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Sensitive assay for midazolam and its metabolite 1′-hydroxymidazolam in human plasma by capillary high-performance liquid chromatography

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Abstract

A sensitive high-performance liquid chromatographic method is described for the quantification of midazolam and 1′-hydroxymidazolam in human plasma. Sample (1 ml plasma) preparation involved a simple solvent extraction step with a recovery of approximately 90% for both compounds. An aliquot of the dissolved residue was injected onto a 3 μm capillary C_{18} column (150 mm×0.8 mm I.D.). A gradient elution was used. The initial mobile phase composition (phosphate buffer–acetonitrile, 65:35) was maintained during 16 min and was then changed linearly during a 1-min period to phosphate buffer–acetonitrile, 40:60. The flow-rate of the mobile phase was 16 μl/min and the eluate was monitored by UV detection. The limits of quantification for midazolam and 1′-hydroxymidazolam were 1 ng/ml and 0.5 ng/ml, respectively. The applicability of the method was demonstrated by studying the pharmacokinetics of midazolam, and its major metabolite 1′-hydroxymidazolam, in human volunteers following i.v. bolus administration of a subtherapeutic midazolam dose (40 μg/kg).

Keywords: Midazolam; 1′-Hydroxymidazolam

1. Introduction

Midazolam, a short-acting benzodiazepine with hypnotic properties, is used for conscious sedation to perform short diagnostic and endoscopic procedures and for induction and maintenance of general anesthesia [1,2]. Midazolam is eliminated in man by metabolism: less than 1% of an intravenous (i.v.) dose is recovered in urine as unchanged drug [3,4]. The major metabolite is 1′-hydroxymidazolam which is pharmacologically active [5]. Other phase I metabolites formed in man are the 4-hydroxymidazolam and 1′,4-dihydroxymidazolam. In vitro studies have shown that phase I metabolism of midazolam in man is mediated almost exclusively by cytochrome P450-3A (CYP3A) isoforms [6,7]. The CYP3A subfamily

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is now recognized to be among the most important of all the human drug metabolizing enzymes and midazolam has been used in recent years as an in vivo and in vitro probe for hepatic and intestinal CYP3A activity [8–10].

This paper describes a very sensitive high-performance liquid chromatography (HPLC) assay for midazolam and its major metabolite 1′-hydroxymidazolam in human plasma. The i.v. clearance of midazolam can be used to compare CYP3A activity among individuals as well as to investigate the effect of known or potential inhibitors and inducers [11]. Therefore, pharmacokinetic studies with a low i.v. dose of midazolam were conducted in healthy subjects before and after treatment with rifampicin, a known CYP3A inducer, to show the applicability of the developed assay.

2. Experimental

2.1. Chemicals and standards

Midazolam, 1′-hydroxymidazolam, 4-hydroxymidazolam and diazepam (internal standard) were kindly provided by Hoffmann-La Roche (Basel, Switzerland). Acetonitrile HPLC ultra gradient grade and cyclohexane ultra resi-analysed were purchased from Baker (Deventer, The Netherlands). Diethyl ether was purchased from Labscan (Dublin, Ireland) and was distilled immediately before use. Water was always demineralized and filtered (Milli-Q Water Purification System, Millipore-Waters, Milford, MA, USA). All other solvents and reagents were of the highest purity available. Blank plasma for the preparation of standard samples was obtained from the university hospital's blood bank.

2.2. Preparation of analytical standards

Stock solutions of midazolam (1 mg/ml), 1′-hydroxymidazolam (560 μg/ml) and diazepam (1 mg/ml) were prepared in methanol and stored at −20°C. Working solutions (in methanol) were prepared from these stock solutions and were also stored at −20°C. Plasma standards were prepared by transferring an aliquot of a methanolic working solution of midazolam and 1′-hydroxymidazolam to a clean tube and by adding blank plasma after evaporation of the methanol. The following series of plasma standards were thus prepared: 1, 2, 4, 10, 20, 50 and 100 ng/ml for midazolam, and 0.5, 1, 2, 4, 7 and 10 ng/ml for 1′-hydroxymidazolam.

2.3. Sample preparation

To a 1-ml aliquot of plasma standard (prepared as described in Section 2.2) or plasma sample (obtained after i.v. injection of midazolam to volunteers) were added: 25 μl of internal standard solution (40 μg diazepam/ml of methanol), 40 μl of 2% NaOH and 3.5 ml of cyclohexane–diethyl ether (31:69). The mixture was extracted on a rotary mixer for 10 min at 4°C and then centrifuged at 2000 *g* for 10 min at 4°C. The organic phase (3.3 ml) was transferred to a clean tube and evaporated to dryness under a stream of nitrogen at 40°C. The residue was dissolved in 300 μl of water–acetonitrile (95:5) and 20 μl were injected onto the HPLC column.

