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Sensitive determination of midazolam and 1′-hydroxymidazolam in plasma by liquid–liquid extraction and column-switching liquid chromatography with ultraviolet absorbance detection and its application for measuring CYP3A activity

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Abstract

This manuscript described a new sensitive determination of midazolam and its metabolite 1′-hydroxymidazolam by automated column-switching high-performance liquid chromatography. The test compounds were extracted from 2 ml of plasma using chloroform-hexane (30:70, v/v) and the extract was injected into a column I (TSK-PW precolumn, 10 μm, 35 mm × 4.6 mm i.d.) for clean-up and column II (C_{18} STR ODS-II analytical column (5 μm, 150 mm × 4.6 mm i.d.) for separation. The mobile phase for separation consisted of phosphate buffer (0.02 M, pH 4.6), perchloric acid (60%) and acetonitrile (57.9:0.1:42, v/v/v) and was delivered at a flow-rate of 0.6 ml/min. The peak was detected using a UV detector set at 254 nm. The method was validated for the concentration range 0.3–100 ng/ml, and good linearity (r > 0.998) was confirmed. Intra- and inter-day coefficient variations for midazolam and 1′-hydroxymidazolam were less than 8.5 and 6.1%, respectively, at the concentrations of 0.5, 5 and 50 ng/ml for the test compounds. Relative errors ranged from −14 to 6% and mean recoveries were 78–85%. The limit of quantification was 0.5 ng/ml for each compound. This method was sensitive enough for pharmacokinetic studies measuring CYP3A activity in human volunteers following an intravenous (1 mg) and a single-oral administration (2 mg) of a subtherapeutic midazolam dose.

Keywords: Midazolam; 1′-Hydroxymidazolam; HPLC; CYP3A4

1. Introduction

The use of in vivo probe substrates is a widely accepted method for evaluating cytochrome P450 (CYP) activity. The plasma clearance of intravenously administered midazolam, a benzodiazepine used clinically for conscious sedation, has been used as an in vivo marker of hepatic CYP3A activity ([1]). The metabolism of orally administered midazolam is catalyzed by CYP3A in small intestine and, to lesser extent, in liver [2]. Although in vitro studies have shown that CYP3A mediates both the 1′-hydroxylation and 4-hydroxylation of midazolam [3], in vivo studies have shown that 4-hydroxylmidazolam is generally not detectable in human plasma [4]. In addition to plasma clearance of midazolam, the ratio of 1′-hydroxylmidazolam to midazolam is also regarded as an in vivo marker of CYP3A activity [5]. The United States Food and Drug Administration advocates use of both oral and intravenous midazolam for the purpose of measuring CYP3A activity for in vivo drug metabolism and drug interaction studies [6].

Several chromatographic methods have been reported for the determination of midazolam and/or its metabolite in plasma [7–21]. Gas chromatographic methods appear more

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sensitive than HPLC; however they require sample derivation for the simultaneous analysis of midazolam and its metabolite [7,8]. HPLC mass spectrometry-based methods are more sensitive and specific, but the necessary equipment is expensive and not always readily available [9–11]. Several HPLC/UV methods have been reported, but the limits of quantification ranged from 2 to 100 ng/ml [12–21]. More sensitive methods are therefore required for measuring CYP3A activity after clinically safe, low-dose administration of midazolam, because high dose of midazolam intravenously injected has a risk of respiratory depression.

This paper describes a new sensitive determination of midazolam and its metabolite 1′-hydroxymidazolam by automated column-switching high-performance liquid chromatography with simple liquid extraction. The method was successfully applied for pharmacokinetic studies measuring CYP3A activity in human volunteers.

2. Experimental

2.1. Chemicals

Midazolam and 1′-hydroxymidazolam were purchased from Daiichi Chemical (Tokyo, Japan) and the internal standard (IS), alprazolam were kindly provided by Pharmacia UpJohn Pharmaceutical. Ltd. (Tokyo, Japan) (Fig. 1). The purity of these materials was more than 98%. Potassium phosphate monobasic, acetonitrile, perchloric acid, *n*-hexane, and chloroform were purchased from Wako Pure Chemical Industries. (Osaka, Japan). Water was deionized and purified using an IWAKI Milli-Q system MP-650 (Asahi Techno Glass Co., Chiba, Japan).

