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Short Communication

## Determination of midazolam and two of its metabolites in human plasma by high-performance liquid chromatography

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### Abstract

A high-performance liquid chromatographic assay coupled with UV detection (254 nm) has been developed for the determination of midazolam and two of its hydroxylated metabolites, 1-hydroxymidazolam (1-OH) and 4-hydroxymidazolam (4-OH), in human plasma. Following a novel solid-phase extraction procedure, midazolam and its metabolites are well recovered from plasma. The analytes were extracted with C<sub>1</sub> cartridges and the extracts were evaporated to dryness. The dry residues were dissolved in 200  $\mu$ l of mobile phase [0.02 M ammonium phosphate monobasic buffer-methanol-acetonitrile (60:35:5, v/v) (300 ml), 600  $\mu$ l of 0.2 M tetrabutylammonium bromide solution, adjusted to a pH (apparent pH) of 4.10]. The separation of the analytes was performed on a Spherisorb C<sub>8</sub> column (10 cm  $\times$  4.6 mm I.D.) maintained at 30°C. The mobile phase was pumped at a flow-rate of 1.5 ml/min. The method has a lower limit of quantitation of 15 ng/ml of plasma for midazolam and proved to be reproducible (inter-assay precision 5.4%) and accurate (94  $\pm$  5%) over the therapeutic range of concentrations.

### 1. Introduction

Midazolam is a weakly basic ( $pK_a$  = 6.0), potent, short-acting imidazobenzodiazepine [1]. It is water soluble at pH < 4, but highly lipophilic at physiological pH [2]. Its plasma elimination half-life ( $t_{1/2}$ ) is 1.5–3.5 h [2,3]. Following intravenous administration, midazolam is eliminated almost exclusively by biotransformation. However, evidence of significant circulating levels of unconjugated metabolite are scarce [4,5]. The principal metabolite, 1-OH, is less pharmacologically active than the parent drug and is rapidly deactivated by conjugation with

glucuronic acid ( $t_{1/2}$  = 11 min). The 1-OH glucuronide ( $t_{1/2}$  = 53 min) makes up 50–70% of the total dose eliminated in urine and is therefore more likely to be found in its unconjugated form in plasma. A second metabolite, 4-OH, also less pharmacologically active than midazolam, is essentially found in its glucuronide conjugated form in plasma. The 4-OH glucuronide makes up 3% of the metabolites found in the urine. The two metabolites can lead to the formation of 1,4-dihydroxymidazolam, which is found exclusively in the urine [2,4,6,7].

Midazolam and its metabolites have been assayed by numerous specific and/or sensitive methods. These include high-performance liquid chromatographic assays (HPLC), gas chromato-

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graphic assays (GC), gas chromatographic–mass spectrometric methods (GC–MS), polarographic detection, and radioimmuno assays [1–19]. To date, however, reported HPLC assays mostly employ liquid–liquid extraction procedures or lack sensitivity. The only method that used a solid-phase extraction procedure had a lower limit of quantitation for midazolam and its metabolites of 50 ng/ml (Sautou *et al.*, [12]). Reported limits of quantitation for midazolam range from 5 to 50 ng/ml.

The method presented here was developed for the assay of midazolam and two of its metabolites, 1-OH and 4-OH, in plasma using solid-phase extraction and reversed-phase HPLC separation with UV detection.

## 2. Experimental

### 2.1. Chemicals and reagents

Midazolam, 1-OH, 4-OH, and the internal standard clonazepam were kindly supplied by Hoffmann-LaRoche (Etobicoke, Canada). Organic solvents and ammonium phosphate monobasic were of HPLC grade (Fisher Scientific, Montreal, Canada) and ammonium phosphate dibasic was reagent grade (Anachemia, Montreal, Canada).

### 2.2. Standard solutions

Standard stock solutions of midazolam and its metabolites were prepared at a concentration of 1 mg/ml in methanol. Dilutions of the 1 mg/ml standards were used to make the appropriate working solutions of midazolam and its metabolites. A standard stock solution of clonazepam was prepared at 1 mg/ml in methanol and further diluted to prepare the working solution at 3  $\mu$ g/ml. Stock and working solutions were stored at  $-10^{\circ}\text{C}$ . Fresh working solutions were prepared every three months.

