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Analysis of omeprazole, midazolam and hydroxy-metabolites in plasma using liquid chromatography coupled to tandem mass spectrometry

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Abstract

A method has been developed and validated for the quantitation of midazolam, alphahydroxy-midazolam, omeprazole, and hydroxyomeprazole from one 250 μL sample of human plasma using high performance liquid chromatography coupled to tandem mass spectrometry. The method was validated for a daily working range of 0.400–100 ng/mL, with limits of detection between 2 and 15 pg/mL. The inter-assay variation was less than 15% for all analytes at four control concentrations and the samples were stable for three freeze–thaw cycles under the analysis conditions and 24 h in the post-preparative analysis matrix. This method was used to analyze samples in support of clinical studies probing the activity of the cytochrome P-450 enzyme system.

Keywords: Omeprazole; Midazolam; Analysis; LCMS; CYP2C19

1. Introduction

The use of probe substrates for evaluating the effects of various factors such as genetics, environment, gender, and xenobiotics on in vivo Cytochrome P-450 (CYP) enzyme activity is an increasingly common practice [1–4], and is an accepted indirect method for evaluating the metabolism and drug interaction potential of new compounds during drug development [4,5]. An ideal probe substrate should be selective for the enzyme being studied, be sensitive to changes in enzyme content or activity, require minimally invasive sampling, be nontoxic, and not directly affect the activity of the enzyme.

The most commonly recommended biomarker for indirectly measuring hepatic CYP3A4 activity is the total body clearance of intravenous midazolam (MDZ) [1,3,4,6]. Following intravenous administration, MDZ is selectively metabolized by the CYP3A subfamily, with CYP3A4 being the predominant catalyst. Thummel et al. [7] demonstrated an excellent correlation ($r = 0.93$, $p < 0.001$) between in vivo total midazolam clearance and hepatic CYP3A content measured ex vivo in liver transplant patients. Midazolam has met most of the putative criteria as a selective and sensitive probe for CYP3A activity [1,6–8], has demonstrated a small degree of intraindividual variability over 3 months, and is not affected by gender or menstrual cycle phase [9]. Midazolam has the advantages of intravenous administration to avoid pre-systemic metabolism, and low cost. In addition, changes in midazolam clearance may be clinically relevant since the drug is a commonly used sedative. The major disadvantages of MDZ as an in vivo CYP3A probe are the sedative effects, and the need to obtain multiple blood samples over 6–8 h. In addition, MDZ may not accurately reflect CYP3A4 activity in patients with a high hepatic extraction ratio, or altered protein binding of the drug [10].

Omeprazole (OPZ) has emerged as the preferred in vivo probe for determining CYP2C19 phenotype [1,4]. Oral

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mephenytoin, the preferred probe substrate in the past, is no longer available and has a risk of sedative adverse effects, especially in poor metabolizers [11]. The ratio of serum OPZ to 5′-hydroxyomeprazole (OH-OPZ) concentrations, also called the hydroxylation index (HI) has excellent concordance with CYP2C19 genotype [11–13], and is not influenced by gender or menstrual cycle phase [14]. In addition, the omeprazole HI is normally distributed among extensive metabolizers, potentially allowing more detailed studies within this group. The OPZ HI has been calculated using area under the curve (AUC) ratios for the parent and metabolite [11], or a single-point measurement at 2–3 h after the dose [11–13]. Omeprazole has the advantages of easy administration, the potential for single point measurement of the HI, and an excellent safety profile. Disadvantages of OPZ for CYP2C19 phenotyping include a high degree of intra-subject variability reported with the 2 h index [14], and a portion of subjects having undetectable parent or metabolite serum concentrations at the single point sampling time [3,14]. Use of a larger oral dose or use of AUC ratios may overcome the limitation of undetectable serum concentrations with single point methods, as well as better analytical methods.

The administration of multiple probe "cocktails" to determine the phenotype for several CYP enzymes in a single study visit is a common practice [2–4]. The development of assay methods for the simultaneous quantification of probe drugs and their major metabolites in plasma has the potential to simplify the performance of multiprobe studies by reducing sample volume requirements, assay time and costs. In this report we describe the development and validation of a sensitive and specific chromatographic method for the simultaneous quantification of MDZ, OPZ and their hydroxyl-metabolites. Structures of the analytes are shown in Fig. 1 [15].

The use of liquid chromatography coupled to tandem mass spectrometry (LC/MS/MS) has emerged as the developmental method of choice in supporting clinical and pre-clinical pharmacokinetic studies [16]. This is based on the ability of this technique to provide superior specificity, speed and detectability in complex matrices, as compared to commonly used high pressure liquid chromatography with ultra-violet absorbance detection (HPLC-UV) methods [16]. When comparing these two techniques, LC/MS/MS is able to analyze more compounds in less time, with a lower limit of quantitation.

