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A highly sensitive LC–MS–MS assay for analysis of midazolam and its major metabolite in human plasma: Applications to drug metabolism

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Abstract

The present report describes a rapid, selective and a highly sensitive assay for midazolam (MDZ) and its major metabolite 1-hydroxymidazolam (1-OH-MDZ) in human plasma employing liquid chromatography–tandem mass spectrometry (LC–MS–MS) detection. The method involves liquid–liquid extraction sample clean-up, separation on a Purospher RP 18-e column and detection with an electrospray interface in the positive ion mode. The overall recoveries were about 100% and 80% for midazolam and 1-hydroxymidazolam, respectively. Accuracy, precision and linearity were acceptable for biological samples with quantitation limits of 0.1–100 ng mL^{-1} plasma for both analytes. The validated method was successfully applied to quantify plasma concentration of midazolam and 1-hydroxymidazolam in authentic samples from a healthy volunteer following a single 15 mg oral dose of midazolam (apparent total clearance: 3.47 L h^{-1} kg^{-1} and $AUC^{0-\alpha}$IOH-MDZ/MDZ: 0.338).

Keywords: Midazolam; LC–MS–MS; Plasma; Pharmacokinetics; CYP3A4

1. Introduction

Midazolam (MDZ) is a widely used short-acting benzodiazepine with hypnotic, sedative, anticonvulsant, muscle-relaxant, amnesic and axiolytic properties. In clinical practice, it is administered intravenously and intramuscularly to treat generalized seizures, refractory status epilepticus and muscle spasms. Midazolan has also been used for conscious sedation and induction of general anaesthesia [1]. MDZ is hydroxylated by CYP3A4 to its primary and pharmacologically active metabolite, 1-hydroxymidazolam (Fig. 1), and minimally to the inactive metabolites 4-hydroxymidazolam and 1,4-dihydroxymidazolam. 1-Hydroxymidazolam (1-OH-MDZ) is produced at higher concentrations following oral administration as a result of extensive first-pass metabolism in the human liver and intestine. Both CYP3A4 and CYP3A5 are capable of catalysing MDZ hydroxylation, although the in vivo contribution of CYP3A5 to MDZ hydroxylation has been questioned [2].

Levels of CYP3A4 activity vary considerably amongst individuals and, in addition, the closely related gene CYP3A5 shows a polymorphism in its expression, with universal expression in intestinal and foetal liver but detectable expression in only 10%–20% of adult livers. CYP3A7 is universally expressed in foetal liver but is also expressed in some adult livers. A fourth CYP3A gene, CYP3A43, has been recently identified but at present there are no data on protein expression. Part of the variation observed in the metabolism of

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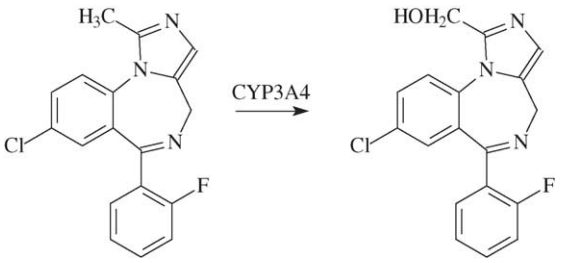

Fig. 1. Chemical structures of midazolam and its major metabolite, 1-hydroxymidazolam.

CYP3A4 substrates may be associated with the variable expression of CYP3A5 and CYP3A7 [3].

Several studies have evaluated the validity of midazolam clearance as a phenotyping measure. The clearance of MDZ administered intravenously correlated closely with hepatic CYP3A4 activity. Since CYP3A is abundant in the intestines, orally administered MDZ is subject to both intestinal and hepatic CYP3A metabolism. However, intravenous MDZ has been proposed for only the hepatic CYP3A phenotyping. This means that simultaneous oral and intravenous administration of MDZ could be used to examine the contribution of both intestinal and hepatic CYP3A [2,4,5].

Thummel et al. [6] reported that the clearance of MDZ after intravenous administration correlated strongly with the 30 min 1-OH-MDZ/MDZ plasma concentration ratio. The total MDZ clearance was highly correlated with the hepatic CYP3A content measured in vitro. In addition, a potential advantage of midazolam is that it is not a substrate for P-glycoprotein like erythromycin, another probe drug widely used as a marker of CYP3A activity. Experiments using a human intestinal cancer cell line (Caco-2) have shown that MDZ is a substrate for an efflux transporter other than P-glycoprotein although the contribution of this transporter to the in vivo disposition of MDZ is not known [7].

