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Short communication

Development and validation of a rapid and sensitive assay for simultaneous quantification of midazolam, 1′-hydroxymidazolam, and 4-hydroxymidazolam by liquid chromatography coupled to tandem mass-spectrometry

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ABSTRACT

Midazolam is an ultra short acting benzodiazepine derivative and a specific probe for phenotyping cytochrome P450 (P450) 3A4/5 activity. A rapid, sensitive, and selective LC–MS/MS method was developed for simultaneous quantitation of midazolam and its metabolites (1′-hydroxymidazolam and 4-hydroxymidazolam). Deuterated (D_5) analog of midazolam was utilized as an internal standard. Sample preparation either from human plasma (100 μL) or liver microsomal incubations involved a simple protein precipitation using acetonitrile (900 μL) with an average recovery of >90% for all compounds. The chromatographic separation was achieved using Zorbax-SB Phenyl, Rapid Resolution HT (2.1 mm × 100 mm, 3.5 μm) and a gradient elution with 10 mM ammonium acetate in 10% methanol (A) and acetonitrile (B). The flow rate was 0.25 mL/min and total run time was 5.5 min. Calibration curves were linear over the concentration range of 0.100–250 ng/mL. The lower limit of quantitation (LLOQ) was 0.1 ng/mL for all three analytes. The accuracy and precision, estimated at LLOQ and three concentration levels of quality control samples in six replicates, were within 85–115%. In conclusion, a robust, simple and highly sensitive analytical method was developed and validated for the analysis of midazolam and its metabolites. This method is suitable for characterizing the P450 3A4/5 activity *in vitro* or in human pharmacokinetic studies allowing administration of smaller doses of midazolam.

1. Introduction

Cytochrome P450 (P450) is a generic term for a superfamily of heme-containing monooxygenases responsible for the biotransformation of both endogenous and exogenous compounds [1]. Various factors including age, gender, disease state or genetic polymorphism of P450 can potentially influence the inter-individual variability in pharmacokinetics or drug response. Therefore, it is important to identify the P450s responsible for the biotransformation reactions and to characterize their relative contribution to the metabolic pathway involved in the overall elimination processes. Phenotyping of drug metabolizing enzymes in animal or human has been proven to successfully predict the potential of drug interactions and other factors governing drug disposition.

The human P450 3A subfamily represents about 30% of the total hepatic P450 content and is believed to be responsible for the biotransformation of almost 55% of all marketed medications [1]. Currently, several probe compounds are used for P450 3A phenotyping; however, the most frequently used *in vivo* test probes include midazolam, erythromycin, and nifedipine [1,2]. Midazolam is available as both oral and intravenous dosage forms so it can be used for phenotyping P450 3A activity of hepatic versus hepatic + gastrointestinal routes. It is therefore one of the most frequently used probes for P450 3A characterization [3]. To date, several chromatographic methods for quantitation of midazolam and its principal metabolite(s) have been published including (*i*) HPLC-UV methods [4–15], (*ii*) GC methods with electron capture detection [16–20], GC/MS methods [21,22] and (*iii*) LC–MS/MS methods [23–27].

The objective of this study was to develop and validate a rapid, simple, selective and sensitive LC–MS/MS method that can be easily implemented routinely for simultaneous analysis of midazolam and its major metabolites, 1′-hydroxymidazolam and

Abbreviations: P450, cytochrome P450 (also termed heme-thiolate P450); HLM, human liver microsomes; HPLC, high performance liquid chromatography; GC, gas chromatography; GC/MS, gas chromatography–mass spectrometry; LC–MS/MS, liquid chromatography–tandem mass-spectrometry.

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4-hydroxymidazolam in clinical pharmacokinetic studies or for *in vitro* characterization of P450 3A activity.