However, with electrospray ionization (ESI) coupled to LC/MS/MS, the issue of unstable instrument response due to ion-suppression must be considered. For reliable quantitation, the belief that very little, if any, sample preparation is needed is typically untrue [17]. Therefore, it is critical that any method developed by LC/MS/MS for quantitation of clinical samples be thoroughly characterized, especially for accuracy in various matrices. This is increasingly important as a greater number of analytes of varying chemical properties are included in one analysis.

For this particular analysis, a review of the literature suggested that solution stability of the compounds should to be given particular consideration. It has been documented that OPZ, sparingly soluble in aqueous media, is unstable unless stored and handled at basic pH [18,19]. In studies investigating the mode of action of OPZ, it was presented that the molecule undergoes acid catalysis, and that the molecule has a half-life of only 1.4 h at pH 5.1, increasing to 38.5 h at pH 7.4 [20,21]. Another study noted that OPZ is stable at −20 °C for 1 month in plasma if the plasma is buffered at pH 8 [22]. This indicated a preferable use of basic media for OPZ analysis.

Second, a review of the literature revealed that MDZ can photo-degrade in aqueous solution, noting a 10% degradation in 1 h at pH 6.4. This decomposition was reduced by half as the media increased in acidity to pH 1.3 [23]. Also, solution pH dictates MDZ residence in open ring (acidic) or closed-ring form (neutral and basic pH). It has been observed that the open-ring form degrades more slowly when exposed to light than the closed ring form [23]. The effect of form on MDZ fragmentation was unknown at the outset of this tandem mass spectrometry development. This indicated a handling of MDZ in amber coated containers, with minimal exposure to light. Thus, consideration of the documented drug stability characteristics dictated the storage and solution conditions within this development.

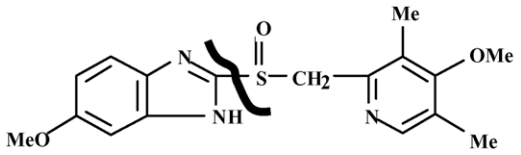
2. Experimental

2.1. Chemicals

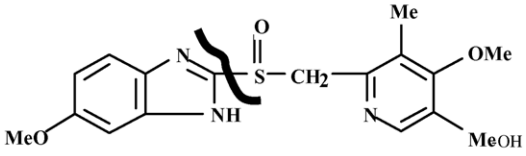
Omeprazole and formic acid were obtained from Sigma–Aldrich (St. Louis, MO, USA) Midazolam, alphahydroxymidazolam (OH-MDZ), and flurazepam were obtained from Lipomed (Cambridge, MA, USA). Hydroxy-omeprazole was donated from Astra–Hassle (Basel, Switzerland). Water, ammonium acetate, methanol, hexanes, and ethyl acetate were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Acetonitrile and ammonium hydroxide was obtained from VWR (South Plainfield, NJ, USA). All solvents used in sample preparation and chromatographic separations were of HPLC grade. Plasma for preparation of standards, quality controls, and blanks was obtained from The Interstate Blood Bank (Memphis, Tenn., USA).

2.2. Instrumentation

The LC/MS/MS system consisted of an Agilent 1100 series autosampler (Foster City, CA, USA) an Agilent 1100 series pump, an Agilent 1100 series degasser, and an Applied Biosystems PE/Sciex, API 3000 mass spectrometer (Foster City, CA, USA) equipped with a Turbo-ionspray source. The system was controlled through Analyst Software, version 1.1 from Applied Biosystems.



Omeprazole
CAS:73590-58-6
345.42g/mol



Hydroxyomeprazole
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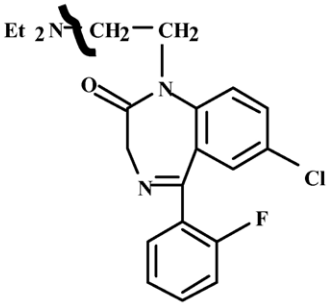
361.42g/mol



Flurazepam
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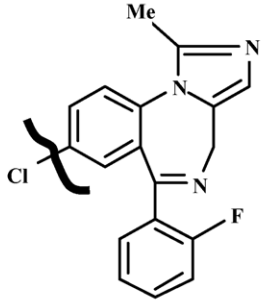
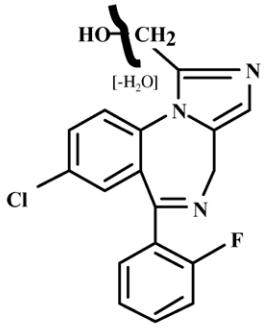
387.88g/mol



Midazolam
CAS:59467-70-8
325.77g/mol



1'-Hydroxymidazolam
CAS:59468-90-5
341.77g/mol

Fig. 1. Structures, neutral masses and molecular weights of the analytes [15]. The proposed fragmentation site is indicated. Definitive fragment confirmation would require multiple stages of tandem mass spectrometry to confirm.