### 2.3. Chromatography

The chromatographic system consisted of a Constametric III pump (LDC Milton Roy,

Riviera Beach, FL, USA), a Model 7125 injector fitted with a 100- $\mu\text{l}$  injection loop (Rheodyne, Cotati, CA, USA), and a Model 441 UV detector (Waters Associates, Milford, MA, USA) operating at a wavelength of 254 nm, connected to a Model CR601 Chromatopac Shimadzu integrator (Scientific Instruments, Columbia, MD, USA). A prepacked 5- $\mu\text{m}$  particle size Spherisorb C<sub>8</sub> column (10 cm  $\times$  4.6 mm I.D.) (Hichrom, Reading, UK) was used for the separation of the analytes. The column temperature was maintained at 30°C by a Model TC-50 column thermostat (Eppendorf, Madison, WI, USA).

The mobile phase consisted of 0.02  $M$  ammonium phosphate monobasic buffer–methanol–acetonitrile (60:35:5, v/v) (300 ml), to which were added 600  $\mu\text{l}$  of a 0.2  $M$  tetrabutylammonium bromide solution. The final solution was adjusted to a pH of 4.10 with phosphoric acid and filtered through a 0.20- $\mu\text{m}$  Teflon pore filter prior to use. The mobile phase was pumped at a flow-rate of 1.5 ml/min and recirculated.

### 2.4. Sample preparation

PrepSep C<sub>1</sub> solid-phase extraction cartridges (12 ml, 500 mg) (Fisher Scientific) were first conditioned with 3 ml methanol and then with 3 ml distilled water. One milliliter of plasma and 300 ng of internal standard working solution were added to the cartridges and then aspirated through the sorbent. The cartridges were sequentially washed with 2  $\times$  3 ml distilled water, and 2  $\times$  1 ml methanol 30% under light vacuum (5 kPa). Analytes were eluted with 2  $\times$  1 ml of 0.05  $M$  ammonium phosphate dibasic (pH 9.0) in methanol (10:90, v/v). The eluents were evaporated using a Model SC210A Speed-Vac concentrator (Savant Instruments, Farmingdale, NY, USA). Prior to HPLC analysis, the dry residues were dissolved in 200  $\mu\text{l}$  of mobile phase and a volume of 100  $\mu\text{l}$  was injected onto the analytical column.

### 2.5. Calibration curves

A pool of plasma (Red Cross) containing 2000 ng/ml of midazolam and its metabolites was

serially diluted with drug-free plasma to yield standards at concentrations ranging from 15 to 1000 ng/ml. Calibration curves were generated by least-squares regression of the analyte/internal standard peak-height ratio *vs.* the concentration of the analyte.

### 2.6. Recovery

The recovery of midazolam and its metabolites from human plasma was determined in triplicate at concentrations within the range of the calibration curves (31.25, 125, and 500 ng/ml). Plasma samples spiked with known amounts of analytes and 300 ng of internal standard were extracted as usual. Blank plasma samples containing only 300 ng of internal standard were extracted and subsequently spiked with the same amounts of analytes prior to evaporation. Recovery was assessed by comparing the peak-height ratios of analyte/internal standard in the two sets of extracts.

### 2.7 Precision and accuracy

Intra-assay precision was assessed from replicate analyses ( $n = 6$ ) of spiked plasma at three different concentrations (31.25, 250, and 1000 ng/ml) on the same day. Inter-assay precision was evaluated from calibration curves that were analyzed on six separate occasions.

Drug-free plasma was spiked with midazolam and its metabolites to yield nine concentrations ranging from 22 to 950 ng/ml. Samples were blindly assayed and concentrations were derived from the calibration curves. The accuracy was evaluated by comparing the estimated concentration with the known concentration of the analyte.

## 3. Results and discussion

The retention times of midazolam, 1-OH, 4-OH, and internal standard were 10.2, 14.5, 7.0, and 12.4 min, respectively. Retention times of all compounds, including the internal standard, were highly dependent on the pH<sup>+</sup> of the mobile phase. At pH<sup>+</sup> values lower than 4.10, the 1-OH

and internal standard peaks gradually merged. At pH<sup>+</sup> values greater than 4.10, merging was observed for the midazolam and internal standard peaks. The assay was therefore carried out at an optimal pH<sup>+</sup> of 4.10 to ensure an adequate chromatographic separation of the analytes.