2. Experimental

2.1. Reagents and chemicals

Midazolam, 1′-hydroxymidazolam, 4-hydroxymidazolam, and D_5-midazolam, all with >99.5% purity, were purchased from TRC (Toronto, Ontario, Canada). HPLC grade acetonitrile, methanol and analytical grade ammonium acetate were obtained from Pharmco Products Inc. (Brookefield, CT, USA) and Acros Organics (Fair Lawn, NY, USA), respectively. Drug free heparin human plasma from healthy donors was obtained from Bioreclamation (Westbury, NY, USA). Individual liver samples from human donors were purchased from XenoTech LLC (Lenexa, KS, USA).

2.2. In vitro study

Microsomes were prepared from human individual liver samples as described previously [28] and stored at −80 °C. Protein concentrations were estimated using a bicinchoninic acid method (Pierce-Fisher, Rockford, IL) with bovine serum albumin as a standard. Concentrations of total P450 were determined as described by Omura and Sato [29]. Incubation mixtures contained 50 μg/mL microsomal proteins and various concentrations of midazolam (0.001–250 μM). The reactions were carried out at 37 °C for 30 min and initiated by the addition of an NADPH-regenerating system [28]. The reactions were terminated by the addition of acetonitrile and followed by extraction (centrifugation at 2000 × *g* for 10 min at 4 °C); the supernatant was evaporated to dryness and was analyzed after reconstitution. D_5-midazolam in acetonitrile (10 ng/mL) was added as an internal standard.

2.3. Chromatographic and mass spectrometric (LC–MS/MS) conditions

The instrumentation consisted of a Shimadzu Prominence HPLC system (SIL-10AD*VP* auto-sampler and LC 20-AB pump, Shimadzu, Kyoto, Japan) coupled to an API 3200 tandem mass spectrometer (AB SCIEX, Toronto, ON, Canada) equipped with electro-spray ionization (ESI) probe. The ionization was set at positive ion mode and at a temperature of 500 °C utilizing nitrogen for the drying and collision gas (curtain gas, 20 psi; collision gas, 3 psi; ionspray voltage, 4500 V; ion source gas 1, 60 psi; ion source gas 2, 70 psi).

Chromatographic separation was achieved using Zorbax SB-Phenyl Rapid Resolution (2.1 mm × 100 mm, 3.5 μm) analytical column (Agilent Technologies, Foster City, CA, USA), preceded by a pre-column filter (0.5 μm) (Supelco, Bellefonte, PA, USA). A gradient elution mode was utilized for optimized separation and sensitivity of analytes from extracted matrix using mobile phase composed of 10 mM ammonium acetate and 10% methanol (A) and HPLC grade acetonitrile (B) at the flow rate of 0.25 mL/min. The following gradient was used: A:B 60:40 (v/v) for 0.01–0.5 min; A:B 40:60 (v/v) for 0.5–1.5 min; A:B 10:90 (v/v) for 1.5–4.3 min; A:B 60:40 (v/v) for 4.4–5.5 min.

2.4. Preparation of calibrators and quality control samples

Separate stock solutions of midazolam, 1′-hydroxymidazolam, 4-hydroxymidazolam, and stable-isotope labeled internal standard (IS) (D_5-midazolam) each containing 1 mg/mL were prepared in methanol. Working standard solutions (10.0, 100 and 1000 ng/mL) of combined analytes were prepared by serial dilution in methanol. Aliquots of the working standard solutions were diluted with human drug free (blank) plasma or human liver microsomal fraction to prepare eight calibration standards ranging from 0.100 to 250 ng/mL (0.100, 0.200, 1.00, 5.00, 25.0, 100, 185 and 250 ng/mL). Quality control standards at four levels representing the Lower Limit of Quantification (LLOQ, 0.100 ng/mL), low (0.250 ng/mL), medium (8.00 ng/mL) and high (160 ng/mL) concentrations were prepared in blank plasma and were stored at −80 °C until analysis.