2.3. Chromatographic conditions

Analytes were separated on a Waters Symmetry Shield RP8 (Milford, MA), which was 3.0 mm inner diameter and 150 mm length, packed with 5 μm sized particles. The injection volume was 20 μL. Since basic conditions were needed to stabilize OPZ and OH-OPZ, the mobile phase was made to accommodate. Isocratic elution using a mobile phase mix of 35% 5 mM ammonium hydroxide/formic acid pH 8.2, and 65% methanol, delivered at a rate of 400 μL/min was used for separation. Prior to entering the electrospray source housing, the flow was split 1:1 using a PEEK tubing splitter (Upchurch Scientific, Oak Harbor, WA, USA), with one split line directed to waste and the other to the Turbo-ionspray source. The ionization source was set at a temperature of 350 °C utilizing nitrogen for the drying and collision gas.

2.4. Optimization of MS/MS detection parameters

Experiments were conducted to discern the optimized detection parameters for MS/MS detection of the analytes. Each of the drugs was dissolved at 1 μg/mL in each of two solutions: a 50/50 (v/v) mix of methanol/5 mM acetate buffer, pH 3.0, or a 50/50 mix of methanol/5 mM ammonium hydroxide solution adjusted with formic acid to a pH of 8.2. To observe how the instrument's potential settings affect primary and fragment ions, analytes were directly infused into the instrument at a flow rate of 7.5 μL/min. Analytes were detected using both positive and negative-mode ionization.

The analyst software "Quantitative Optimization" wizard was used to discern the optimal parameters.

2.5. Preparation of stocks, standards, control, and internal standard solutions

Two 1 mg/mL stock solutions of each analyte and internal standard were prepared in methanol. Stock solutions were protected from light and stored at −70 °C for up to 2 months. Standards were made by combining one set of stock solutions and diluting serially in 80/20 methanol/5 mM ammonium acetate adjusted with formic acid to pH 8.2. Then 50 μL of each standard was combined with 250 μL of plasma. The resulting 10 standard concentrations of 0.40, 1.0, 2.0, 4.0, 8.0, 10, 20, 40, 80, and 100 ng/mL were used to construct daily working curves for each analyte. Control solutions were made from the second set of stock solutions, and dissolved in plasma. Before utilizing using any plasma, it was previously tested for the presence of analyte interference. Standards and control solutions (in plasma) were made ahead of time and stored at −70 °C for up to 2 months. A 500 ng/mL internal standard solution (flurazepam) was diluted from the 1 mg/mL stock solution in methanol. The internal standard solution was stored at −20 °C for up to 2 months.

2.6. Preparation of samples

On the day of analysis, 50 μL of 80/20 methanol/ammonium acetate and formic acid, pH 8.2 was added to 250 μL of each control (quality control) and unknown sample. 300 μL of each standard was used for analysis. After 50 μL of internal standard was added to all standards, controls and samples, 3 mL of 75:25% ethylacetate:hexane was added for extraction. Each sample was shaken on high speed using a shaker (Eberbach Instrument Apparatus, Ann Arbor, MI, USA) for 25 min. Samples were then centrifuged for 10 min at 3000 × *g*, leaving two layers within each test tube. The upper layer was removed, placed into a clean test tube and evaporated to dryness with air using a Zymark Turbo Vap LV (Hopkinton, MA, USA) for 30 min at 50 °C. Samples were reconstituted in 100 μL of mobile phase mix, placed into amber vials to protect from any photodegradation, and injected into the LC/MS/MS system for analysis.

2.7. Recovery

The recovery of the analytes from plasma was determined and optimized using two experiments. In the first experiment, five solvent systems were used for extraction. For each, three replicate samples and one control were prepared. To each replicate, a known amount of analyte was added prior to preparation. Extraction time on the shaker was 15 min for experiment 1. To measure recovery, the same concentration of analyte was placed into methanol, which was evaporated to dryness and reconstituted in mobile phase. By comparing the analyte signal from samples where the analytes underwent extraction to samples where the analytes did not, the recovery of each of the species could be determined. The second experiment analyzed 10 replicate samples of a known addition of analyte, and compared these to five control samples, using one extraction solvent system chosen as a result of the first experiment. For this experiment, time on the shaker was increased from 15 to 25 min.

2.8. Lower limit of quantitation and limit of detection

The lower limit of quantitation (LOQ) was defined as the lowest concentration for which analytes could be determined reproducibly within 20% of the targeted value while producing a signal-to-noise ratio of at least 5. Six analyses were completed for each analyte at the LOQ on 3 validation days. The lowest three standard concentrations were utilized for determination of the LOQ. The limit of detection (LOD) was defined as the concentration that produced a signal that was three times the noise level of a blank preparation. Experimentally, the concentration was sequentially decreased below the LOQ to 100 pg/mL. From the 100 pg/mL chromatogram obtained, the LOD was estimated through extrapolation versus the measurement of a corresponding blank.