2.4. Instrumentation

The HPLC system consisted of a 7125 Rheodyne injector with a 20-μl loop (Cotati, CA, USA), a Kontron Instruments Model 325 pump (Milan, Italy), and a Kontron 335 UV detector equipped with a UZ capillary flow cell (LC Packings, Amsterdam, The Netherlands), operated at 240 nm between 0 and 17.6 min and at 300 nm between 17.6 and 28.0 min. Data processing was performed by using the Kontron PC integrator pack. The HPLC pump was converted to deliver a microflow by interfacing the pump with a microflow processor (Accurate, LC Packings).

2.5. Operating conditions

A guard column filled with C_{18} packing (μ-Precolumn cartridge, 5 mm×0.8 mm I.D., LC Packings) was used. The separation was performed on a Hypersil C_{18} BDS 3 μm capillary column (150 mm×0.8 mm I.D., LC Packings) at ambient temperature. To reduce the analysis time, a gradient elution was necessary. The initial mobile phase composition was phosphate buffer (10 m*M* Na_2HPO_4 buffer adjusted to pH 7.0 with phosphoric acid)–acetonitrile (65:35). This mobile phase composition was

maintained during 16 min following injection at which time the composition was changed linearly during a 1-min period to phosphate buffer–acetonitrile (40:60). The mobile phase was continuously degassed with helium. The flow-rate through the column was 16 μl/min.

2.6. Assay validation

Calibration curves in the range of 1 to 100 ng/ml for midazolam and 0.5 to 10 ng/ml for 1′-hydroxymidazolam were constructed as the peak area ratios of midazolam and 1′-hydroxymidazolam to internal standard. Least-squares linear regression analysis was used to determine the slope, intercept and correlation coefficient. Inter-day reproducibility was determined by calculating the coefficients of variation of the slopes and intercepts of five calibration curves. The accuracy of the assay was determined by calculating the relative error (R.E.) on the standards of the calibration curves.

Recovery of midazolam and 1′-hydroxymidazolam was measured by comparing the chromatographic peak areas of plasma standards following extraction to those obtained from direct injection of aqueous (water–acetonitrile, 95:5) solutions of both compounds. Recoveries were determined at 1, 50 and 100 ng/ml plasma for midazolam and at 0.5, 6.25 and 10 ng/ml plasma for 1′-hydroxymidazolam.

Intra-day and inter-day accuracy and precision of the midazolam and 1′-hydroxymidazolam assays were determined at 1, 5, 50 and 100 ng midazolam/ml plasma and at 0.5, 2, 6.25 and 10 ng 1′-hydroxymidazolam/ml plasma (see Tables 1 and 2, respectively).

2.7. Pharmacokinetic study of i.v. midazolam in healthy volunteers

A 40 μg/kg dose of midazolam was administered intravenously to healthy volunteers ($n=9$) on two different occasions: (1) without rifampicin (Rifadine, Marion Merrell Dow) pretreatment (control study), and (2) following a 7-day rifampicin (600 mg/day) treatment period (induction study). The study was approved by the Human Ethics Committee of University Hospital. Blood samples (10 ml) were collected before (blank) and at the following times after drug administration: 5, 10, 15, 20, 30, 45, 60, 90, 120, 180, 240 and 300 min. Plasma samples were stored at −20°C until analyzed.

3. Results and discussion

3.1. Method evaluation

Because midazolam is a commonly used drug and in addition has been proposed as an in vitro and in vivo probe to measure CYP3A activity in man, many quantitative assays have been developed. Plasma (or serum) concentrations of midazolam and its major metabolite, 1′-hydroxymidazolam, have been determined by numerous techniques including gas chromatographic (GC) assays with electron capture or nitrogen–phosphorus detection (e.g., Refs. [12,13]),

Table 1
Precision (repeatability) and accuracy of the HPLC assay for midazolam in plasma

	Midazolam added (ng/ml)	Midazolam found (mean±S.D.) (ng/ml)	C.V. (%)	R.E. (%)
Intra-day	1	0.95±0.04	4.65	−5.0
($n=4$)	5	4.90±0.13	2.65	−2.0
	50	49.8±0.42	0.84	−0.4
	100	100.0±0.21	0.21	0.0
Inter-day	1.0	1.10±0.08	7.66	+10.0
($n=5$)	5.0	4.93±0.14	2.89	−1.4
	50.0	49.8±0.40	0.79	−0.4
	100.0	100.0±0.11	0.11	0.0

S.D.=Standard deviation; C.V.=coefficient of variation; R.E.=relative error [(found−nominal)/nominal].