2.5. Sample extraction

Plasma (calibrators, QCs, double blank, control blank) or microsomal fraction samples were thawed at room temperature and were vortex-mixed for 20 s. Acetonitrile (900 μL) containing IS (2.5 ng/mL) was added to 100 μL of each plasma sample except double blank to which only acetonitrile was added. The samples were vortex-mixed for 30 s and centrifuged at 2000 × *g* for 10 min. The supernatant was transferred to clean glass tubes and evaporated to dryness at 65 °C using SPD 1010 Speedvac system (Thermosavant, Holbrook, NY, USA) followed by reconstitution with 100 μL of water:methanol (80:20, v/v). The tubes were again vortex-mixed, centrifuged and 20 μL of clear supernatant was injected onto the analytical column. The column was maintained at 55 °C using Flatron Systems TC-50 temperature controller and CH-30 column heater (ASTEC, Whippany, NJ, USA).

2.6. Method validation

Method validation with respect to accuracy and precision was performed according the procedure described in detail elsewhere (www.fda.gov/cder/guidance/4252fnl.htm).

2.6.1. Selectivity and specificity

LC–MS/MS methods generally have high selectivity since only certain ions produced from selected precursor ions are monitored. The ion transitions were midazolam (326 → 291), 1′-hydroxymidazolam (342 → 203), 4-hydroxymidazolam (342 → 325), and D_5-midazolam (331 → 296) with retention times 4.0, 3.1, 2.6, and 4.0, respectively.

2.6.2. Linearity, accuracy and precision

Inter-day and intra-day precision and accuracy were assessed by analyzing three individual batches consisting of calibration standards, six replicates of quality control standards at four concentration levels, double blank (without IS) and control blank (with IS) in three different runs. The eight-point calibration curve was constructed using peak area ratios of analyte over IS using weighted ($1/x^2$) quadratic regression.

2.6.3. Stability study

Three cycles of freeze and thaw, bench top (room temperature for 6 h), and auto-sampler (room temperature, 24 h) stability for all the three analytes was carried out using low and high QCs in triplicate.

2.6.4. Matrix effect

The possibility of ion suppression or enhancement by extracted matrices was investigated by infusing neat solutions of midazolam and its metabolites into the MS, post column, using a syringe pump at 20 μL/min via a mixing tee. After a constant response was established, blank plasma or microsomal fraction samples that had been extracted and reconstituted, as described in 2.5 were injected. The values of absolute matrix effect [%] was calculated by subtracting average count per second values (CPS) of the baseline before and after the injection of the matrix divided by average CPS before matrix injection.

Table 1
Optimized compound parameters used for estimation of midazolam and its metabolites in human plasma using LC–MS/MS technique and electrospray ionization at positive mode.

Analyte	Molecular mass	Precursor (*m*/*z*)	Product (*m*/*z*)	DP (V)	EP (V)	CEP (V)	CE (V)	CXP (V)
Midazolam	325	326	291	59	5	35	36	5
D_5-midazolam	330	331	296	63	5	38	47	5
1′-hydroxy midazolam	341	342	203	54	6	17	39	7
4-hydroxy midazolam	341	342	325	56	4	10	33	5

CXP, Collision cell exit potential; CE, Collision energy; CEP, Collision entrance potential; DP, Declustering potential; EP, Entrance potential.

Table 2
Summary of standards and calibration curve parameters from three individual runs.

Analyte	Matrix	STD [ng/mL]	STD1 0.100	STD2 0.200	STD3 1.00	STD4 5.00	STD5 25.0	STD6 100	STD7 185	STD8 250	R^2
Midazolam[a]	Plasma	% CV	8.8	6.6	4.4	5.9	2.4	5.1	2.1	3.5	0.986
1′-hydroxy midazolam[a]	Plasma	% CV	8.6	9.7	5.5	6.8	8.1	2.9	0.7	3.7	0.999
4-hydroxy midazolam[a]	Plasma	% CV	7.9	9.7	6.5	7.6	6.0	3.3	3.2	5.0	0.993
Midazolam[b]	HLMs	% CV	5.3	7.6	2.0	3.0	1.1	9.0	2.3	1.0	0.998
1′-hydroxy midazolam[b]	HLMs	% CV	3.5	7.0	0.0	2.0	1.0	7.2	4.1	1.1	0.998
4-hydroxy midazolam[b]	HLMs	% CV	3.5	9.1	7.3	2.1	3.4	4.1	3.8	2.1	0.991