2.9. Calibration procedures and acceptability criteria

Calibration curves were constructed on a daily basis using an internal standard (Flurazepam). Unknown and control samples were quantitated using a linear regression of the calibration samples, as calculated by the Analyst program. For all of the analytes, calibration curves were weighted by a factor of 1/(analyte concentration)^2. To accept the calibration two criteria had to be satisfied. First, it was required that at least six standard concentrations be included within the calibration curve. Any back-calculated standards that did not fall within 15% of the nominal value were excluded and the curve was recalculated. Second, at least two-thirds of the standard concentrations' back-calculated values were required to be with 15% of their nominal values. For example, working curves with 10 standard concentrations were only acceptable if seven of those 10 met the criteria for accuracy. Otherwise, the entire analysis was repeated. Calibration samples were randomized throughout the batch of injections and peak areas were used for all measurements.

For each analyte, the within- and between-day precision was determined using six replicate samples of each control concentration on 4 days. Four representative control concentrations were used. The lowest control sample concentration was targeted to be twice the LOQ. To satisfy this requirement the lowest control had to be re-prepared for OH-MDZ, OPZ and OH-OPZ during the validation (after the LOQ had been determined in replicate). For the assay to be considered

valid, at least two-thirds of the samples of each control concentration had to be within 15% of the target value. Control samples were randomized throughout the batch of injections.

2.10. Reliability of the method in independent sources of plasma matrix

Due to the variability of plasma drawn from different individuals, the effect of varying plasma on quantitation is critical, especially where ion-suppression is a possibility. One way to accomplish this is to obtain and compare the results of a known amount of analyte added to independent sources of plasma [16,17]. Each analyte was added to each of five lots of blank plasma, quantitated and evaluated in terms of precision and accuracy. Three repetitions were prepared in each matrix. For comparison, a blank of each matrix was also analyzed. The matrix utilized for quality control preparation was excluded from this experiment. It should be noted that either heparin or EDTA was used as an additive in all of the plasma lots.

2.11. Stability of the analytes in the post-preparative matrix

Stability for these analytes under various storage, pH and lighting is fairly well-documented. Thus, measures to assure assay integrity within our own methodology considerations were implemented or tested. First, for samples that quantitated above our working curve upper limit of 100 ng/mL, plasma dilution by 10 was utilized and reanalysis was conducted. On each assay batch that included dilution an additional two replicates of quality controls diluted prior to analysis by the same factor as the samples were included. These additional control samples were subjected to the same acceptability criteria as routine controls (quality controls). Second, instrumental break-down can also cause a loss of results. Ideally, reinjecting samples after repair would be desirable in terms of time and cost. To measure the variation of storing samples in the post-preparative extract matrix, controls were prepared and analyzed, then reanalyzed after 3 weeks residence at −20 °C.

3. Results

3.1. Optimization of MS/MS detection parameters

For optimization of the mass spectrometer's potential settings for each drug, analytes were infused directly into the mass spectrometer. During this infusion, sequential changes of electrode potentials allowed for identification of the optimum settings for detection of precursor and product ions. Because of the pH dependent stability of these analytes optimization was conducted using solutions of either pH 3.0 (acetate buffer) or pH 8.2 (formic acid and ammonium hydroxide) components. A summary of the results is shown in Table 1. For all of the analytes, positive ionization provided the best detectability. The molecular ion was the primary ion observed for each analyte. In terms of pH, no appreciable signal for omeprazole or hydroxyomeprazole was observed in solutions containing the pH 3.0 acetate based buffer. Midazolam and OH-MDZ produced ions in both buffers of comparable intensities.

For the duration of method development, two fragment ions were chosen for optimization for OPZ, OH-MDZ, and MDZ. Both fragments had comparable signal during initial development. For OH-OPZ, only one dominant fragment was observed. Monitoring of the transitions between the precursors and these fragments was maintained throughout the optimization of separation parameters. The final proposed sites of fragmentation according to mass loss calculations are indicated in Fig. 1.

Coupling chromatography to mass spectrometry provides several advantages. First, retention of analytes allows diversion of interfering unretained species prior to entering the mass spectrometer, decreasing the chance of instrument contamination and electrospray ion suppression. Second, separation of retained matrix components from the target analyte minimizes matrix effects, which can affect quantitation through ion suppression [17]. It was found that retention and resolution were obtained utilizing isocratic elution with a mobile phase composition consisting of 35% 5 mM ammonium hydroxide adjusted with formic acid to pH 8.2 and 65% methanol. Once the separation was optimized, the ion transition that produced the highest signal

Table 1
Detection parameters for analyte precursor and fragment ions

Analyte	Omeprazole	Midazolam	OH-OPZ	OH-MDZ	Flurazepam			
Ionization mode of highest intensity	Positive	Positive	Positive	Positive	Positive			
pH for observation of analyte species	8.5	Both	8.5	Both	–			
	Mass/charge:precursor/fragment							
	346.2/198.1[a]	346.2/136.2	326.2/291.2[a]	326.2/102.1	362.1/214.1	342.1/324.1[a]	342.1/168.1	388.2/314.9
Declustering potential (V)	36.0		46.0		21.0	46.0		31.0
Focusing potential (V)	170.0		180.0		140.0	260.0		230.0
Collision energy (V)	45.0	11.0	37.0	83.0	17.0	31.0	51.0	31.0
Collision cell exit potential (V)	12.0	14.0	10.0	8.0	16.0	12.0	10.0	18.0

[a] Indicates highest intensity ion transition after chromatographic optimization.