Drug metabolism studies require a sensitive, selective and rapid analytical method to determine the concentrations of MDZ and its major metabolite in plasma samples. A number of assays have been developed for this purpose employing chromatographic techniques with different detection systems [8–16]. Among them, HPLC coupled to mass spectrometry (LC–MS) [12–14] or tandem mass spectrometry (LC–MS–MS) [17–20] has almost completely replaced other detection systems in the bioanalytical field because of its high sensitivity and specificity, although GC–MS has been used in most of the studies assessing CYP3A activity. The GC–MS technique is highly sensitive and specific for the determination of MDZ and its metabolites, the only drawback being the necessity of including a derivatisation procedure before chromatographic analysis with a long total analytical time [8,9]. The latest LC–MS [14] method described for the determination of MDZ and 1-OH-MDZ in plasma samples is rapid, selective and sensitive, with detection limits of 0.65 and 0.68 ng mL^{-1}, respectively, although calibration curves for both drugs were constructed over the 6.5–208.0 ng mL^{-1} range.

This paper describes a highly sensitive LC–MS–MS assay for MDZ and 1-OH-MDZ in human plasma with LOQ of 100 pg mL^{-1} for both drugs. The validated method was applied to determine the plasma concentrations of MDZ and 1-OH-MDZ up to 6 h after a single 15 mg oral dose of MDZ although lower MDZ doses have been proposed for CYP3A phenotyping justifying the high sensitivity of this method.

2. Materials and methods

2.1. Reagents and standard solutions

Midazolam maleate (purity >99.5%) was kindly supplied by Hoffmann-La Roche (Rio de Janeiro, Brazil) and 1-hydroxymidazolam base (no information available on the purity) was kindly donated by Dr. J. Martens (Otto von Guericke Universität Magdeburg, Medizinische Fakultät, Institut für Klinische Pharmakologie, Magdeburg, Germany).

Acetonitrile and methanol, HPLC grade, were purchased from Merck (Darmstadt, Germany). The analytical grade reagents used were sodium hydroxide pellets (Mallinckrodt Baker, Inc., Xalostoc, Mexico) and ammonium acetate (J.T. Baker, Xalostoc, Mexico). All water was distilled and purified with a Milli-Q Plus System (Millipore, Bedford, MA, USA).

Stock standard solutions were prepared at 1 mg mL^{-1} in methanol and stored at −20 °C for a maximum of three months. Working solutions of MDZ and 1-OH-MDZ were prepared by appropriate dilution with methanol in the concentration range of 0.04–4.0 μg mL^{-1} and the internal standard (IS), clobazam (purity >98.5%), was prepared at a concentration of 0.1 μg mL^{-1}. Spiked plasma samples were obtained by the addition of known aliquots of these working standard solutions to drug-free plasma prior to extraction.

Drug-free pooled human plasma samples from blood donors were supplied by the Blood Center of the University Hospital, Faculty of Medicine of Ribeiráo Preto (University of São Paulo, Ribeiráo Preto, Brazil).

2.2. Instrumentation

The liquid chromatography system consisted of an LC10AD pump and a CTO-10AS column oven from Shimadzu (Kyoto, Japan). The separation of MDZ, 1-OH-MDZ and IS was performed on a reverse phase Purospher® RP 18-e column, 150 mm × 4.6 mm I.D., 5 μm particle size, with a LiChrospher® 100 RP 18-e precolumn, 4 mm × 4 mm I.D., 5 μm particle size, both from Merck. A 20 μL aliquot of each sample was injected and the column was kept at 27 ± 1 °C. The mobile phase consisting of a mixture of acetonitrile and 10 mmol L^{-1} ammonium acetate aqueous solution (50:50, v/v) was pumped at a flow-rate of 0.7 mL min^{-1}.

Detection was obtained with a Quattro Micro LC triple quadrupole mass spectrometer (Micromass, Manchester, UK) fitted with an electrospray interface (ESI) source and tandem mass operated in the positive ion mode. The LC flow was split so that approximately 150 μL min^{-1} entered the mass spectrometer. The desolvation temperature was maintained at 275 °C, the source at 120 °C and the capillary voltage was 3.0 kV. Nitrogen and argon were used as nebuliser/desolvation and collision gas, respectively. Cone voltage and collision energy were optimised for each analyte by performing full scan acquisitions.