Table 2
Precision (repeatability) and accuracy of the HPLC assay for 1′-hydroxymidazolam (1′-OH-MDZ) in plasma

	1′-OH-MDZ added (ng/ml)	1′-OH-MDZ found (mean±S.D.) (ng/ml)	C.V. (%)	R.E. (%)
Intra-day	0.5	0.50±0.06	11.46	0.0
(n=4)	2	2.01±0.08	4.19	−0.5
	6.25	6.23±0.10	1.57	−0.3
	10	10.02±0.03	0.33	+0.2
Inter-day	0.5	0.53±0.07	13.73	+6.0
(n=5)	2	1.95±0.11	5.55	−2.5
	6.25	6.28±0.07	1.08	+0.5
	10	10.05±0.15	1.51	+0.5

S.D.=Standard deviation; C.V.=coefficient of variation; R.E.=relative error [(found−nominal)/nominal].

gas chromatography–mass spectrometry (GC–MS) methods using electron impact or negative ion chemical ionization techniques (e.g., Refs. [14,15]), and HPLC (e.g., Refs. [16–19]). In general, GC methods are more sensitive than HPLC methods for the quantification of midazolam and 1′-hydroxymidazolam in biological fluids. With certain GC methods the lower limits of quantification have been reported to be in the subnanogram range. However, most of these GC methods are time consuming (sample preparation/derivatization) and/or require expensive equipment such as GC–MS. Therefore, HPLC methods are preferred but these, in general, lack the necessary sensitivity to measure plasma concentrations of midazolam and 1′-hydroxymidazolam following a small, subtherapeutic i.v. bolus dose to healthy volunteers. Indeed, limits of quantification of most HPLC methods are not below 5–10 ng/ml for either midazolam or 1′-hydroxymidazolam, which is not sufficient to characterize the pharmacokinetics of midazolam and its major metabolite following i.v. administration of a small dose.

Therefore, a sensitive and relatively simple capillary HPLC method was developed for midazolam and 1′-hydroxymidazolam. Fig. 1 shows chromatograms of blank plasma, plasma spiked with midazolam, 1′-hydroxymidazolam and internal standard, and plasma obtained from a volunteer following i.v. injection of a 40 μg/kg dose of midazolam. Sample preparation consisted of one single extraction step. Recoveries for both midazolam and 1′-hydroxymidazolam were approximately 90% (Table 3). Plasma extracts did not show interfering peaks from endogenous substances. Preliminary results showed that it was absolutely necessary to use freshly distilled diethylether for extracting plasma and that the extraction procedure was best carried out at 4°C to avoid interfering peaks from endogenous plasma constituents. Another metabolite, 4-hydroxymidazolam, elutes from the column just before the 1′-hydroxymidazolam with a retention time of 9.6 min, but was not detectable in human plasma following i.v. administration of a 40 μg/kg midazolam dose.

Calibration curves for midazolam and 1′-hydroxymidazolam were linear within the ranges studied, i.e. 1–100 ng/ml for midazolam and 0.5–10 ng/ml for 1′-hydroxymidazolam. The coefficients of variation of the slopes of five standard curves were 5.1% and 6.7% for midazolam and 1′-hydroxymidazolam, respectively, and all intercepts were close to zero (Table 4). The intra-day and inter-day precision of the midazolam and 1′-hydroxymidazolam assay were determined at different concentrations including the extremes of the calibration curves. The intra-day and inter-day precision for both compounds were good as indicated by the coefficients of variation which were always smaller than 5%, except for the inter-day precision of the lowest midazolam standard (i.e., 1 ng/ml) for which a coefficient of variation of 7.7% was found, and for the lowest 1′-hydroxymidazolam plasma standard (i.e., 0.5 ng/ml) for which coefficients of variation of 11.5% (intra-day) and 13.7% (inter-day) were found (Tables 1 and 2).