[a] $n = 3$ (1 replicate for each of the three validation runs).
[b] $n = 2$ (1 replicate for each of the two validation runs).
%CV calculated as 100 × Std Dev/Mean.
HLMs: human liver microsomes.

3. Results

3.1. Specificity

Fig. 1 displays an extract of a plasma spiked with midazolam (Fig. 1A), 1′-hydroxymidazolam (Fig. 1B), and 4-hydroxymidazolam (Fig. 1C) at a concentration of 0.100 ng/mL (LLOQ). A typical chromatogram of midazolam and its hydroxy metabolites and D_5-midazolam extracted from a human plasma sample is shown in Fig. 1D. The peak shapes were symmetrical and the peaks were baseline resolved. Comparison of the chromatograms of the control blank and the spiked human plasma from six different individuals and microsomal fraction from 24 liver samples indicated no significant interference at the expected retention time of analytes and IS from endogenous substrates in plasma and microsomal fraction. The optimized compound parameters used for estimation of midazolam and its metabolites in human plasma are given in Table 1.

3.2. Validation characteristics

The measurement variance over the range of 0.100–250 ng/mL increased proportionally with the midazolam concentration. Therefore, a weighting factor was applied inversely proportional to the variance at the given concentration. The correlation coefficients for midazolam and its metabolites are summarized in Table 2.

Table 4
Results of matrix effect for plasma and human liver microsomes (HLMs). Means of all samples (3 replicates) were within 15% of the target values.

Analyte	Matrix	Absolute matrix effect [%]		
		Low QC [%]	Medium QC [%]	High QC [%]
Midazolam	Plasma	2.5	6.4	4.8
1′-hydroxy midazolam	Plasma	3.1	4.5	3.2
4-hydroxy midazolam	Plasma	3.2	5.9	4.7
Midazolam	HLMs	4.7	6.3	1.5
1′-hydroxy midazolam	HLMs	5.2	−2.0	4.1
4-hydroxy midazolam	HLMs	5.8	2.7	4.3

The intra and inter-day accuracy for all three analytes at four concentration levels of QCs were >85%. The result of intra-day accuracy is presented in Table 3. The recovery of all analytes and IS were found to be >90%. Stability assessment indicated that all the three analytes, as well as the IS, were stable in matrix at the end of three consecutive freeze–thaw cycles and on the bench at room temperature, and the extracted samples were stable during resident time in the auto-sampler.

Furthermore, a slight matrix effect was noted 2 minutes after the injection of the extracted matrices. The mean values of absolute matrix effect ranged from −3.2 to 6.4%, suggesting a minimal matrix effect on the ionization of compounds of interest under the experimental conditions (Table 4).

Table 3
Summary of quality control samples from three individual runs.

Analyte		QCs in heparin human plasma			
Midazolam	QCs [ng/mL]	0.100	0.250	8.00	160
	% accuracy	111	105	96.1	102
	%CV	6.5	4.8	9.1	5.0
1′-hydroxy midazolam	QCs [ng/mL]	0.100	0.250	8.00	160
	% accuracy	97.5	106	95.3	100
	%CV	4.2	8.1	9.6	7.3
4-hydroxy midazolam	QCs [ng/mL]	0.100	0.250	8.00	160
	% accuracy	94.0	105	90.9	104
	%CV	2.7	11.3	9.8	10.8

$n = 18$ (6 replicates for each validation run for three separated runs).
%CV calculated as 100 × Std Dev/Mean.