MS conditions were optimised by direct infusion of standard solutions (10 μg mL^{-1}) prepared in the mobile phase and delivered by a syringe pump at a flow-rate of 10 μL min^{-1}.

For quantitation, the MS/MS was operated in the multiple reaction monitoring (MRM) mode to monitor IS, MDZ and 1-OH-MDZ, with the dwell time set at 0.1 s for each mass transition. Data acquisition and quantitative analysis were performed using a MassLynx (Micromass) data acquisition system, Version 3.5.

2.3. Sample preparation

Aliquots of 1.0 mL of human plasma were supplemented with 25 μL IS (0.1 μg mL^{-1} clobazam solution) and alkalinised with 100 μL 0.1 mol L^{-1} sodium hydroxyde solution. MDZ and 1-OH-MDZ were extracted from plasma samples with 4.0 mL toluene–isoamylic alcohol (100:1, v/v) by horizontal mechanical shaking for 30 min. After centrifugation at 2000 × *g* for 5 min, the organic phases were collected and evaporated to dryness in a centrifugal evaporator vacuum system (RCT90 and RC 10.22 model) from Jouan AS (St. Herblain, France). The residues were dissolved in 50 μL acetonitrile–10 mmol L^{-1} ammonium acetate (50:50, v/v) and vortexed for 20 s. A 20 μL aliquot from each sample was injected into the analytical column.

2.4. Method validation

The human drug-free plasma employed for the validation of the analytical method was initially used to determine the absence of interference peaks. The calibrations curves were constructed by analysing in duplicate 1.0 mL samples of drug-free plasma spiked with known amounts of MDZ and 1-OH-MDZ. The linear regression equations and the correlation coefficients were obtained from the areas of the peaks plotted against their respective concentrations (0.1–100 ng mL^{-1} plasma for MDZ and 1-OH-MDZ).

The efficiency of the extraction procedure was assessed by analysing in triplicate drug-free 1.0 mL plasma aliquots spiked with seven different concentrations of MDZ at 0.1, 0.2, 1.0, 4.0, 10, 20 and 50 ng mL^{-1} plasma, and of 1-OH-MDZ at 0.25, 0.5, 1.0, 5.0, 10, 25 and 50 ng mL^{-1} plasma. Recoveries were measured by comparing the peak areas of spiked plasma standards following the extraction procedure to those obtained from direct injection of MDZ and 1-OH-MDZ standard solutions.

Intra-day and inter-day accuracy and precision were evaluated at five concentrations by analysing plasma samples spiked at 0.1, 0.4, 4.0, 16 and 50 ng mL^{-1} for MDZ and at 0.1, 0.5, 4.0, 10.0 and 50 ng mL^{-1} for 1-OH-MDZ. Aliquots of these plasma samples were stored at −20 °C and analysed in replicate experiments ($n = 10$) using a single calibration curve for intra-assay evaluation, and in triplicate on five consecutive days for inter-assay evaluation.

The stabilities of MDZ and 1-OH-MDZ in plasma were evaluated (same concentrations as used for the accuracy and precision studies) by three freeze-thaw cycles at −20 °C for 24 h. The short-term stability of MDZ and 1-OH-MDZ in plasma was also assessed on the benchtop for 3 h at room temperature (25 °C). Aliquots of spiked samples were stored at −20 °C in glass tubes. One set of duplicate samples of each concentration was analysed immediately after spiking, which served as the reference concentration. The other samples were analysed after one, two and three freeze-thaw cycles, with thawing being performed at room temperature for approximately 1 h, followed by refreezing. The change in concentration during the freeze-thaw cycles was determined by comparing the concentrations after thawing with the reference concentrations and was expressed as percent deviation from the reference concentration.

The quantitation limit (LOQ) was obtained by quintuplicate analysis of plasma samples spiked with MDZ and 1-OH-MDZ at concentrations as low as 0.1 ng mL^{-1}. The LOQ was defined as the lowest plasma concentration of each analyte analysed with a relative error of 20% or lower.