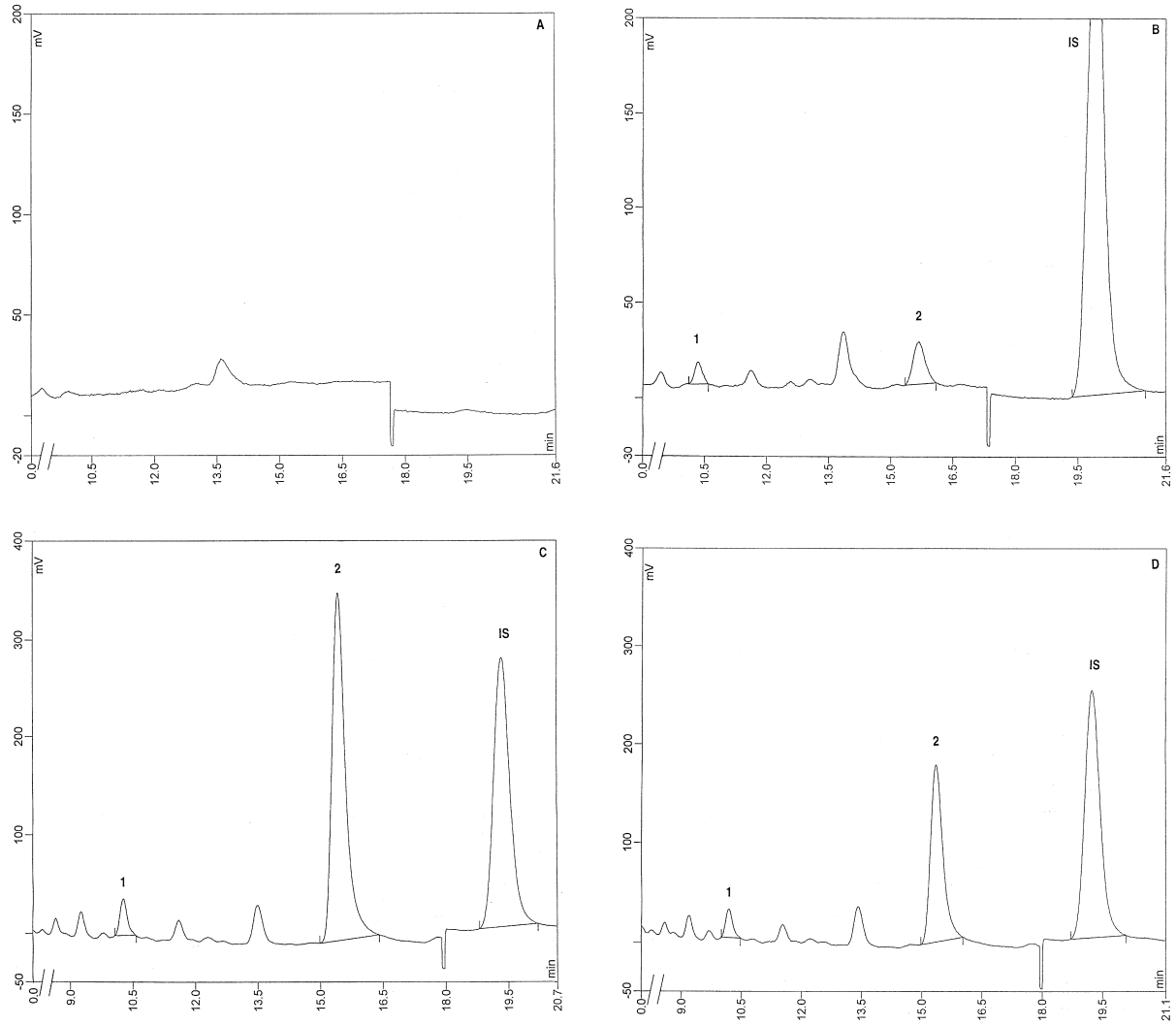

Fig. 1. Chromatograms of (A) blank human plasma, (B) blank human plasma spiked with midazolam (1 ng/ml), 1′-hydroxymidazolam (1 ng/ml) and internal standard (1 μg/ml), (C) plasma sample obtained 30 min following i.v. injection of a 40 μg/kg midazolam dose to a healthy volunteer (control), and (D) plasma obtained 30 min after i.v. injection of a 40 μg/kg midazolam dose to a healthy volunteer following pretreatment with rifampicin (1=1′-hydroxymidazolam, 2=midazolam, I.S.=internal standard).

These values are in accordance with the guidelines formulated by a group of experts during the 1990 conference on ''Analytical Methods Validation: Bioavailability, Bioequivalence and Pharmacokinetic Studies'' [20], which state that the precision around the mean value should not exceed ±15% (±20% for the limit of quantification, LOQ) and accuracy should be within ±15% (±20% for the LOQ) of the actual value.

