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Journal of Chromatography B, 783 (2003) 303–307

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Short communication

Determination of midazolam and 1′-hydroxymidazolam by liquid chromatography–mass spectrometry in plasma of patients undergoing methadone maintenance treatment

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Received 27 March 2002; received in revised form 9 September 2002; accepted 9 September 2002

Abstract

A rapid, sensitive and selective LC–MS method was developed for the simultaneous determination of midazolam (MDZ) and 1′-hydroxymidazolam (1′-OHMDZ) in plasma taken from 54 patients undergoing methadone maintenance therapy, most of whom were multidrug users. Samples spiked with prazepam, the internal standard, and were extracted into diethyl ether. Compounds were separated on a Phenomenex Luna C_{18} column and a mobile phase of acetonitrile–ammonium acetate buffer (10 m*M*, pH 4.7) (52:48, v/v) at a flow-rate of 1 ml/min. The limit of detection was 0.65 and 0.68 (ng/ml) for MDZ and 1′-OHMDZ, respectively. Within-day relative standard deviations were less than 8%.

Keywords: Midazolam; 1′-Hydroxymidazolam

1. Introduction

Midazolam is a short-acting benzodiazepine that is used in clinical practice for induction of anesthesia [1,2]. It is also widely used as a probe to measure the activity of CYP3A4, an important member of the cytochrome P450 superfamily of drug metabolizing enzymes [3–9]. This benzodiazepine undergoes oxidative metabolism to one major metabolite, 1′-hydroxymidazolam, in humans, a pathway which seems to be mediated almost exclusively by CYP3A isoforms [10,11]. The published techniques for the analysis of midazolam and its metabolites during pharmacokinetic studies have mainly involved high-performance liquid chromatography (HPLC) with UV detection [12–20] although GC–MS is used in most of studies assessing CYP3A4 activity [7,21–24].

Currently we are studying the activity of CYP forms in an opiate-user population. Many of these individuals are using concurrent medication, particularly antidepressants and benzodiazepines. Because of the potential for the co-elution of other drugs with midazolam and it metabolites during chromatography, HPLC with UV detection was not

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1570-0232/02/$ – see front matter
PII: S1570-0232(02)00673-6

be suitable for the analysis of plasma samples from opiate users. We have now developed a LC–MS method using selected ion monitoring for the determination of MDZ and 1′-OHMDZ in human plasma.

2. Experimental

2.1. Chemicals

Midazolam HCl was obtained from Antigen Pharmaceuticals (Roscrea, Ireland), 1′-hydroxymidazolam base from Ultrafine (Manchester, UK) and the internal standard, prazepam from Sigma (Poole, UK). Other chemicals were of HPLC or analytical grade and were used without any further purification.

2.2. LC–MS

The equipment comprised a Waters™ 2690 separation module (Waters, Watford, UK) coupled to a platform LC single quadruple mass spectrometer (Micromass, Altrincham, UK). The latter was equipped with atmospheric pressure ionization (APCI) source, and a crossflow counter electrode. An APCI pin voltage of 3.88 kV and a cone voltage of 35 V were used. The source heater was set at 120 °C and the APCI heater at 500 °C, and drying nitrogen flow-rate was 240 l/h. The chromatographic separation was performed on a Luna C_{18} analytical column (3 μ*M* particle size, 100×4.6 mm I.D.) (Phenomenex, Cheshire, UK) coupled to a security guard C_{18} precolumn (Phenomenex), using an isocratic mobile phase of acetonitrile–10 m*M* ammonium acetate buffer (pH 4.7) (52:48, v/v), delivered at a flow-rate of 1 ml/min.

2.3. Sample preparation

Plasma was treated according to the method of Carrillo et al. [15] with some modifications. To 1 ml plasma, 20 μl of internal standard (prazepam, 68 ng/ml), 1 ml 0.75 *M* glycine buffer (pH 9) and 4 ml diethyl ether were added. The samples were mixed for 30 min and centrifuged for 10 min at 2500 *g*. A 1-ml volume of 0.1 *M* acetate buffer (pH 4.7) was added to the organic phase after separation, and after a second extraction and centrifugation for 5 min the organic phase was transferred to conical glass tubes and evaporated to dryness using a Buchler vortex evaporator (Genetic Research Instrumentation, Dunmow, UK) at 37 °C. The residue was dissolved in 150 μl of mobile phase and 100 μl was injected onto the LC column.