Fig. 1 illustrates chromatograms obtained after the extraction of blank plasma (A), and plasma standard containing 250 ng/ml of midazolam and its metabolites and 300 ng of internal standard (B). Peaks of interest were free of any interfering peaks, with the exception of 4-OH; these small peaks were observed in all plasma samples and were due to coextracted endogenous substances. The actual peak height of 4-OH was determined by setting the integrator to plot background-compensated chromatograms. In this mode, the integrator has memorized the baseline of a blank plasma sample and this baseline is subsequently subtracted from each sample. This technique yielded excellent precision without compromising the sensitivity of the assay.

The lower limit of quantitation was 15 ng/ml of plasma for midazolam and its two metabolites, 1-OH and 4-OH. The mean recovery of midazolam, 1-OH, and 4-OH for the three

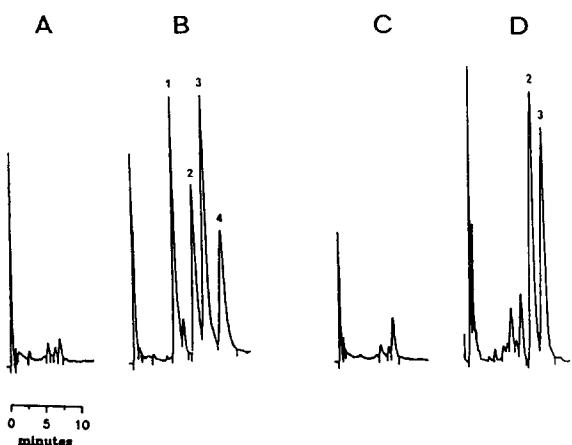


Fig. 1. HPLC following the extraction of samples containing: (A) drug-free plasma; (B) plasma spiked with 250 ng/ml midazolam and its metabolites and 300 ng internal standard; (C) plasma collected from a patient before the injection of midazolam; (D) plasma collected from a patient 2 min following the administration of an i.v. bolus of 0.05 mg/kg midazolam. Peaks: 1 = 4-OH, 2 = midazolam, 3 = internal standard, 4 = 1-OH.

**Table 1**  
Recovery of midazolam and its metabolites from human plasma

Analyte	Concentration (ng/ml)	Recovery (mean $\pm$ S.D., $n = 3$ ) (%)	Coefficient of variation (%)
Midazolam	31.25	89.6 $\pm$ 9.2	10.3
	125	76.7 $\pm$ 1.3	1.7
	500	73.1 $\pm$ 5.9	8.1
1-OH	31.25	85.1 $\pm$ 8.0	9.4
	125	80.5 $\pm$ 3.3	4.1
	500	74.9 $\pm$ 5.6	7.5
4-OH	31.25	88.8 $\pm$ 10.4	11.7
	125	83.2 $\pm$ 3.2	3.8
	500	78.1 $\pm$ 5.3	6.7

concentrations studied was 80, 80, and 83%, respectively (Table 1).

The calibration curves for midazolam and its metabolites were linear in the concentration range investigated. The regression equations for midazolam, 1-OH, and 4-OH, were  $y = 0.003x -$

0.004 ( $r^2 = 0.994$ ),  $y = 0.002x - 0.003$  ( $r^2 = 0.995$ ), and  $y = 0.004x - 0.014$  ( $r^2 = 0.997$ ), respectively.

The intra-assay precision for samples of midazolam and its metabolites were in all cases less than 10% (Table 2). The inter-assay precision gave mean coefficients of variation of 5.2, 6.4, and 4.9% for midazolam, 1-OH, and 4-OH, respectively (Table 3).

Blindly assayed spiked samples of midazolam, 1-OH, and 4-OH at concentrations within the range of the calibration curves showed mean accuracy values of 94, 100, and 85, respectively (Table 4).

