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Rapid and simultaneous measurement of midazolam, 1′-hydroxymidazolam and digoxin by liquid chromatography/tandem mass spectrometry: Application to an in vivo study to simultaneously measure P-glycoprotein and Cytochrome P450 3A activity

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ABSTRACT

In order to simultaneously determine in vivo P-glycoprotein (P-gp) and Cytochrome P450 3A (CYP3A) activity, a new, rapid and sensitive liquid chromatography/tandem mass spectrometry (LC–MS/MS) method has been developed and fully validated to simultaneously determine midazolam (MDZ, as CYP3A substrate), 1′-hydroxymidazolam (1′-OHMDZ) and digoxin (DG, as P-gp substrate) in rat plasma using digitoxin as the internal standard (IS). After a single step liquid–liquid extraction with tert-butyl methyl ether/dichloromethane (75:25, v/v), analytes were subjected to LC–MS/MS analysis using positive electrospray ionization (ESI^+) under selected reaction monitoring mode (SRM). Chromatographic separation was performed on an XTerra MS C18 column (50 mm × 2.1 mm, i.d. 3.5 μm). The MS/MS detection was conducted by monitoring the fragmentation of 326.05 → 244.00 (m/z) for MDZ, 342.02 → 168.01 (m/z) for 1′-OHMDZ, 798.33 → 651.36(m/z) for DG and 782.67 → 635.24 (m/z) for IS. The method had a chromatographic running time of 3 min and linear calibration curves over the concentrations of 2–400 ng/mL for MDZ and 1′-OHMDZ and 0.5–100 ng/mL for DG. The recoveries of the method were 86.8–96.3% for MDZ, 84.6–86.4% for 1′-OH MDZ, and 81.7–85.1% for DG. The lower limit of quantification (LLOQ) of the method was 2 ng/mL for MDZ and 1′-OHMDZ and 0.5 ng/mL for DG. The intra- and inter-batch precision were less than 15% for all quality control samples at concentrations of 5, 50 and 320 ng/mL for MDZ and 1′-OHMDZ and 1, 10 and 80 ng/mL for DG. The validated LC–MS/MS method has been successfully used to analyze the concentrations of MDZ, 1′-OH MDZ and DG in rat plasma for simultaneous measurement of in vivo P-gp and CYP 3A activity.

1. Introduction

Cytochrome P450 3A (CYP3A) is the most important subfamily of drug-metabolizing enzymes in humans and metabolizes more than 50% of the clinically used drugs [1]. P-glycoprotein (P-gp) is an efflux transporter that is highly expressed in the intestine, liver, kidney, and blood–brain barrier [1]. CYP3A and P-gp are important in the disposition of a large number of drugs. Many drugs that are substrates of CYP3A enzymes are also substrates of P-gp [1,2]. Because of the wide substrate selectivity of CYP3A and P-gp, drug interactions with substrates, inducers or inhibitors of CYP3A and P-gp are frequent. Therefore, to determine the mechanism and extent of such interactions and to predict such clinical interactions, it is important to measure the in vivo activity of CYP3A and P-gp.

Midazolam (MDZ), a short-acting water-soluble benzodiazepine, is extensively metabolized by CYP3A [3]. The major metabolite of MDZ is 1′-hydroxymidazolam (1′-OHMDZ) and the lesser metabolites are 4-hydroxymidazolam (4-OHMDZ) and 1′, 4-dihydroxymidazolam (1′, 4-diOHMDZ) [4]. MDZ is the most widely accepted probe drug to measure in vivo and in vitro activity of CYP3A [5,6]. Importantly, MDZ is not a substrate of P-gp [6]. Digoxin (DG) is a cardiac glycoside which is commonly used to treat heart failure and arrhythmias [7]. DG has been shown to be a substrate of P-gp in vivo and in vitro [8], and has been widely used as an in vivo and in vitro probe to measure P-gp activity [6,9,10].

Usually, to measure in vivo CYP3A and P-gp activity, MDZ and DG are administered separately with and without the interacting drug, and the substrates are extracted and detected separately. Thus, the duration of an interaction study in vivo tends to be long. Recently, it is reported that MDZ and DG could be co-administered to simul-

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taneously determine P-gp and CYP3A activity in vivo, but MDZ and DG were still separately extracted and detected [6].

There are numerous methods to determine MDZ and its metabolites in plasma, including high performance liquid chromatography (HPLC) methods and liquid chromatography/tandem mass spectrometry (LC–MS/MS) methods [11–16]. Several methods for the determination of DG in bio-matrix also have been reported, including immunoassay techniques [17,18], HPLC methods [19,20] and LC–MS/MS methods [21,22]. However, to our knowledge, no analytical method has been reported that is able to simultaneously determine MDZ, 1′-OHMDZ and DG in bio-matrix after co-administration of MDZ and DG. Therefore, it is necessary and urgent to develop a rapid and sensitive method for simultaneous determination of DG, MDZ, and its major metabolite 1′-OHMDZ to further reduce the duration of study to measure both CYP3A and P-gp activities.

Thus, the purpose of the current study was to develop a rapid and sensitive LC–MS/MS method for simultaneous determination of MDZ, 1′-OHMDZ and DG in rat plasma, and to apply the developed method to simultaneously study in vivo P-gp and CYP3A activity.

2. Experimental

2.1. Chemicals and reagents

Midazolam (MDZ, purity, 99%, Fig. 1) and 1′-hydroxymidazolam (1′-OHMDZ, purity, 98%, Fig. 1) were purchased from IL Co. (San Bruno, USA). Digoxin (DG, purity, ≥95%, Fig. 1) and digitoxin (IS, purity, ≥92%, Fig. 1) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Methanol and acetonitrile of HPLC–MS grade were purchased from Tedia Inc. (Fairfield, USA). All other solvents were of analytical grade or HPLC grade when appropriate. Ultra-pure water was obtained from a Milli Q-plus system (Billerica, MA).