2.2. Drug solutions

Stock solutions of midazolam and 1′-hydroxymidazolam and IS for generating standard curves were prepared by dissolving an appropriate amount of each compound in methanol to yield concentrations of 0.2 mg/ml. High working standard solutions of midazolam and 1′-hydroxymidazolam and IS (2.0 μg/ml) were obtained by diluting each stock solution 100 times with 0.001 M hydrochloride. Low (200 ng/ml) working standard solution of each compound was obtained by further diluting the working standard solution 10 times with purified water. Stock solutions were stable at 4 °C for at least 6 months. Drug-free plasma from healthy donors was used for validation studies. Calibration curves were prepared by spiking 3, 10 and 30 μl of low working solutions (200 ng/ml), and 10, 30 and 100 μl of high working solutions (2.0 μg/ml) in 2 ml of blank plasma (final volume) to yield the final concentrations 0.3, 1, 3, 10, 30 and 100 ng/ml for each analysis. Standard curves were prepared daily and constructed by linear regression analysis of the compounds/internal standard peak-area ratio versus the respective concentration of midazolam and 1′-hydroxymidazolam. Stock solution of each compound was separately prepared for quality controls in the same manner as for standard curves. Working plasma solutions were obtained by dilution of stock solutions 1000 times with blank plasma (200 ng/ml). Quality control samples were obtained by spiking 5–500 μl of working plasma solutions in 2 ml of blank plasma (final volume) to yield the final concentrations range of 0.5, 5 and 50 ng/ml, and kept at −20 °C until analysis. All standard curves were checked using these quality control samples.

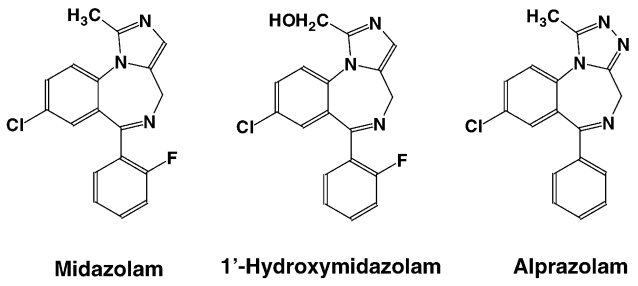

Fig. 1. Chemical structures of midazolam, 1′-hydroxymidazolam and alprazolam.

2.3. In vivo study

The subjects were 12 healthy volunteers (6 males and 6 females). The mean (and range) of age and body weight were 24 years (21–32 years) and 57 kg (42–78 kg), respectively. This study was approved by the Ethics Committee of Hirosaki University School of Medicine and written informed consents were obtained from all subjects. This study was conducted in crossover-randomized manner. They took either 1 mg midazolam by intravenous injection (IV) for 5 min or 2 mg midazolam in apple juice (PO) with at least a 2-week interval between testing. Blood samplings (10 ml) were performed before and 5, 15, 30, 60, 120, 180, 240, 300 and 360 min after the IV dosing and before and 15, 30, 60, 90, 120, 180, 240, 300 and 360 min after the PO dosing. Plasma samples were frozen and kept at −20 °C until analysis.

2.4. Apparatus

The column-switching HPLC system consisted of two Shimadzu LC-10AT high-pressure pumps (for eluent A and B), and a Shimadzu CTO-10A column oven and a Shimadzu Work station CLASS-VP chromatography integrator (Kyoto, Japan) and a Shimadzu SPD-10AVP (Kyoto, Japan) and Shimadzu autosampler SIL-10ADVP (500 μl injection volume) (Kyoto, Japan). A TSK gel PW precolumn (a hydrophilic metaacrylate polymer column) for sample clean-up (column I; 35 mm × 4.6 mm i.d., particle size 10 μm; Tosoh, Tokyo, Japan) and a C_{18} STR ODS-II column as an analytical column (column II; 150 mm × 4.6 mm i.d., particle size 5 μm; Shinwa Chemical Industry, Kyoto, Japan) were used.

2.5. Extraction procedure

IS (alprazolam) 200 μl of 250 ng/ml and 0.5 ml NaOH (0.5 M) were added to 2 ml of plasma. The tubes were vortex-mixed for 10 s and 5 ml of *n*-hexane-chloroform (70:30, v/v) was added as extraction solvent. After 10 min of shaking, the mixture was centrifuged at 1700 × *g* for 10 min at 4 °C, and the organic phase was evaporated in vacuo at 45 °C to dryness (TAITEC VC-960, Shimadzu, Kyoto, Japan). The residue was dissolved with 100 μl of eluent A and used as an extract.

