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Determination of midazolam and its metabolites in serum microsamples by high-performance liquid chromatography and its application to pharmacokinetics in rats

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Abstract

A single-solvent extraction step high-performance liquid chromatographic method is described for quantitating midazolam and its two hydroxy metabolites in rat serum microsamples (50 μl). The separation used a 2 mm I.D. reversed-phase Symmetry C_{18} column with an isocratic mobile phase consisting of methanol–acetonitrile–14.9 m*M* sodium acetate in water at pH 3.0 (10:23:67, v/v). The detection limit was 10 ng/ml for all the compounds using an ultraviolet detector operated at 230 nm. The method was used to study the pharmacokinetics of midazolam after an intravenous bolus dose (0.75 mg/kg).

Keywords: Midazolam

1. Introduction

Midazolam is a potent, weakly basic (pK_a 6.0) imidazobenzodiazepine with sedative, hypnotic, anticonvulsant, muscle-relaxant and anxiolytic activities [1]. In humans, midazolam is extensively metabolized in the liver by hydroxylation to the active metabolites, α-hydroxymidazolam and 4-hydroxymidazolam [2]. Radioreceptor assay [3], gas chromatography (GC) [4–10], GC–mass spectrometry (MS) [11–13], high-performance liquid chromatography (HPLC) [14–22], and HPLC–MS [23] have been used for assaying midazolam and its metabolites. Of these methods, HPLC is the most attractive and versatile technique for the determination of drugs in biological samples since it provides the possibility of adjusting the selectivity of the resolution over a wide range. Moreover, owing to its milder working conditions, HPLC is the most suitable technique for the analysis of thermally labile compounds.

This paper describes a rapid and sensitive HPLC method capable of determining midazolam and its metabolites in small samples (50 μl). Sample size is critical when the animal species used is small, especially when repeated blood sampling is necessary to trace the temporal changes in drug levels in individual animals. The convenience of our method is facilitated by its use of a single-solvent extraction procedure and the commercially available 2 mm I.D. column. An added advantage of using the 2 mm I.D. column is a reduction in solvent consumption by up to 80%, compared to the 4.6 mm I.D. column. This method is hereby applied to evaluate the phar-

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macokinetics of midazolam after intravenous (i.v.) bolus midazolam administration.

2. Experimental

2.1. Instrumentation

The HPLC system consisted of a Waters Associates Model 510 pump (Milford, MA, USA), a 7725 Rheodyne injector with 20-μl loop (Cotati, CA, USA), and a 785A programmable absorbance UV detector, operated at 230 nm (Applied Biosystems Instruments, Foster City, CA, USA). The separation was performed on a symmetry C_{18} column, 150×2.1 mm I.D. (Waters Associates) with a 2 μm precolumn filter (Rheodyne). The data were collected using a PE Nelson 900 series interface, Turbochrom 4.1 software (Norwalk, CT, USA) and an IBM-type 486 microcomputer workstation.

2.2. Reagents and standards

Midazolam maleate, α-hydroxymidazolam and 4-hydroxymidazolam were supplied by Hoffmann-La Roche (Nutley, NJ, USA). HPLC-grade methanol, acetonitrile, chloroform, sodium acetate and diethyl ether were purchased from Fisher Scientific (Springfield, NJ, USA). The 1 *M* borate–sodium carbonate–potassium chloride buffer (pH 9.0) was prepared by the method of de Silva and Puglisi [24]. All other chemicals were reagent grade.

Midazolam, α-hydroxymidazolam and 4-hydroxymidazolam were dissolved in methanol individually to make 1 mg/ml stock solution. Dilutions of the 1 mg/ml standards were used to make the working standards (0.1, 0.5, 1.0 μg/ml) containing the three compounds.

The HPLC analyses were performed using an isocratic mobile phase consisting of methanol–acetonitrile–14.9 m*M* sodium acetate in water at pH 3.0 (10:23:67, v/v). Mobile phases were degassed and filtered through a solvent filtration apparatus (Alltech Associates, Deerfield, IL, USA).