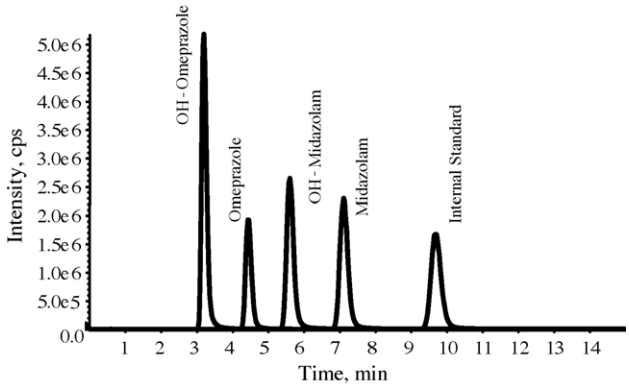


Fig. 2. Optimized separation of omeprazole, midazolam, the hydroxy-metabolites, and the internal standard flurazepam. This is a standard sample corresponding to a sample concentration of 200 ng/mL of each analyte.

for each analyte was chosen and utilized for method validation and sample analysis. A chromatogram of a standard solution extracted from a plasma matrix is shown in Fig. 2.

3.2. Recovery of analytes

The extraction procedure was developed to quantify all four analytes at the expected concentrations from one aliquot of clinical sample. Prior to clinical analysis it was postulated that midazolam would be present at lower concentrations than typically found in plasma due to administration of decreased dose for anticipated clinical investigations utilizing healthy volunteers. Both liquid–liquid and solid-phase extraction were available. A review of the literature showed that liquid–liquid extraction was more likely to produce a higher recovery for all four analytes in a simple preparation for the four analytes from plasma [22,24–26]. Once tested, the liquid–liquid methodology was found to be reproducible in studies of single concentration in one matrix, and within multiple concentrations in multiple matrices in a timely fashion. Therefore, solid-phase extraction procedures were not assessed.

Pretreatment of plasma to extract analytes was the result of testing six liquid–liquid extraction procedures. Results are summarized in Table 2. First, shaking the samples for 25 min markedly improved the reproducibility over shaking for 15 min as can be observed in comparing experiments 1–2. When using a diethylether/2-propanol extraction solvent, no peaks were obtained and a precipitate was observed in the extraction solvent. Of the remaining solvents, recovery generally decreased with increased percent hexane, although there was no statistical difference in many of the comparisons due to poor reproducibility. It was not apparent as to why greater than 100% recovery was observed for omeprazole using 75% ethylacetate/25% hexane with 15 min of shaking. This solvent was chosen for further optimization since higher recovery was obtained, particularly for OH-OPZ where recovery was otherwise less than 10%. Omeprazole did not produce an average recovery of higher than 100% on repeat with the 25 min shaking time. The order of average recovery, from highest to lowest, was omeprazole > midazolam > OH-MDZ > OH-OPZ, with no statistical difference between MDZ and OH-MDZ, as determined by a *t*-test. Further testing of the method in multiple matrices, as well as inter- and intra-day precision shows reproducibility of the recovery. A standard determination of the specific three control concentrations designating the exact recovery was not explicitly assessed.

3.3. Limits of detection and quantitation

Results for the limits of detection and quantitation are shown in Table 3. The lowest limit of detection was obtained for OH-OPZ, at a level of 2 pg/mL. A chromatogram of a 100 pg/mL sample versus a blank preparation can be seen in Fig. 3. It can be seen that a signal for omeprazole is observed in the plasma blank. Omeprazole, being a common medication, can be observed in purchased plasma. The plasma blank chosen in this example was of a typical magnitude. Regardless, all blank plasma is tested prior to its use. The 100 pg/mL sample shown in Fig. 3a produces a signal appreciably greater than the blank signal.

Replicates of the three lowest standard concentrations were utilized to determine the lower limit of quantitation, 0.400, 0.800, and 1.00 ng/mL. Replicate samples were prepared at each concentration over the course of 3 days. Quantitation produced results within 20 % of the target value for all of the analytes at each level, except for OPZ and OH-OPZ for

Table 2
Determination of extraction solution via recovery measurement

Solvent system	Average % recovery (±S.D.)			
	Omeprazole	Midazolam	OH-omeprazole	OH-midazolam
Experiment 1				
75% ethylacetate/25% hexane	139.5 (47.2)	86.7 (22.7)	34.7 (18.5)	74.2 (21.2)
50% ethylacetate/50% hexane	67.8 (20.2)	51.9 (9.02)	6.03 (2.00)	49.6 (11.7)
25% ethylacetate/75% hexane	52.1 (14.3)	70.3 (12.2)	0.81 (0.16)	60.9 (13.4)
100% diethylether	71.3 (9.59)	78.2 (7.11)	7.40 (2.19)	66.0 (7.26)
50% diethylether/50% 2-propanol	No peak/precipitate observed			
Experiment 2				
75%ethylacetate/25% hexane, $n = 10$ shaken for 25 min	90.7 (11.3)	76.6 (7.2)	40.8 (3.4)	71.0 (8.21)