2.6. Chromatographic condition

Column-switching chromatographic condition was set based on our previous report [22]. A 0.1-ml portion of the extract was automatically injected into the HPLC system. The column-switching system was operated. From 0 to 5.0 min after the sample injection, midazolam, 1′-hydroxymidazolam and IS were separated from the interfering substances present in the extract on column I with a mobile phase (eluent A) of KH_2PO_4 buffer (0.02 M, pH 4.6) and acetonitrile (86:14, v/v). Between 5.0 and 9.0 min after the injection, two analytes and IS retained on column I were eluted with a mobile phase (eluent B) of KH_2PO_4 buffer (0.02 M, pH 4.6), perchloric acid (60%) and acetonitrile (58.9:0.1:41, v/v/v), and effluent from column I was switched to column II. Then midazolam and 1′-hydroxymidazolam were separated on column II by eluting with eluent B (between 9.0 and 15.0 min). Thus, the injection interval for this system was 15 min. The flow-rates of eluents A and B were 1.2 and 0.6 ml/min, respectively. The temperatures of column I and II were 40 °C. The peak was detected using a UV detector set at 254 nm. The peak area was used for the quantification of these two compounds.

3. Results and discussion

3.1. Chromatography

The retention of midazolam, 1′-hydroxymidazolam and internal standard was influenced by the pH of mobile phase. In addition to pH influence, separation of each compound in mobile phase (pH 2.3) adjusted by perchloric acid was much better than those adjusted by other acids. The retention behavior of compounds was less affected by concentration of methanol and 2-propanol than acetonitrile. Based on these findings, the concentration of the organic modifiers (acetonitrile) and perchloric acid content was defined to achieve the optimal separation of compounds.

A representative chromatogram of an unextracted working aqueous solution containing 25 ng each of midazolam and 1′-hydroxymidazolam and alprazolam (internal standard) is shown in Fig. 2A. The chromatogram of an extracted blank plasma sample is shown in Fig. 2B, while the chromatogram