2.5. Pharmacokinetic study

The study was approved by the local Ethics Committee of the University Hospital, Faculty of Medicine of Ribeirão Preto (University of São Paulo, Ribeirão Preto, Brazil).

The developed method was used to investigate plasma profiles of MDZ and its major metabolite, 1-OH-MDZ, after a single 15 mg oral dose of MDZ. Plasma samples were obtained from a healthy volunteer (a 24-year-old woman, self reported as white, 70 kg and 1.66 m tall), who was informed about the study, agreed to participate and signed a written consent form. After clinical examination and biochemical tests for the confirmation of normal hepatic, renal and cardiac functions, the volunteer received orally one tablet of 15 mg MDZ (Dormonid® 15 mg tablets, Roche, Rio de Janeiro, Brazil) after a 12 h fast. Blood samples were collected through an intravenous catheter at times zero, 15, 30, 45, 60, 90, 120, 150, 180, 210, 270 and 360 min after MDZ administration. The blood samples were transferred to heparinized tubes (Liquemine® 5000IU, Roche, Rio de Janeiro, Brazil) and centrifuged at 2000 × *g* for 10 min. The plasma samples were separated and stored at −70 °C until chromatographic analysis. The kinetic disposition of MDZ and 1-OH-MDZ was analysed by the WinNonlin

Professional software Version 4.0 using a one-compartment model. The area under the plasma concentration time curve ($AUC^{0-\alpha}$) was calculated by the trapezoid method with infinite extrapolation. The plasma $AUC^{0-\alpha}$ 1-OH-MDZ/$AUC^{0-\alpha}$ MDZ ratio was used to investigate in vivo CYP3A activity. The apparent total body clearance (CL/*f*) was calculated from the $CL/f = Dose/AUC^{0-\alpha}$ equation, where *f* is the fraction of the drug dose reaching the systemic circulation.

3. Results and discussion

3.1. Method optimisation and validation

The resolution of MDZ, 1-OH-MDZ and IS was obtained by reverse-phase LC using a Purospher® RP 18-e column (150 mm × 4.6 mm I.D., 5 μm particle size). The final LC conditions were optimised to ACN–10 mmol L^{-1} ammonium acetate (50:50, v/v) at a flow-rate of 0.7 mL min^{-1}. The optimised LC conditions described in Section 2.2 permitted a typical analysis time of less than 6 min, in which the major metabolite, 1-OH-MDZ, IS and MDZ were separated as symmetric peaks eluting from the LC column in this order (Fig. 2). This run time is 50% lower than the previously described methods [11,12].

The optimisation of MS detection was carried out in the electrospray (ES) positive ion mode due to the tertiary amines in the MDZ and 1-OH-MDZ structures. The mobile phase consisting of a mixture of ACN–10 mmol L^{-1} ammonium acetate (50:50, v/v) was found to be suitable to obtain a simultaneous electrospray response for clobazam, used as IS, and for both MDZ and 1-OH-MDZ. The cone voltage optimisation yielded best results at 38 V. The product ions were generated through fragmentation of the molecular ions by collision-activated dissociation using argon as the collision gas. Varying the collision energy, which was 30 eV for all analytes, optimised the intensity of a selected product ion peak *m/z* in the MS–MS spectrum of each compound. The protonated species $[M + H]^+$ and their respective product ions were monitored at 342 > 203 (0.0–2.5 min) for 1-OH-MDZ, at 301 > 259 (2.5–3.5 min) for the IS and at 326 > 291 (3.5–5.0 min) for MDZ.

The liquid–liquid extraction procedure that gave the best results involved a fast alkalinisation step followed by extraction with a mixture of toluene–isoamylic alcohol (100:1, v/v). This procedure completely and simultaneously extracted MDZ and its major metabolite from human plasma. The validity of the sample preparation procedure with an extraction efficiency of about 104 ± 14.373 and 82 ± 6.072% was obtained for MDZ and 1-OH-MDZ, respectively, in the concentration range of 0.1–50 ng mL^{-1} (regarding accuracy, the relative error was less than 10%).

