)

Timed plasma (min)	MDZ (μg/L)	1′-OH MDZ (μg/L)	4-OH MDZ (μg/L)	1′,4-diOH MDZ (μg/L)
10	2758.0 ± 186.0	65.0 ± 10.0	69.0 ± 7.7	27.5 ± 6.7
20	1672.0 ± 122.0	116.0 ± 9.5	145.0 ± 12.4	76.2 ± 9.5
40	1098.0 ± 47.0	69.0 ± 8.3	102.2 ± 12.2	56.2 ± 10.2
60	413.0 ± 29.0	61.2 ± 10.6	42.3 ± 15.0	53.7 ± 10.2
90	205.0 ± 21.0	n.d.	43.0 ± 9.0	18.5 ± 2.8
120	90.0 ± 6.5	n.d.	n.d.	n.d.
180	22.5 ± 3.2	n.d.	n.d.	n.d.
240	n.d.	n.d.	n.d.	n.d.

Each value is expressed as mean ± S.D.; n.d.: not detectable.

4. Conclusion

The new isocratic HPLC method for the determination of MDZ and its hydroxylated metabolites was demonstrated to be simple, with short retention times and acceptable limits of detection. As mentioned above, hydroxylation of midazolam may be used for evaluation of either human CYP3A4/5 [9,10,11] or rat CYP3A1/2 [7,8] activity. With the assessment of all major metabolites of MDZ, this method is applicable for pharmacokinetic studies of MDZ and for measuring CYP3A1/2 activity in rats and CYP3A4/5 activity in humans. This method may find useful applications in modeling and description of possible pharmacological interactions on rat CYP3A1/2 or human CYP3A4/5 enzymes. The determination of 1′,4-diOH MDZ can be used to study the mechanism its formation, i.e., for answering the question of whether 1′,4-diOH MDZ originates from 4-OH MDZ or 1′-OH MDZ.

Acknowledgments

This work was supported by the Ministry of Education (projects no. MSM 0021622404 and LC06023), and by the Grant Agency of the Czech Republic (grant no. GA 203/06/0047). The authors gratefully acknowledge Hoffmann-LaRoche (Basel, Switzerland) for the supply of midazolam and Dormicum®. The authors also thank to Professor Igor Linhart (Department of Organic Chemistry, Faculty of Chemical Technology, Insti-

tute of Chemical Technology, Prague) for the synthesis of the hydroxylated derivatives of midazolam.