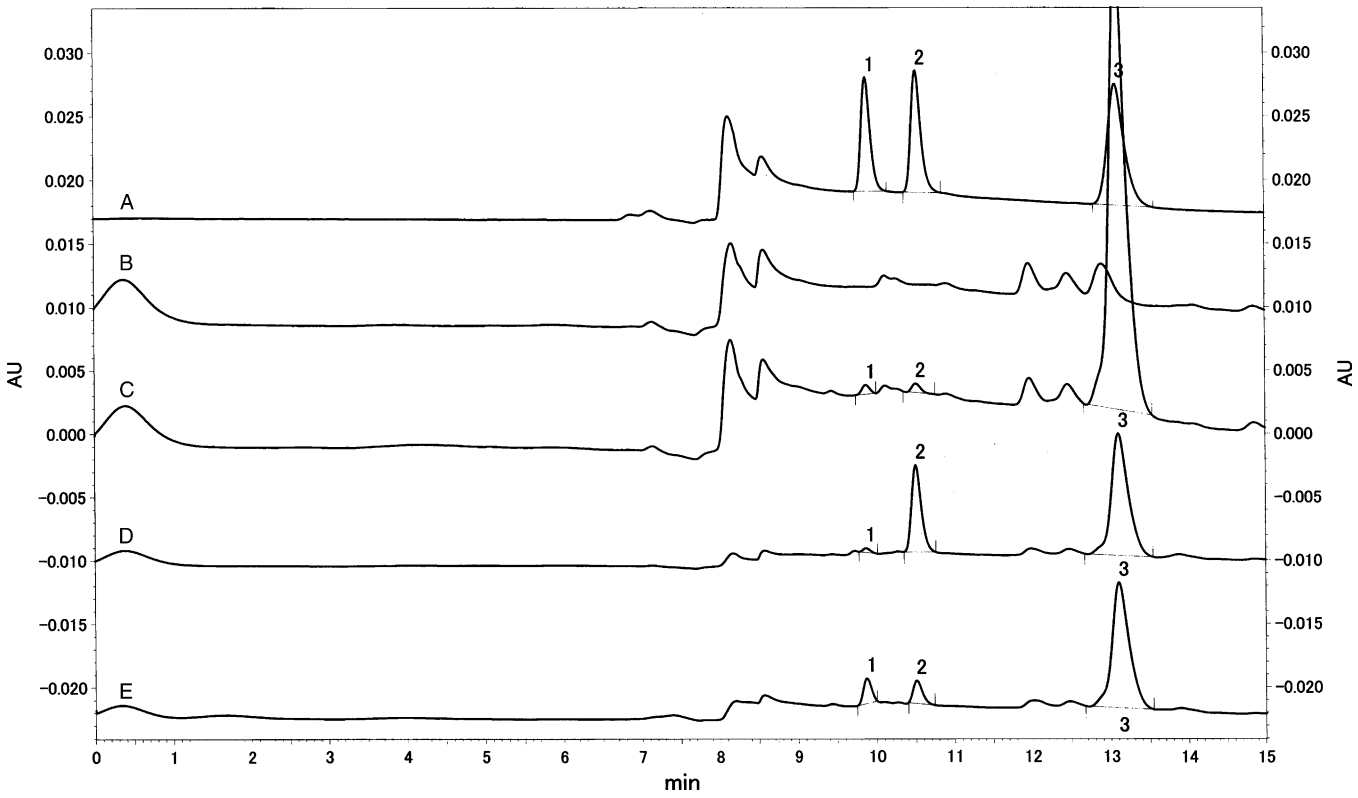

Fig. 2. Chromatograms of (A) direct injection of a working solution of reference compounds, (B) blank plasma extract, (C) extract from spiked analytes (0.5 mg/ml) on blank plasma and plasma extract from a healthy volunteer at 0.25 h after (D) the IV and at 0.5 h after (E) the PO dosing.

Table 1
Analytical recoveries of midazolam, 1′-hydroxymidazolam and internal standard, alprazolam

Compound	Concentration (ng/ml)	Recovery (%)	CV (n = 6) (%)
Midazolam	0.5	75	9.4
	5	72	6.5
	50	67	5.9
1′-Hydroxymidazolam	0.5	79	8.7
	5	75	5.7
	50	72	6.4
Alprazolam	25	85	3.4

of an extracted sample spiked with 2.0 ng (1.0 ng/ml) of midazolam and 1′-hydroxymidazolam and IS is shown in Fig. 2C. All compounds were well separated from each other and from the front of the solvent peaks. The chromatograms of extracted plasma samples obtained from a volunteer receiving 1 mg of intravenous dose and 2 mg of oral dose of midazolam did not show interference peaks (Fig. 2D and E). Plasma concentrations of midazolam and 1′-hydroxymidazolam were 31.0 and 0.8 ng/ml (15 min after IV injection) and 7.2 and 7.7 ng/ml (30 min after oral administration), respectively.

3.2. Recovery and linearity

Recovery from plasma was calculated by comparing the peak heights of pure standards prepared in purified water, and injected directly into the analytical column, with those of extracted plasma samples containing the same amount of the test compound (n = 6 each). Recoveries and their CV values were determined at three different concentrations ranging from 0.5 to 50 ng/ml (Table 1). Calibration curves were linear over the concentrations range from 0.3 to 100 ng/ml (r = 0.998 for all compounds) (Table 2).

Table 2
Individual and mean values for slopes, intercepts and correlation coefficients of five calibration curves of midazolam and 1′-hydroxymidazolam

Compound	Curve	Slope	Intercept	r
Midazolam	1	0.0285	0.0143	0.9985
	2	0.0254	0.0018	0.9989
	3	0.0225	0.0055	0.9991
	4	0.0255	0.0012	0.9996
	5	0.0263	0.0014	0.9999
	Mean	0.0256	0.0048	0.9992
	S.D.	0.0019	0.0050	0.0005
1′-Hydroxymidazolam	1	0.0202	0.0011	0.9991
	2	0.0198	0.0016	0.9992
	3	0.0223	0.0045	0.9995
	4	0.0188	0.0066	0.9999
	5	0.0222	0.0012	0.9994
	Mean	0.0207	0.0030	0.9994
	S.D.	0.0014	0.0022	0.0003

3.3. Sensitivity

The limit of detection defined as analyte responses was at least three times the response as compared to blank response. The lowest standard for the validation was defined as the limit of quantification for which analyte peaks for both compounds were identifiable, discrete, and reproducible with a precision of 20% and accuracy of 80–120%. The limits of detection and quantification were 0.2 and 0.5 ng/ml, respectively, for both midazolam and 1′-hydroxymidazolam. This method is more sensitive than previous reports in which the limits of quantification ranged from 2 to 100 ng/ml [12–21].

3.4. Precision and accuracy

Intra- and inter-day precision and accuracy were evaluated by assaying quality controls with three different concentrations of midazolam and 1′-hydroxymidazolam. Intra- and inter-day precisions were assessed by analyzing six quality control samples at each concentration on the same day and mean values of a quality control for 6 days, respectively. These extracts underwent the same column-switching procedure. Intra- and inter-day relative standard deviations were less than 5.6 and 8.5% for midazolam and 5.1 and 6.1% for 1′-hydroxymidazolam, respectively, in the concentration range of 0.5 to 50 ng/ml (Table 3). Accuracy was expressed as percent error (relative error) [(measured concentration−spiked concentration)/spiked concentration] × 100 (%), while precision was quantitated by calculating intra- and inter-CV values.