Typical chromatograms of a spiked plasma sample are shown in Fig. 2. During the recovery studies, six spiked plasma samples from different subjects were used in order to verify the ion suppression effect. It should be mentioned that no ion suppression was noted because the peak areas

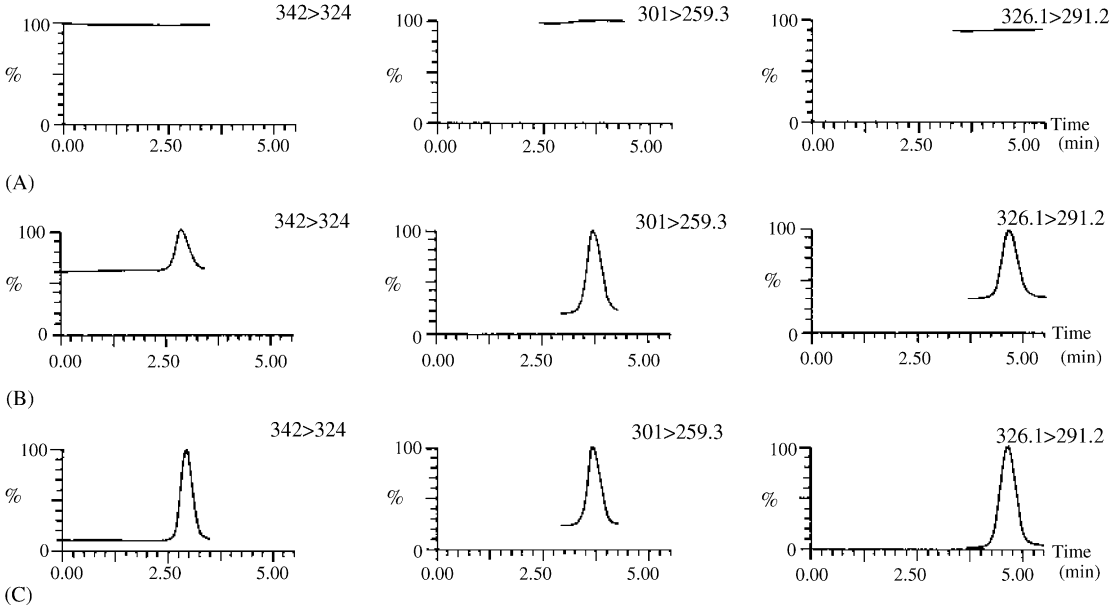

Fig. 2. Mass chromatograms of midazolam, its major metabolite 1-hydroxymidazolam, and the internal standard clobazam, from (A) a drug-free plasma sample, (B) a spiked plasma sample of 0.5 ng mL^{-1} of midazolam and 1-hydroxymidazolam and (C) a plasma sample from a healthy volunteer 3 h after drug administration. Chromatographic conditions: Purospher® column, 150 mm × 4.6 mm I.D., 5 μm particle size, with the mobile phase consisting of ACN–10 mmol L^{-1} ammonium acetate solution (50:50, v/v) at a flow-rate of 0.7 mL min^{-1}. Mass detection conditions: source temperature: 120 °C, desolvation temperature: 275 °C, desolvation gas flow: 350 L h^{-1}, cone voltage: 38 V, capillary voltage: 3.0 kV, collision energy: 30 eV.

Table 1
Precision and accuracy of the LC–MS–MS method for midazolam and 1-hydroxymidazolam analysis in human plasma

	Nominal concentration (ng mL^{-1})	Found concentration (ng mL^{-1})	CV (%)	RE (%)
Midazolam				
Intra-day (n = 10)	0.1	0.09	13.4	−10.0
	0.4	0.45	9.1	12.5
	4	3.79	4.1	−5.2
	16	13.75	2.3	−14.1
	50	46.66	2.7	−6.7
Inter-day (n = 5)	0.1	0.11	14.1	10.0
	0.4	0.43	8.4	7.5
	4	3.96	8.2	−1.0
	16	15.30	6.5	−4.4
	50	46.46	6.1	−7.1
1-Hydroxymidazolam				
Intra-day (n = 10)	0.1	0.11	12.0	10.0
	0.5	0.49	8.3	−2.0
	4	4.26	4.6	6.5
	10	9.66	7.7	−3.4
	50	46.81	3.7	−6.4
Inter-day (n = 5)	0.1	0.11	13.8	10.0
	0.5	0.47	8.9	−6.0
	4	4.15	8.6	3.7
	10	9.35	9.2	−6.5
	50	45.6	5.4	−8.8

CV: coefficient of variation, RE: relative error (%) = ((found concentration − nominal concentration)/nominal concentration) × 100.

of both drugs did not change significantly after the extraction plasma procedure. Regarding costs, this liquid–liquid extraction is less expensive than the solid-phase extraction involved in human plasma sample preparation reported for other LC–MS–MS systems [18,19], and also shows good reproducibility.