Table 3
Summary of assay accuracy and variability

Analyte calibration range (ng/mL): (regression)	Control concentrations													
	Omeprazole, midazolam, OH-OPZ, OH-MDZ: 0.800 ng/mL				Omeprazole, midazolam, OH-OPZ, OH-MDZ: 4.00 ng/mL				Omeprazole, midazolam, OH-OPZ, OH-MDZ: 20.0 ng/mL				Omeprazole, midazolam, OH-OPZ, OH-MDZ: 100.0 ng/mL			
	Daily means	Number of days	R.S.D. (%)		Daily means	Number of days	R.S.D. (%)		Daily means	Number of days	R.S.D. (%)		Daily means	Number of days	R.S.D. (%)	
Within-assay variability, $n = 6$ on each day																
Omeprazole: 0.400-100	0.707–0.747	3	≤4.55		3.61–3.93	4	≤7.85		17.5–19.8	4	≤7.67		86.2–94.5	4	≤13.8	
Midazolam: 0.400-100	0.810–0.897	4	≤12.1		3.47–4.30	4	≤7.81		19.0–21.9	4	≤6.01		91.6–107.5	4	≤7.39	
OH-OPZ: 0.400-100	0.806–0.897	3	≤6.47		4.00–4.52	4	≤13.0		18.8–22.0	4	≤16.7		92.9–104.9	4	≤16.4	
OH-MDZ: 0.400-100	0.790–0.850	3	≤11.4		3.99–4.51	4	≤9.25		21.2–22.0	4	≤6.91		100.0–104.5	4	≤7.90	
Analyte and Calibration Range (ng/mL)	Mean	% Error	R.S.D. (%)		Mean	% Error	R.S.D. (%)		Mean	% Error	R.S.D. (%)		Mean	% Error	R.S.D. (%)	
Between-assay variability																
Omeprazole: 0.400-100	0.697	−12.9	12.6		3.83	−4.25	6.63		18.6	−7.00	7.50		89.4	−10.7	9.40	
Midazolam: 0.400-100	0.855	6.88	9.38		3.94	−1.50	10.1		21.0	5.00	8.71		98.4	−1.60	7.79	
OH-OPZ: 0.400-100	0.860	7.50	12.4		4.23	5.75	12.4		20.6	−3.00	10.4		99.4	−0.60	10.6	
OH-MDZ: 0.400-100	0.851	6.38	14.2		4.32	8.00	8.00		21.7	8.50	5.44		102.1	2.10	6.14	

one sample. A level of 0.400 ng/mL was chosen as the LOQ, and adopted as the lowest standard for routine analysis.

3.4. Assay calibration, accuracy and variation

Working curves for each analyte from five separate preparations produced coefficients (r^2) greater than 0.994. The calibration range for each analyte was 0.400–100 ng/mL utilizing 10 standards. For evaluation of accuracy and variation, control samples were analyzed within each validation batch in replicates of six. Table 3 shows a summary of the independent analysis of calibration and control samples. Also represented is the within- and between-analysis variation. Means of all control samples were within 15% of the target values. Also, at least four of each set of six replicates was required to be acceptable on all 4 days. Within- and between-day accuracy and precision was under 15% for all analytes on all days, with the exception of OH-OPZ on 1 day, where the precision was above 16% for two concentrations (making the highest R.S.D. above 16%). Since the accuracy of that day was acceptable (within 15% for two-thirds of the replicates) and the problem could not be reproduced, no remedial experiments were performed. Due to the adjustment of the low-control concentration for OPZ, OH-OPZ, and OH-MDZ after determination of the LOQ only 18 replicates (3 days) are represented at the concentration of 0.8 ng/mL for these analytes. The first validation day utilized a low control at a concentration of 1.25 ng/mL, rather than 0.8 ng/mL; results were within the acceptability criteria (data not shown). Since an LOQ of 0.4 ng/mL proved quantifiable the low control sample was adjusted to be within twice the LOQ. The concentrations shown in Table 4 were utilized as control concentrations for daily samples analysis.

3.5. Accuracy and precision in various matrices

The accuracy and precision of the method was tested in plasma from five different sources to observe the utility of quantitation in varying patient plasma. Results are shown in Table 5. Precisions in all matrices were less than 7%. In addition, blank preparations of each matrix showed no appreciable interfering signals. Additionally, all of the matrices had no more than one replicate which produced a result outside of 15% of the target value. One replicate was lost in plasma 3 due to a instrument error. No appreciable blank signal was detected within these plasma lots, and the internal standard peak areas were consistent with standards and controls (Table 6).