3.2. Applicability of the method: pharmacokinetic study

Fig. 2 shows the semi-logarithmic plasma concentration–time profiles for midazolam and 1′-hydroxymidazolam in a healthy volunteer following i.v. administration of a 40 μg/kg midazolam dose. 1′-Hydroxymidazolam is the major metabolite of midazolam in man and was the only one detectable

Table 3
Extraction recoveries of midazolam and 1′-hydroxymidazolam

Compound	Concentration (ng/ml)	Recovery (%) (mean±S.D.)	C.V. (%)
Midazolam	1	89.7±4.5	5.0
	50	91.3±2.0	2.2
	100	94.9±2.5	2.7
1′-Hydroxymidazolam	0.5	90.7±4.5	4.9
	6.25	91.7±3.2	3.5
	10	89.8±3.2	3.5

S.D.=Standard deviation; C.V.=coefficient of variation.

Table 4
Individual and mean values for the slopes, intercepts and correlation coefficients of five calibration curves for midazolam and 1′-hydroxymidazolam

Compound	Curve	Slope	Intercept	*r*
Midazolam	1	0.0144	0.0037	0.999
	2	0.0136	0.0046	0.989
	3	0.0153	0.0021	0.995
	4	0.0152	0.0013	0.999
	5	0.0153	0.0030	0.997
	Mean ±S.D.	0.0148 ±0.0008	0.0029 ±0.0013	
1′-Hydroxymidazolam	1	0.0136	0.0004	0.997
	2	0.0150	0.0005	0.987
	3	0.0163	0.0007	0.995
	4	0.0152	0.0005	0.999
	5	0.0144	0.0008	0.999
	Mean ±S.D.	0.0149 ±0.0010	0.0006 ±0.0002	

in the plasma samples. As already pointed out before, 4-hydroxymidazolam elutes from the HPLC column just in front of 1′-hydroxymidazolam and does not interfere with the quantification of the 1′-hydroxymidazolam. In the nine volunteers who received a 40 μg/kg i.v. bolus dose of midazolam, plasma concentrations of midazolam fluctuated between 123 ng/ml, at 5 min following injection, and 2 ng/ml at 360 min following injection. Plasma concentrations of 1′-hydroxymidazolam were much lower and did not exceed 8.5 ng/ml in any of the volunteers studied. In a number of volunteers 1′-hydroxymidazolam plasma concentrations decreased below the LOQ (0.5 ng/ml) before the end of the sampling period. In all cases, however, the AUC for the 1′-hydroxymidazolam plasma concentration–time profile could be measured with the AUC-extrapolated representing less than 12% of the total AUC. Induction with rifampicin is associated with an increased systemic midazolam clearance (Fig. 2).

The developed method can be easily adapted, and is now also being used in our laboratory, to quantify 1′-hydroxymidazolam and 4-hydroxymidazolam in microsomal suspensions prepared from rat liver and intestinal mucosa.

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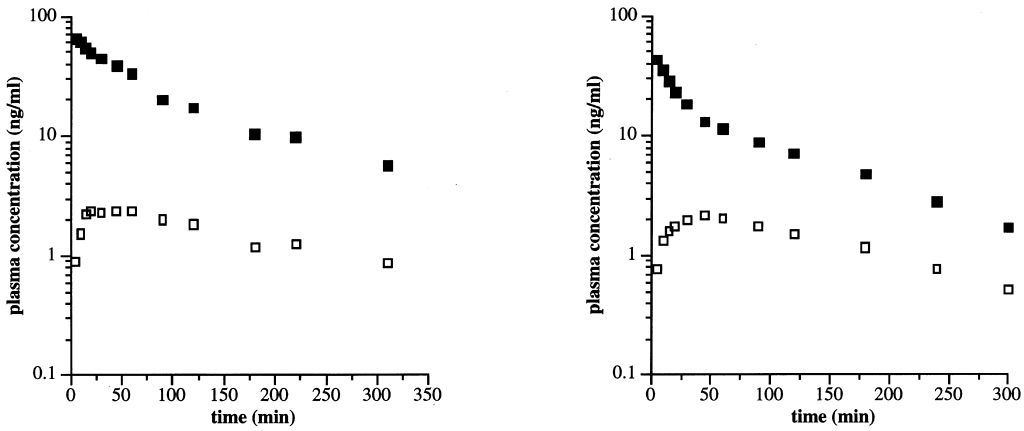

Fig. 2. Semi-logarithmic plasma concentration–time profiles of midazolam (■) and 1′-hydroxymidazolam (□) after an i.v. bolus injection of 40 μg/kg to a healthy volunteer: control (day 0), following a one week of 600 mg/day rifampicin pretreatment (day 7).

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