References

[1] J.G. Reves, R.J. Fragen, H.R. Vinik, D.J. Greenblatt, Anesthesiology 62 (1985) 310.
[2] M. Gerecke, Br. J. Clin. Pharmacol. 16 (1983) 111.
[3] H. Allonen, G. Ziegler, U. Klotz, Clin. Pharmacol. Ther. 30 (1981) 653.
[4] A. Ghosal, H. Satoh, P.E. Thomas, E. Bush, D. Moore, Drug Metab. Dispos. 24 (1996) 940.
[5] P. Heizmann, M. Eckert, W.H. Ziegler, Br. J. Clin. Pharmacol. 16 (1983) 43.
[6] P. Heizmann, W.H. Ziegler, Arzneimittel-Forsch. 31 (1981) 2220.
[7] K. Kobayashi, K. Urashima, N. Shimada, K. Chiba, Biochem. Pharmacol. 63 (2002) 889.
[8] M.M. Cotreau, L.L. von Moltke, M.C. Beinfeld, D.J. Greenblatt, J. Pharmacol. Toxicol. Methods 43 (2000) 41.
[9] J.C. Gorski, S. Vannaprasaht, M.A. Hamman, W.T. Ambrosius, M.A. Bruce, B. Haehner-Daniels, S.D. Hall, Clin. Pharmacol. Ther. 74 (2003) 275.
[10] J.F. Rogers, M.L. Rocci, D.B. Haughey, J.S. Bertino, Clin. Pharmacol. Ther. 73 (2003) 153.
[11] K.E. Thummel, D.D. Shen, T.D. Podoll, K.L. Kunze, W.F. Trager, P.S. Hartwell, V.A. Raisys, C.L. Marsh, J.P. McVicar, D.M. Barr, J.D. Perkins, R.L. Carithers, J. Pharmacol. Exp. Ther. 271 (1994) 549.
[12] P. Soucek, I. Gut, Xenobiotica 22 (1992) 83.
[13] C.B. Eap, G. Bouchoux, K. Powell Golay, P. Baumann, J. Chromatogr. B 802 (2004) 339.
[14] F. Rubio, B.J. Miwa, W.A. Garland, J. Chromatogr. 233 (1982) 167.
[15] S.R. Harris, J.I. Gedge, A.N.R. Nedderman, S.J. Roffey, M. Savage, J. Pharmaceut. Biomed. 35 (2004) 127.
[16] M.R. Shiran, A. Gregory, A. Rostami-Hodjegan, G.T. Tucker, M.S. Lennard, J. Chromatogr. B 783 (2003) 303.
[17] H. Kanazawa, A. Okada, E. Igarashi, M. Higaki, T. Miyabe, T. Sano, R. Nishimura, J. Chromatogr. A 1031 (2004) 213.
[18] N. Yasui-Furukori, Y. Inoue, T. Tateishi, J. Chromatogr. B 811 (2004) 153.
[19] K.M. van Rij, D. Compas, E.L. Swart, P.N. de Goede, D.J. Touw, Ther. Drug Monit. 21 (1999) 416.
[20] N. van Brandt, P. Hantson, P. Mahieu, R.K. Verbeeck, Ther. Drug Monit. 19 (1997) 352.
[21] F. Ma, C.E. Lau, J. Chromatogr. B 682 (1996) 109.
[22] K. Chan, R.D. Jones, J. Chromatogr. 619 (1993) 154.
[23] V. Sautou, J. Chopineau, M.P. Terrisse, P. Bastide, J. Chromatogr. 271 (1991) 298.
[24] E.J. Portier, K. de Blok, J.J. Butter, C.J. van Boxtel, J. Chromatogr. B 723 (1999) 313.
[25] R. Lauber, M. Mosimann, M. Buhrer, A.M. Zbinden, J. Chromatogr. B 654 (1994) 69.
[26] V. Mastey, A.C. Panneton, F. Donati, F. Varin, J. Chromatogr. B 655 (1994) 305.
[27] P. Odou, H. Robert, M. Luyckx, C. Brunet, T. Dine, B. Gressier, M. Cazin, J.C. Cazin, Biomed. Chromatogr. 11 (1997) 19.
[28] L. Yuansheng, W. Yutian, Z. Jing, Z. Zhenxing, Q. Zhanxi, G. Shen, K. Qinghong, W. Xinhua, Microchem. J. 53 (1996) 130.
[29] J. Martens, P. Banditt, J. Chromatogr. B 692 (1997) 95.
[30] Y. Gaillard, J.P. Gay-Montchamp, M. Ollagnier, J. Chromatogr. 622 (1993) 197.
[31] T. Sano, K. Sato, R. Kurihara, Y. Mizuno, T. Kojima, Y. Yamakawa, T. Yamada, A. Ishii, Y. Katsumata, Legal Med. 3 (2001) 149.
[32] A. Bugey, C. Staub, J. Pharmaceut. Biomed. 35 (2004) 555.
[33] A. Mahjoub, C. Staub, J. Pharmaceut. Biomed. 23 (2000) 447.
[34] H.R. Ha, K.M. Rentsch, J. Kneer, D.J. Vonderschmitt, Ther. Drug Monit. 15 (1993) 338.
[35] A. Walser, L.E. Benjamin, T. Flynn, C. Masson, R. Schwartz, R.Y. Fryer, J. Org. Chem. 43 (1978) 936.
[36] L.L. Miller, C.G. Bly, M.L. Watson, W.F. Bale, J. Exp. Med. 94 (1951) 431.