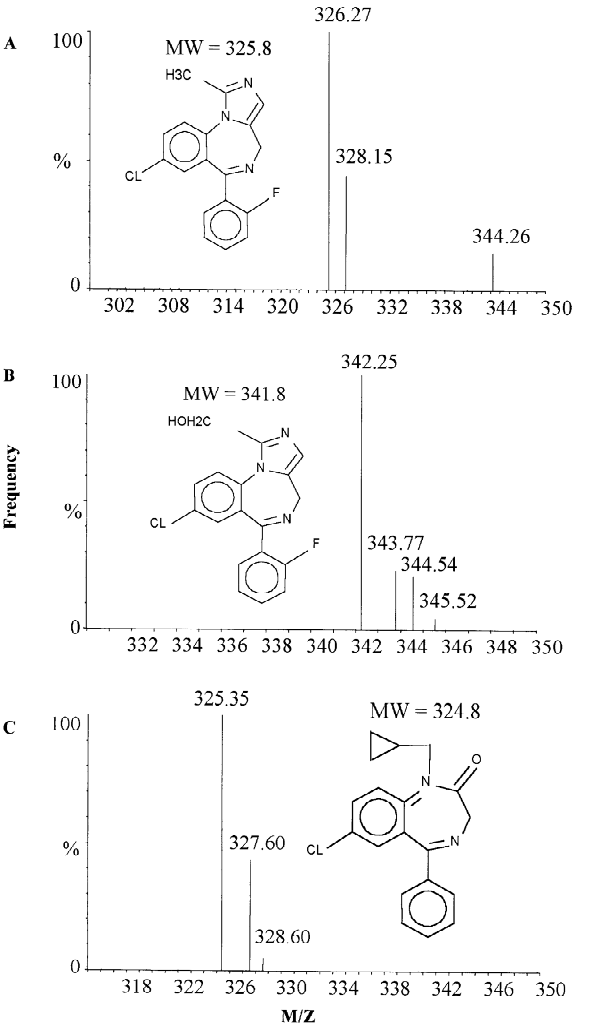

Fig. 1. Mass spectra of (A) MDZ, (B) 1′-OHMDZ and (C) prazepam (I.S.).

2.4. Calibration curves

Known quantities of midazolam and 1-hydroxymidazolam in the concentration range 6.5–208 ng/ml were added to blank plasma samples. Calibration curves were constructed by plotting the peak area ratios of drug or metabolite peaks to that of the I.S. against known concentrations.

2.5. Method validation

Within-day precision and accuracy were determined by preparing and analysing on the same day five replicates at two concentrations. Between-day precision and accuracy were assessed by analysing each day for 5 days a set of 1-ml samples spiked with 6.5, 13, 26, 52, 104 and 208 ng/ml of drug and metabolite. Relative standard deviation (RSD) values were used as the index of precision, and an estimate of accuracy was obtained by comparing the mean experimental concentration of assayed standards with their theoretical target values.

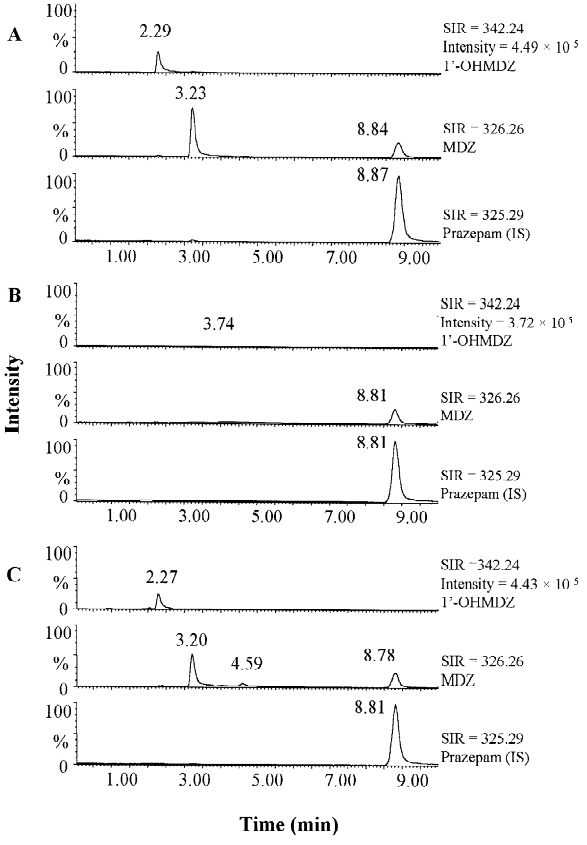

Fig. 2. Reconstructed mass chromatograms following the analysis of plasma spiked with 26 and 27 ng/ml of MDZ and 1′-OHMDZ, respectively (A), blank plasma of a patient (B), and the plasma of a patient taken 2 h after oral administration of 7.5 mg MDZ (C). The concentration of the latter was 33 and 34 ng/ml for MDZ and 1′-OHMDZ, respectively.