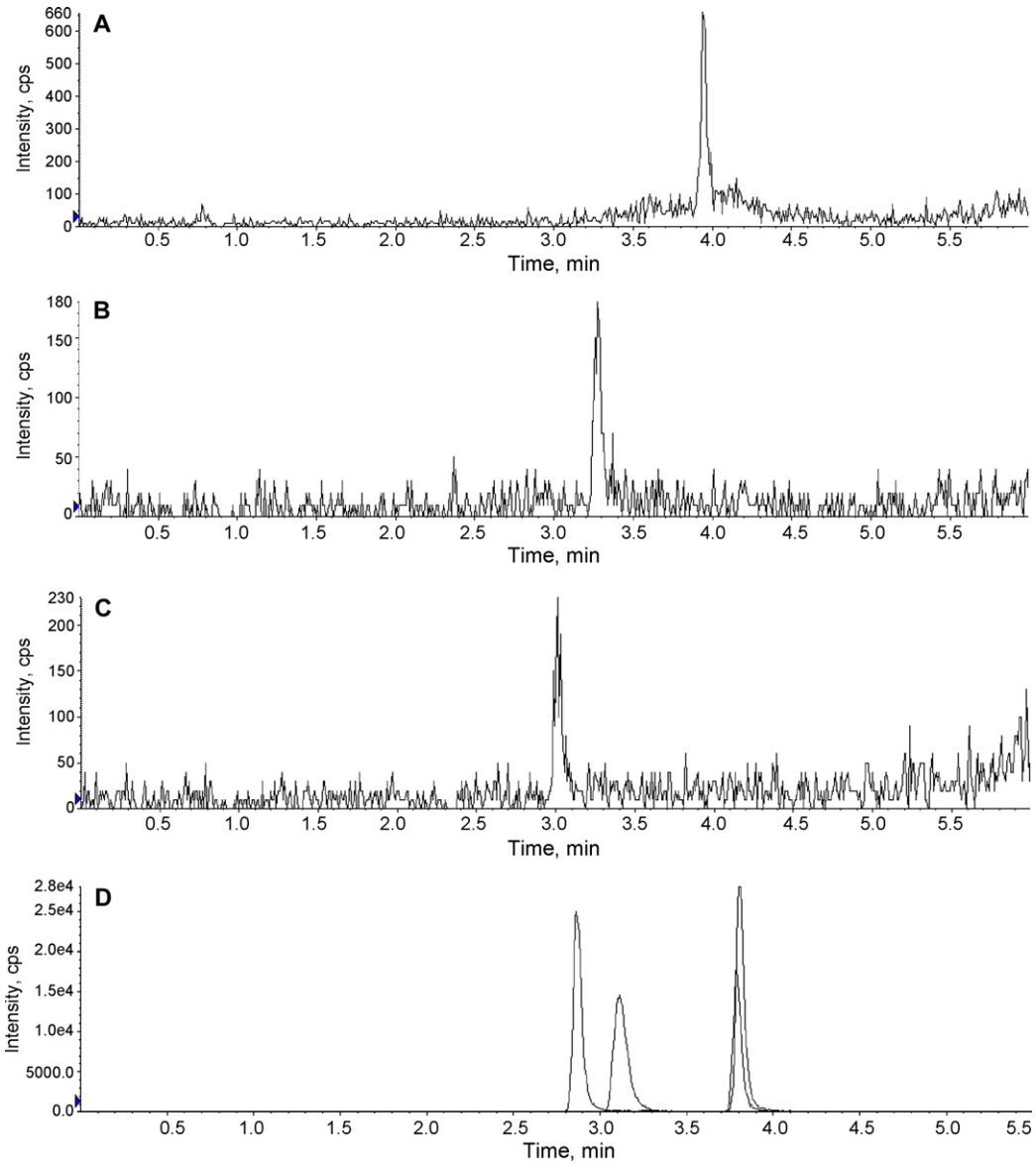

Fig. 1. MRM tracing of 100 μL plasma spiked with midazolam (A), 1′-hydroxymidazolam (B), and 4-hydroxymidazolam (C) at a concentration of 0.1 ng/mL. A typical chromatogram of midazolam, internal standard (D_5-midazolam) and hydroxy metabolites of midazolam at 0.25 ng/mL (D).