3.6. Methodological considerations in the analysis of patient samples

This method is utilized in support of clinical studies investigating these analytes in patients. Analysis of samples from these studies has provided us with the verification that the appropriate analytical parameters have been chosen for this

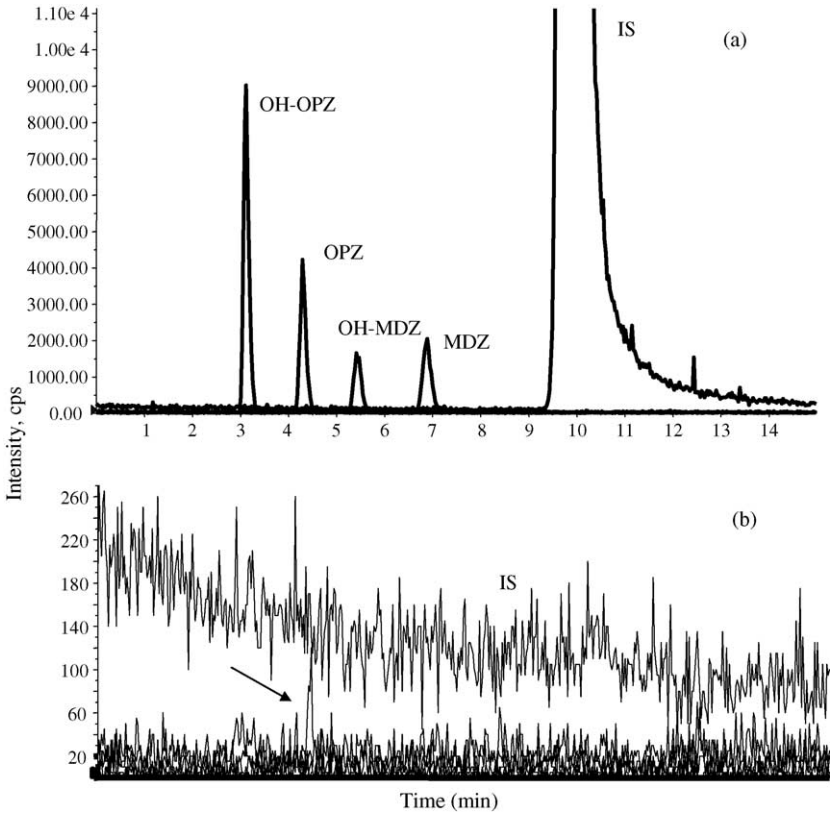


Fig. 3. (a) Standard sample prepared at a concentration of 100 pg/mL prior to extraction from plasma for each analyte and, (b) a corresponding plasma blank. In the plasma blank, omeprazole was observed, indicated with an arrow. Limits of detection were calculated for OPZ, MDZ, OH-OPZ, and OH-MDZ to be 10, 10, 2, and 10 pg/mL in the post-extract sample, respectively.

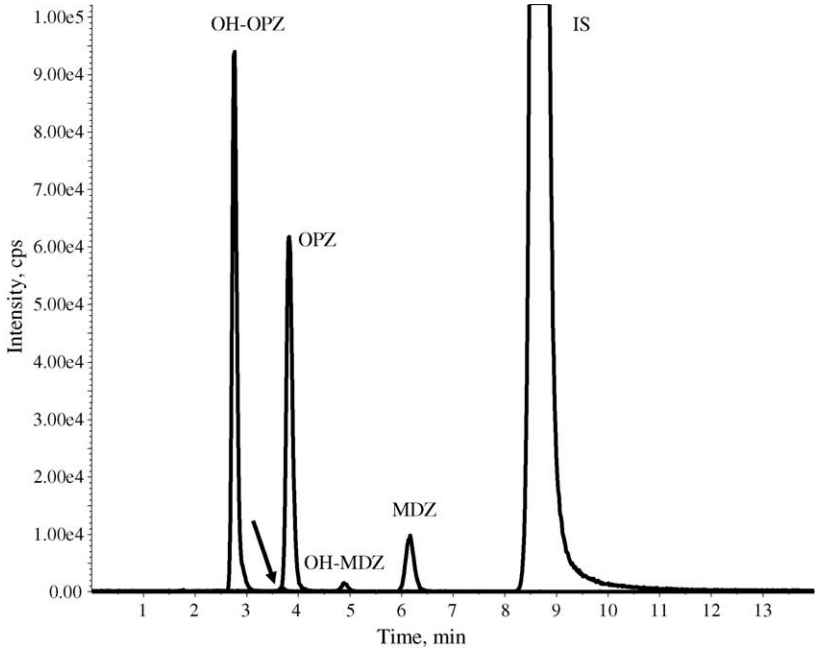


Fig. 4. Analysis of a patient sample at 2 h post-OPZ and MDZ ingestion. In this sample the results indicated concentrations of OPZ: 21.6 ng/mL; OH-OPZ: 31.8 ng/mL; MDZ: 7.27 ng/mL; OH-MDZ 0.94 ng/mL. In spite of the selectivity of tandem mass spectrometry, another peak elutes correspondent to the mass transition pattern of OH-OPZ, indicated with an arrow. This was not observed in all patient samples. Although this peak produces a much smaller area than OH-OPZ, separation of it prevents it from contributing to quantitation of OH-OPZ.