The peak area ratios of MDZ and 1-OH-MDZ to IS in human plasma samples varied linearly with the concentration in the range of 0.1–100 ng mL^{-1} for both analytes using linear least-square regression. The detection and quantitation limits of 0.04 and 0.1 ng, respectively, for the two analytes were established using a 1.0 mL plasma sample. The present method was found to be more sensitive than recently described LC–MS methods [15,16] for human plasma samples. The high sensitivity of the method allows its additional application for CYP3A phenotyping in humans following the administration of low oral dose of MDZ in order to prevent its side-effects.

The coefficient of variation and relative error values at the five concentrations obtained in the intra- and inter-day assays are presented in Table 1. The results show that the analytical method is accurate (±20% different from the nominal concentration at the quantitation limit) and the precision values expressed as coefficient of variation are within the accepted limits of 20% or less for the quantitation limit and of 15% or less for other concentration levels studied. Three freeze-thaw cycles did not affect the stability of MDZ or 1-OH-MDZ in plasma, as seen by the deviation from nominal concentrations, which were within the acceptable limits of ±15% at all concentration levels. The samples were found to be stable for 4 h at room temperature prior to extraction.

3.2. Method application

Fig. 3 shows the plasma concentration-time profiles for MDZ and 1-OH-MDZ in a healthy volunteer following a single 15 mg oral dose of MDZ. The resulting pharmacokinetic parameters are shown in Table 2. These parameters were compatible with those obtained by Tateishi et al. [21] using a clinical protocol performed after oral MDZ administration to European-American men.

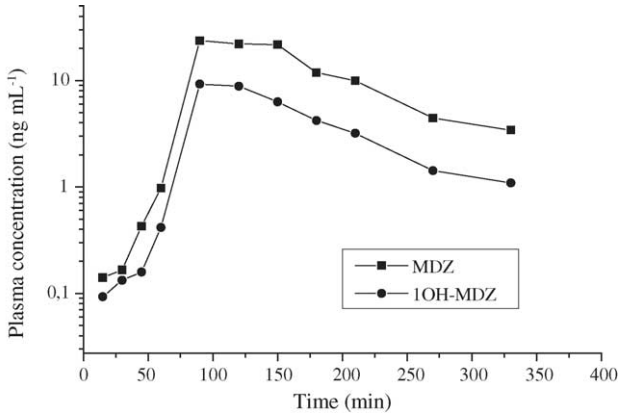

Fig. 3. Plasma concentration-time curves for MDZ and 1-OH-MDZ following the administration of a single 15 mg oral dose of MDZ to a healthy volunteer.

Table 2
Kinetic disposition of MDZ and 1-OH-MDZ following the administration of a single 15 mg oral dose MDZ to a healthy volunteer

	Midazolam	1-Hydroxymidazolam
C_{max} (ng mL^{-1})	23.73	9.28
t_{max} (h)	1.5	1.5
$t_{1/2}\beta$ (h)	1.6	1.1
β (h^{-1})	0.43	0.56
AUC$^{0-\infty}$ (μg h^{-1} L^{-1})	61.74	20.87
Vd/f (L kg^{-1})	8.02	–
CL/f (L h^{-1} kg^{-1})	3.47	–
AUC$^{0-\infty}_{1\text{-OH-MDZ}}$/AUC$^{0-\infty}_{MDZ}$		0.338

4. Conclusion

The present study reports the quantitation of MDZ and its major hydroxylated metabolite, 1-OH-MDZ, in human plasma employing LC–MS–MS. The sample clean-up involved a simple solvent extraction step and showed excellent reproducibility. The advantage of this method is demonstrated by its shorter run time (6 min) without mobile phase gradient elution and by its higher sensitivity.

The validated method, which shows good accuracy and reproducibility, is being used in our laboratory to quantify MDZ concentrations as low as 0.1 ng mL^{-1} and the concentrations of its major metabolite in human plasma samples from patients treated with a single oral dose of MDZ. The method has proved to be adequate and reliable for routine application to drug metabolism studies.

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