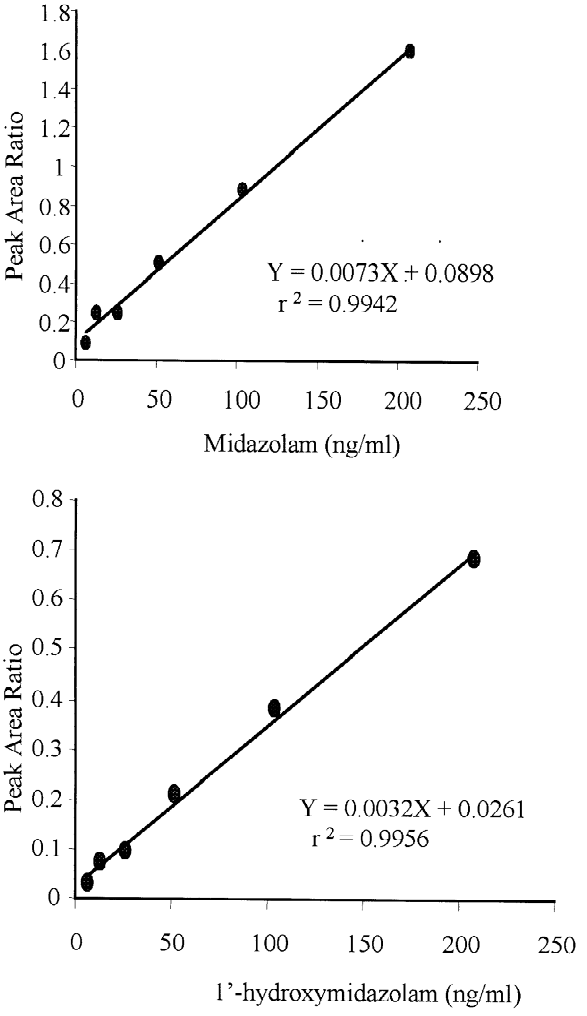

Fig. 3. Representative calibration curves for MDZ and 1′-OHMDZ.

Table 1
Within- and between-day precision, accuracy and linearity data for the LC–MS determination of midazolam and 1′-hydroxymidazolam in human plasma

Concentration (ng/ml)	Within-day precision		Between-day ($n=5$)			
	($n=5$) RSD (%)		Precision (RSD, %)		Accuracy (%)	
	MDZ	1′-OHMDZ	MDZ	1′-OHMDZ	MDZ	1′-OHMDZ
6.5 (MDZ) 6.8 (1′-OHMDZ)	7	8	20	22	124	109
208 (MDZ) 219 (1′-OHMDZ)	3	3	1.5	4	101	101
Mean correlation coefficient					$r^2 = 0.99$ $n = 5$	$r^{2=}0.99$ $n = 5$

Precision is expressed as relative standard deviation (RSD) and accuracy as the assayed concentration relative to the actual concentration (%).

3. Results

Mass spectra of midazolam, 1′-hydroxymidazolam and prazepam are shown in Fig. 1, and ions at m/z of 326.26, 342.27 and 325.34, respectively, were selected for subsequent quantitative analysis. Under the chromatographic conditions used, midazolam (retention time 3.20 min), 1′-hydroxymidazolam (2.27 min) and prazepam (8.80 min) gave rapidly eluting, fully resolved and essentially symmetrical peaks (Fig. 2).

Calibration curves for both drug and metabolite over the range 6.5–208.0 ng/ml were linear ($r^2 >$ 0.98) and passed through the origin (95% confidence interval for intercept −0.003–0.006, $P = 0.07$) (Fig. 3). Assay precision and accuracy data are shown in Table 1. The limit of determination, defined as the lowest concentration yielding a signal-to-noise ration higher than 3, was 0.65 and 0.68 ng/ml for midazolam and 1′-hydroxymidazolam, respectively.

4. Discussion and conclusions

We had previously used HPLC techniques to measure MDZ and 1′-OHMDZ in pediatric patients undergoing minor surgery [25]. However, when we attempted to use the same technique for measuring MDZ and 1′-OHMDZ in methadone maintenance patients, we failed to obtain chromatograms with adequate separation of MDZ or 1′-OHMDZ from other drugs taken by the patients. However, following the analysis by LC–MS of plasma samples taken before dosing with midazolam, from more than 50 opiate users, no significant peaks co-eluting with drug, metabolite or internal standard were found on the reconstructed chromatograms (Fig. 2B).

Previously published GC–MS and liquid chromatography/tandem mass spectrometry (LC–MS–MS) procedures yielded a lower limit of detection than the present technique (<0.3 ng/ml) [25–27]. However, our LC–MS method is considerably more sensitive than most of the HPLC assays, the latter having detection limits generally higher than 1 μg/l [17,20,28–31], It is also more sensitive than a recently published LC–MS technique, which had a lower limit of detection of 1–2 ng/ml [32]. Furthermore, the present technique is two times more rapid than the LC–MS–MS method published by Ayrton et al. [33] when the system used a 250×4.6 mm I.D. column packed with 5 μm Zobax (Hichrom, Reading, UK) RX-C_8 stationary phase [33]. Originally diazepam was chosen as the I.S. However, because many of the patients in our studies were taking the drug, it was replaced by prazepam which is not marketed in the UK.

In summary, we have developed a rapid, sensitive and selective LC–MS method for the analysis of midazolam and 1′-hydroxymidazolam in plasma. The method is particularly suitable for use in large clinical studies of patients who are also likely to be taking other drugs.

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