Table 4
Limits of detection and quantitation

Analyte	Extrapolated limit of detection (pg/mL)	Lower limit of quantitation (pg/mL) $n = 6$	Accuracy at limit of quantitation: average % error (R.S.D., %)	Calibration regression
Omeprazole	10	400.0	6.00 (10.0), $n = 17$	$y = 0.00294x - 2.07e^{-6}$, $r = 0.996$
Midazolam	10	400.0	−2.00 (9.56), $n = 18$	$y = 0.00188x - 5.11e^{5}$, $r = 0.999$
OH-OPZ	2	400.0	3.50 (11.8), $n = 17$	$y = 0.00245x + 10.1e^{5}$, $r = 0.998$
OH-MDZ	10	400.0	6.50 (11.0), $n = 18$	$y = 0.0018x + 6.28e^{5}$, $r = 0.995$

Table 5
Quantitation of analytes in independent sources of matrix

	Hydroxymidazolam	Hydroxyomeprazole	Midazolam	Omeprazole
Plasma 1				
Exp. val.	39.7	39.7	39.7	39.7
Obt. val.	40.0	43.4	42.8	42.3
R.S.D. (%)	1.8	1.5	2.5	2.3
No within 15%	3/3	3/3	3/3	3/3
Plasma 2 (one sample was lost due to technical error)				
Exp. val.	39.7	39.7	39.7	39.7
Obt. val.	41.2	43.7	40.6	41.0
No within 15%	2/2	1/2	2/2	2/2
Plasma 3				
Exp. val.	39.7	39.7	39.7	39.7
Obt. val.	38.3	42.0	37.4	39.4
R.S.D. (%)	3.3	6.4	4.6	3.4
No within 15%	3/3	3/3	3/3	3/3
Plasma 4				
Exp. val.	39.7	39.7	39.7	39.7
Obt. val.	42.1	43.4	43.6	43.8
R.S.D. (%)	2.7	1.5	3.4	4.1
No within 15%	3/3	3/3	3/3	3/3
Plasma 5				
Exp. val.	39.7	39.7	39.7	39.7
Obt. val.	45.9	45.0	42.5	43.1
R.S.D. (%)	1.2	1.5	2.6	1.8
No within 15%	2/3	3/3	3/3	3/3

method. To our knowledge and review of literature, published analytical methods for quantitation of these analytes is plasma have not utilized as low an LOQ as 400 pg/mL. Twelve healthy volunteers, participating in a study evaluating changes in CYP enzyme activity, received single doses of midazolam 0.025 mg/kg intravenously and omeprazole 40 mg orally as components of a multiple drug "cocktail" on two separate occasions. Blood samples were collected just prior to the medications and 5, 30 and 60 min, then 2, 4, 6, and 8 h after the time of the midazolam injection. Fig. 4 shows a 2 h post-dose sample containing 21.6 ng/mL OPZ. The 2 h omeprazole HI was used to evaluate CYP2C19 activity in this study. The 2 h post dose samples for all 12 volunteers on two study days ($n = 24$) were between 0.448 and 754 ng/mL for OPZ, and 0.94 and 372 ng/mL for OH-OPZ. None of the 24 samples fell below our limit of quantitation, allowing calculation of the 2 h HI for each study period.

Additional measures to investigate stability of the analytes in the post-preparative reconstitution matrix were taken in anticipation of instrumental errors. To measure the variation of storing samples in the post-preparative extract matrix, controls were prepared and analyzed, then reanalyzed after 3 weeks residence at −20 °C. These results are also shown in Table 4. For a routine assay batch, which would include two controls at each concentration, the results were within acceptability for all analytes.

Table 6
Dilution of the analytes prior to analysis

Analyte	Residence at −20 °C in post-preparative matrix (number of controls within acceptability criteria after 3 weeks)
Omeprazole	8 out of 8
OH-OPZ	8 out of 8
Midazolam	8 out of 8
OH-MDZ	7 out of 8

4. Conclusions

We have developed and validated a method for the analysis of OPZ, OH-OPZ, MDZ, OH-MDZ using ESI-LC/MS/MS. Liquid–liquid extraction was used to isolate analytes from

the samples; recoveries ranged from 40 to 91%. Through validation, the method proved to be accurate and reliable. Using a 250 μL plasma sample, a limit of quantitation of 0.400 pg/mL could be achieved. This allows better observation of patient samples across the pharmacokinetic profile, including the 2 h omeprazole HI sample time, with fewer results below the LOQ. Validation included testing the accuracy in various matrices, within- and between-day reliability, reproducibility of standard curves, quantitation of unknowns, and quantitation at the lower limit of detection, and stability in the post-preparative analysis matrix after 3 weeks. Accuracies and coefficients of variation were acceptable for all validation tests performed. It has been observed that OPZ and MDZ have contrasting stability issues and are typically assayed separately. Through the use of HPLC and MS/MS operated at basic conditions protecting the final samples from light, the simultaneous analysis of these species is possible.

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