4. Discussion

The development and validation of an LC–MS/MS assay for quantification of midazolam and two of its metabolites is described. The novelty of the assay is in the fact that 0.1 ng/mL LLOQ was obtained on an AB SCIEX 3200 mass spect without the need for a more sensitive UPLC–MS/MS instrumentation. This level of sensitivity was achieved because of the use of a newer column chemistry resulting in very sharp peaks. Only 100 μL of plasma is needed for this assay which makes the assay suitable for human pharmacokinetic or drug–drug interaction studies where several compounds must be measured serially on limited amount of blood sample. Furthermore, we have validated this assay for quantification of midazolam and two of its metabolites both in human plasma and *in vitro* metabolism studies in human liver microsomes. Most comparable assays are only validated for the quantification of midazolam and 1′-hydroxymidazolam in human plasma.

This method is significantly more sensitive than all of the HPLC based assays published previously, with detection ranges between 2 and 100 ng/mL [4,5,7,9,10,12,14,15,30–33]. GC and GC/MS methods involve time consuming extraction and derivatization steps before chromatographic analysis with the LLOQ greater than 2 ng/mL [16–20]. In addition, the current method is more sensitive than a recently published LC–MS/MS method, which had a lower limit of quantitation of 1–2 ng/mL, comparable to those of HPLC-UV or GC–MS/MS techniques [34] with LLOQ of 1.5 ng/mL [24] and 6.5 ng/mL [25]. Marquet et al. [35] reported a LLOQ of 0.5 ng/mL; however, this method requires 2 mL plasma, which can be a limiting factor. Moreover, the microbore system used in this method requires specialized equipment making the instrumentation expensive. Lepper et al. [10] reported LLOQ of 1.00 ng/mL, using 0.6 mL of human plasma sample and additionally, the run time of this method is significantly longer (15 min) compared to the method described in this article. The LLOQ reported in this article is comparable to a method reported by Li et al. [36]; however, this method has a somewhat more complex extraction process and is not validated for determination of 4-hydroxymidazolam.

Petsalo et al. [37] reported the most rapid and sensitive midazolam assay till date utilizing ultra performance liquid chromatography coupled to tandem mass-spectrometry

(UPLC–MS/MS). The current assay provides an alternative to some of these methods with higher degree of sensitivity using only 100 μL sample aliquots.

The present technique is more rapid than most of the previously published methods that require long run time for analysis of a single sample. Moreover, the sample preparation procedure is based on a simple protein precipitation step thereby eliminating the need of performing multiple steps involved in solid phase extraction and liquid–liquid extraction procedures as published previously [10,14,24,34,38,39].

In conclusion, a validated LC–MS/MS method was developed for quantitation of midazolam and P450 3A specific metabolites in human plasma. Because this method is highly sensitive, it can be implemented for P450 3A phenotyping when lower dose of midazolam is administered to reduce drowsiness and other side effects. Moreover, since complete chromatographic separation of midazolam and its two metabolites is achieved, this method can potentially be adapted for HPLC-UV quantitation of three compounds, albeit with higher LLOQ, when mass spectrometer based detection is not available. This method is an economical tool for quantitative analysis of midazolam and its metabolites because it utilizes low flow rate, requires no solid phase cartridges and its lower limit of quantitation is comparable to UPLC–MS/MS [37]. In the future, this method can be implemented in drug metabolism experiments *in vitro* and in clinical pharmacokinetic studies involving midazolam as a P450 3A probe.

Conflict of interest

The authors indicate that no potential conflict of interest exists with any commercial entity whose products are described in the manuscript.

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