2.3. Sample preparation

Standards and serum samples were prepared as previously described [25]. Briefly, a 50-μl working serum standard was added to a 15-ml conical centrifuge tube. Borate buffer (1 *M*, pH 9.0, 100 μl) was added and the solution was mixed well. A 1-ml volume of chloroform-diethyl ether (95:5) was added and the sample mixture was vortex-mixed for 1 min and centrifuged for 5 min at 1100 *g*. The 1.15-ml sample mixture rose to 2 cm below the rim of the 15-ml conical centrifuge tube during vortex-mixing, a procedure which ensured vigorous mixing for the extraction of midazolam and its metabolites to the organic solvent. The organic layer was carefully transferred to a 5-ml conical centrifuge tube and evaporated to dryness in an evaporator (Pierce, Rockford, IL, USA) at 40°C under nitrogen. The residue was resuspended in 50 μl of the mobile phase and injected onto the column. Samples for serum drug analysis were prepared identically except that standards were not added.

2.4. Extraction recovery

The assay recoveries of midazolam and its metabolites were assessed at concentrations of 0.1, 0.5 and 1.0 μg/ml. Six replicates of each concentration, containing the three compounds, extracted according to the method described above, were injected onto the column. Six replicates of each concentration were computed using the following equation:

$$\text{Recovery} = \frac{\text{(peak height extract)}}{\text{(mean peak height direct injection)}} \times 100\%$$

2.5. Midazolam administration and serum sampling

Four adult male, Holtzman albino rats held to 80% of their normal, adult starting weight (mean 382 g, range 380–386 g) were used. Right jugular vein cannulation and blood sampling have been described previously [26]. The catheter was flushed with 0.9% saline containing 20 units of heparin per ml and sealed with fishing line when not in use. Animals were allowed to recover for two days and then received an i.v. bolus midazolam maleate dose (0.75 mg/kg). Injections were given in a volume of 1 ml/kg. Blood samples (100 μl) from the jugular catheter were collected at 5, 15, 30, 45, 60 and 120 min, were centrifuged for 10 min at 13,700 *g* and stored frozen until analysis. The experimental proto-

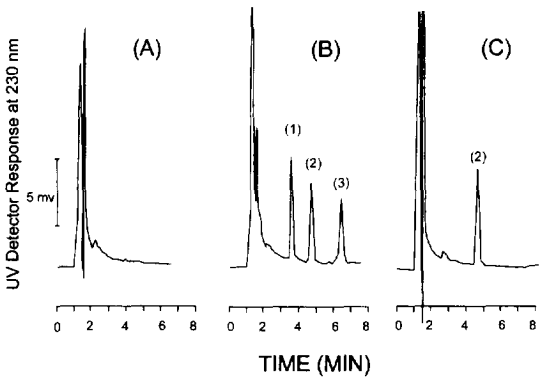

Fig. 1. Chromatograms of (A) rat serum blank, (B) rat serum containing 0.25 μg/ml 4-hydroxymidazolam, midazolam and α-hydroxymidazolam, (C) a 50-μl rat serum sample obtained 15 min after 10 mg/kg midazolam s.c. administration. Peaks: 1 = 4-hydroxymidazolam; 2 = midazolam; 3 = α-hydroxymidazolam.

col was reviewed and approved by the Rutgers University Animal Care and Facilities Committee.

2.6. Pharmacokinetic analysis

After i.v. midazolam maleate administration, a biexponential function of the following form was fitted to the experimental data.

$$C_t = A\,e^{-\alpha t} + B\,e^{-\beta t}$$

$$Cl = \text{dose/AUC}$$

$$V_c = \text{dose}/(A + B)$$

where C_t is the concentration in serum at any time t, α and β are the hybrid rate coefficients for the distribution and elimination phases, A and B are the preexponential coefficients, Cl is the clearance and V_c is the volume of distribution for the central compartment. The area under the curve (AUC) was calculated by the trapezoidal method. Midazolam dose was corrected to base to calculate Cl and V_c.

3. Results and discussion

3.1. Method evaluation

Fig. 1 shows chromatograms of (A) a serum blank with no interfering peaks, (B) a spiked serum sample containing a working standard (0.25 μg/ml) which was extracted by the liquid–liquid extraction procedure, and (C) a representative rat serum sample (50 μl) obtained 15 min after 10 mg/kg midazolam subcutaneous (s.c.) administration. In a previous study, an ion-pairing agent was needed in the mobile phase to separate midazolam using a reversed-phase Ultrasphere C_{18} column [21]. In the present study, the ion-pairing agent could be omitted from the mobile phase and the shapes of the peaks for the three compounds were symmetrical using the Symmetry column.