3.5. Specificity

Potential interference from co-administered drugs was investigated by determining their retention times in this system. No peaks were observed until 30 min after injection of ex-

Table 3
Precision and accuracy

Compound	Concentration added (ng/ml)	Concentration found (ng/ml)	CV (%)	RE (%)
Intra-assay (n = 6)				
Midazolam	0.5	0.47	5.6	−6
	5	4.92	4.4	−1.6
	50	51.1	1.5	2.18
1′-Hydroxymidazolam	0.5	0.46	5.1	−8
	5	5.16	4.3	3.2
	50	52.3	0.5	4.6
Inter-assay (n = 6)				
Midazolam	0.5	0.43	8.5	−14
	5	5.22	6.6	4.4
	50	51.6	4.4	3.2
1′-Hydroxymidazolam	0.5	0.45	6.1	−10
	5	5.28	4.8	5.6
	50	51.5	4.3	3

CV is coefficient of variation. RE is relative error: (concentration found−concentration added) × 100/(concentration added).

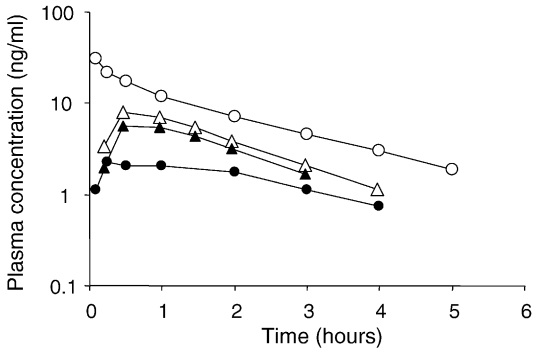

Fig. 3. Mean time–plasma concentration curves of midazolam (opens) and 1′-hydroxymidazolam (closed) after an intravenous dosing (1 mg) (circles) and a single-oral dose of midazolam (2 mg) (triangle).

Table 4
Pharmacokinetics of midazolam and 1′-hydroxymidazolam after 1 mg intravenous injection and 2 mg oral administration of midazolam in 12 healthy volunteers

	Parameters	Mean ± S.D.	CV (%)
1 mg intravenous injection			
	AUC (ng h/ml)	44.9 ± 12.2	27
Midazolam	CL (ml/min)	400 ± 110	27
	Vd (l/kg)	0.94 ± 0.17	18
	$t_{1/2}$ (h)	1.5 ± 0.4	27
1′-Hydroxymidazolam	C_{max} (ng/ml)	2.6 ± 1.3	50
	t_{max} (h)	0.6 ± 0.3	60
	AUC (ng h/ml)	8.2 ± 2.9	35
	$t_{1/2}$ (h)	1.9 ± 0.4	22
2 mg oral administration			
	C_{max} (ng/ml)	8.2 ± 4.0	49
	t_{max} (h)	0.6 ± 0.2	32
Midazolam	AUC (ng h/ml)	17.9 ± 8.1	45
	$t_{1/2}$ (h)	1.2 ± 0.4	35
	F	0.2 ± 0.06	32
1′-Hydroxymidazolam	C_{max} (ng/ml)	5.9 ± 2.1	36
	t_{max} (h)	0.7 ± 0.24	35
	AUC (ng h/ml)	13.8 ± 6.2	45
	$t_{1/2}$ (h)	1.1 ± 0.3	24

tracts from plasma samples of healthy volunteers receiving CYP3A inhibitors, erythromycin, clarithromycin, itraconazole and CYP3A inducers, carbamazepine, rifampicin and St. John's wort.

3.6. *In vivo study*

Mean time–plasma concentration curves of midazolam and 1′-hydroxymidazolam in 10 healthy volunteers after 1 mg of intravenously injected administration and 2 mg of oral administration of midazolam are shown in Fig. 3. Midazolam was detectable in plasma until 6 h after the IV dosing and 5 h after the IV dosing, while 1′-hydroxymidazolam were detectable in plasma until 5 h after the IV dosing and 4 h after the oral dosing. Pharmacokinetic parameters are shown in Table 4.

4. Conclusion

The HPLC procedure described is selective and sensitive enough for simultaneous determination of midazolam and its major metabolite, 1′-hydroxymidazolam. Satisfactory validation data were achieved for linearity, precision and recovery. The limit of quantification obtained allows measurement of CYP3A activity after safe and minimum administration of midazolam.

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