The analytical method presented here was recently applied to the determination of plasma levels of midazolam following a single intraven-

**Table 2**  
Intra-assay precision of midazolam and its metabolites ( $n = 6$ )

Concentration (ng/ml)	Coefficient of variation (%)		
	Midazolam	1-OH	4-OH
31.25	4.5	7.7	8.8
250	8.7	5.9	4.1
1000	4.2	2.9	2.3

**Table 3**  
Inter-assay variability of midazolam and its metabolites ( $n = 6$ )

Spiked Concentration (ng/ml)	Midazolam		1-OH		4-OH	
	Estimated concentration (mean $\pm$ S.D.) (ng/ml)	Coefficient of variation (%)	Estimated concentration (mean $\pm$ S.D.) (ng/ml)	Coefficient of variation (%)	Estimated concentration (mean $\pm$ S.D.) (ng/ml)	Coefficient of variation (%)
15.625	15.43 $\pm$ 1.42	9.2	14.94 $\pm$ 1.47	9.8	14.97 $\pm$ 1.48	9.9
31.25	31.84 $\pm$ 0.81	2.5	33.74 $\pm$ 2.80	8.3	31.65 $\pm$ 1.61	5.1
62.5	63.66 $\pm$ 2.97	4.7	64.84 $\pm$ 6.85	10.5	67.65 $\pm$ 6.17	9.1
125	124.03 $\pm$ 2.83	2.3	122.51 $\pm$ 5.05	4.1	128.63 $\pm$ 4.51	3.5
250	246.01 $\pm$ 12.74	5.2	236.65 $\pm$ 2.81	1.2	243.60 $\pm$ 3.23	1.3
500	516.44 $\pm$ 36.20	7.0	523.11 $\pm$ 36.31	6.9	501.85 $\pm$ 15.67	3.1
1000	966.00 $\pm$ 55.27	5.7	954.62 $\pm$ 37.44	3.9	925.75 $\pm$ 23.16	2.5

Table 4  
Accuracy of the assay for midazolam and its metabolites

Spiked concentration (ng/ml)	Estimated concentration (ng/ml)		
	Midazolam	1-OH	4-OH
22	20.9	19.1	19.9
42	37.5	48.2	43.9
42	41.0	54.6	33.2
90	81.9	90.0	79.4
130	124.0	132.1	95.7
240	206.4	216.7	206.5
850	798.7	782.4	680.5
920	954.2	977.1	855.1
950	864.9	893.4	769.6
Accuracy (%)	94	100	85
Coefficient of variation (%)	5	10	9

ous (i.v.) dose of 0.05 mg/kg midazolam to a patient scheduled for elective surgery under regional anaesthesia. Briefly, arterial blood samples were drawn in heparinized tubes for up to three hours, the samples were centrifuged, and the plasma frozen until assayed for midazolam and its metabolites. Fig. 1 illustrates chromatograms obtained from an extracted plasma sample before (C) and 2 min following (D) the i.v. bolus. Fig. 2 illustrates the midazolam plasma concentration–time profile. Neither 1-OH nor 4-OH were detected throughout the study time-

course. The lack of detectable levels of either metabolite is consistent with published HPLC assays for the determination of midazolam and its metabolites in human plasma in which a single i.v. dose of midazolam was administered to human subjects [2,14,15,17].

#### 4. Conclusion

The method presented here for the determination of midazolam and its metabolites is simple and reproducible and is therefore readily applicable to pharmacokinetic studies in humans. The advantages of this HPLC assay are its sensitivity and the easy extraction of the analytes from plasma. Specifically, the assay enables the quantitation of each of the analytes with a limit of detection of 15 ng/ml. In addition, the extraction of midazolam and its metabolites using solid-phase extraction cartridges is a convenient alternative to liquid–liquid extraction.

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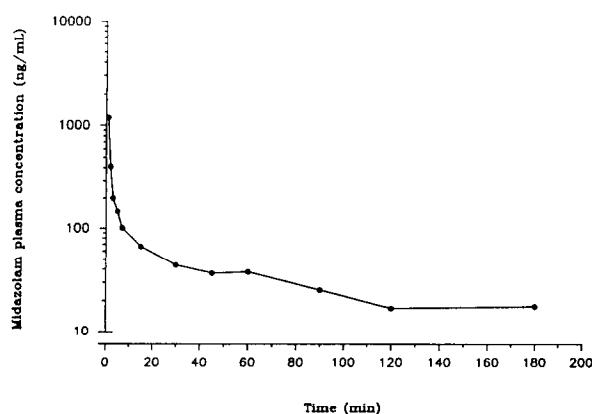


Fig. 2. Midazolam plasma concentration versus time curve in a patient following the administration of an i.v. bolus of 0.05 mg/kg midazolam.

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