The external standard method was used in the calibration and evaluation of the unknown samples. Table 1 shows the within-day and between-day precisions of midazolam and its metabolites which

Table 1
Precision data for midazolam and its metabolites in serum

Compound	Within-day ($n=6$)		Between-day ($n=6$)	
	Concentration (mean±S.D.) (μg/ml)	C.V. (%)	Concentration (mean±S.D.) (μg/ml)	C.V. (%)
Midazolam	0.1005±0.002	2.34	0.1053±0.004	3.82
	0.5017±0.009	1.72	0.4971±0.019	3.77
	0.9964±0.015	1.47	0.9930±0.030	2.97
α-Hydroxymidazolam	0.0997±0.003	2.49	0.1003±0.004	3.48
	0.5052±0.008	1.48	0.4991±0.014	2.89
	0.9998±0.013	1.28	1.0186±0.028	2.74
4-Hydroxymidazolam	0.0992±0.002	1.67	0.0994±0.004	3.93
	0.4973±0.006	1.22	0.5080±0.019	3.68
	1.0002±0.015	1.52	0.9943±0.029	2.94

were established at three different concentrations (0.1, 0.5 and 1.0 μg/ml) by adding these compounds to blank serum. Both within-day and between-day precisions for midazolam and its metabolites were markedly high as indicated by the coefficients of variation (C.V.), which ranged from 1.22 to 2.49% and from 2.89 to 3.93% respectively. Although the internal standard technique is widely used in chromatography, limitations of this technique have been found in the analysis of drugs in biological materials, and external calibration offers better precision in the absence of a suitable internal standard [27].

Calibration curves for midazolam and its two hydroxy metabolites are linear within the ranges (0.1–1.0 μg/ml) examined. For each of the six regression lines, the correlation coefficients are larger than 0.998. The coefficients of variation of the slopes ($n=6$) of the regression lines ranged from 1.43 to 1.69 with intercepts all close to zero (Table 2). The detection limit was 0.2 ng (10 ng/ml) for all the compounds, with a signal-to-noise ratio of 4.

The extraction recoveries (mean±S.D.) for midazolam, α-hydroxymidazolam and 4-hydroxymidazolam at the three concentrations (0.1, 0.5 and 1.0 μg/ml) were above 86.83% (Table 3). By using chloroform–diethyl ether (95:5) instead of diethyl ether as the extraction solvent, and vortex-mixing the samples in the extraction solvent longer (1 min), markedly improved recoveries for the three compounds were attained [21].

Table 3
Recovery of midazolam and its metabolites

Concentration (μg/ml)	Recovery (%) (mean±S.D.)	C.V. (%)
Midazolam		
0.1	91.72±5.31	5.79
0.5	89.38±3.12	3.49
1.0	86.83±3.80	4.38
α-Hydroxymidazolam		
0.1	89.13±5.39	6.05
0.5	91.68±2.88	3.14
1.0	90.86±2.24	2.47
4-Hydroxymidazolam		
0.1	89.93±5.85	6.51
0.5	90.73±6.56	7.23
1.0	88.45±3.35	3.79

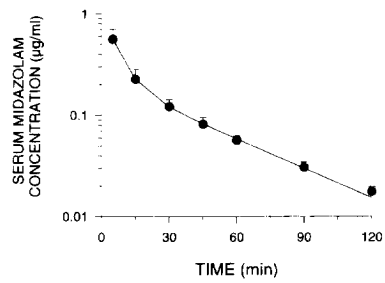

Fig. 2. Serum midazolam concentration-time profile following an i.v. bolus of 0.75 mg/kg midazolam maleate administration ($n=4$).

3.2. Pharmacokinetic results

Fig. 2 shows the serum midazolam concentration-time profile after 0.75 mg/kg midazolam maleate i.v. administration. Metabolites were not detectable by both routes of administration (i.v. and s.c.). The distribution and elimination half-lives were 4.0±3.1 and 39.7±16.1 min, respectively. These values for midazolam were consonant with the values obtained in rats by a GC method, where 0.4 ml of plasma were used [9]. *Cl* for midazolam was 11.5±3.4 ml/min and V_c was 0.18±0.1 l in the present study.

Table 2
Mean of calibration equations for midazolam and its metabloites over the concentration range 0.1–1.0 μg/ml

Compound	Equation	Correlation coefficient	C.V. of slope (%)
Midazolam	$y=26\,959\ (\pm960)\ x-142\ (\pm269)$	0.9980	3.56
α-Hydroxymidazolam	$y=21\,572\ (\pm689)\ x-244\ (\pm221)$	0.9992	3.19
4-Hydroxymidazolam	$y=32\,656\ (\pm1234)\ x-320\ (\pm190)$	0.9990	3.78

y = peak-height, x = concentration of each compound.

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