2.2. Liquid chromatographic and mass spectrometric conditions

A Finnigan Surveyor MS pump (San Jose, CA, USA) and a Finnigan Surveyor autosampler were used for solvent and sample delivery. Chromatographic separation was achieved by using a XTerra MS C_{18} column (50 mm × 2.1 mm, i.d., 3.5 μm, Waters, USA) at room temperature. The mobile phase consisted of methanol–water (containing 5 mM ammonium formate and 0.1% formic acid) (80:20, v/v), pumped at a flow rate of 200 μL/min. The total running time was 3.0 min for each sample.

A Finnigan TSQ Quantum triple quadrupole mass spectrometer equipped with an ESI source was used for mass analysis and detection. Mass spectrometric analysis was performed in the positive ion mode (ESI^+) and set up in the selected reaction monitoring (SRM) mode. Nitrogen was used as the sheath gas (35 psi) and the auxiliary gas (35 psi). The capillary temperature was 350 °C. The spray voltage was 3500 V. Collision induced dissociation (CID) studies were performed and argon was used as the collision gas with a collision cell gas pressure of 1.5 mTorr (1 Torr = 133.3 Pa). The optimized source CID was 10 V. The optimized collision energy was 36, 30, 12 and 14 V for MDZ, 1′-OHMDZ, DG and digitoxin, respectively. On the basis of the full-scan mass spectra of each analyte, the mass spectrometer was set to monitor the transitions of the precursors to the product ions as follows: 326.05 → 244.00 (m/z) for MDZ, 342.02 → 168.01 (m/z) for 1′-OHMDZ, 798.33 → 651.36 (m/z) for DG and 782.67 → 635.24 (m/z) for IS. The scanning time for each analyte was set to 0.2 s. Typical product ion spectra of MDZ, 1′-OHMDZ, DG and digitoxin are shown in Fig. 2. Data acquisition was performed with the Finnigan Xcalibur 1.3 software. Peak integration and calibration were performed with the Finnigan Lcquan software.

2.3. Preparation of standard and quality control samples

The stock standard solutions of MDZ, 1′-OHMDZ and DG were prepared by dissolving the accurately weighed individual compounds in methanol–water (50:50, v/v) to give a final concentration of 800 μg/mL, 100 μg/mL and 200 μg/mL, respectively. The solutions were then serially diluted with methanol–water (50:50, v/v) to obtain working solutions over concentrations range of 0.020–4.0 μg/mL for MDZ and 1′-OHMDZ, 0.0050–1.0 μg/mL for DG. A standard stock solution of digitoxin (IS) at 300 μg/mL was also prepared in methanol–water solution (50:50,v/v) and then diluted to obtain a working solution at 0.15 μg/mL. The stock solutions were stored at −80 °C, and the working solutions were stored at −20 °C and were brought to room temperature before use. Calibration standard samples were prepared by spiking blank rat plasma with standard working solutions to give concentrations of 2, 5, 10, 20, 50, 100, 200, 400 ng/mL for MDZ and 1′-OHMDZ, and 0.5, 1, 2, 5, 10, 20, 50, 100 ng/mL for DG. Quality control (QC) samples were prepared at concentrations of 5, 50, and 320 ng/mL for MDZ and 1′-OHMDZ, and 1, 10, and 80 ng/mL for DG.

2.4. Sample preparation

A 80 μL of blank rat plasma was added to a 2 mL test tube followed by spiking with the standard working solutions (10 μL MDZ and 1′-OHMDZ mixture, 10 μL DG and 10 μL IS). This mixture was then vortexed for 1 min using a vortex mixer. Then, 50 μL ammonia hydroxide solution (pH = 11.2) was added and vortexed for 1 min. The extraction was carried out with 1 mL tert-butyl methyl ether/dichloromethane (75:25, v/v) by vortexing-mixed for 2 min and standing at room temperature for 5 min. After centrifugation at 10,000 × g for 5 min, the organic phase was then removed to a new 1.5 mL centrifuge tube and evaporated to dryness. The residues were reconstituted in 120 μL mobile phase and 10 μL was injected directly into the LC–MS/MS system for analysis.

2.5. Application to an in vivo pharmacokinetics study to simultaneously phenotype CYP3A and P-gp activity

Male Sprague–Dawley rats weighing between 280 and 330 g were supplied by the Laboratory Animal Service Center in Sun Yat-sen University. The animals were kept in a 22–24 °C room with a light/dark cycle of 12:12 h and 55–60% relative humidity. They had free access to standard rodent food and water. The rats were fasted for 12 h before the pharmacokinetics study. All studies were in accordance with the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of People's Republic of China.

The rats were randomly divided into three groups with five rats in each group. The rats were orally administered with MDZ (15 mg/kg) and DG (0.25 mg/kg) dispersion. About 0.2 mL of blood samples via the right jugular vein were collected into a pre-heparinized tube at pre-dosing and at 5, 10, 15, 30, 45 min, 1, 1.5, 2, 3, 4, 5, 6, 8, 12 h post-dosing. Blood samples were centrifuged immediately to separate 100 μL of plasma which were kept at −80 °C until analysis.

3. Results and discussion

3.1. Method development

The positive-ion mode (ESI^+) was chosen as the ionization source in this study. In order to make DG and IS form predominant ammonium adduct ion $[M+NH_4]^+$ which was easily fragmented during MS/MS detection, ammonium formate was selected to add

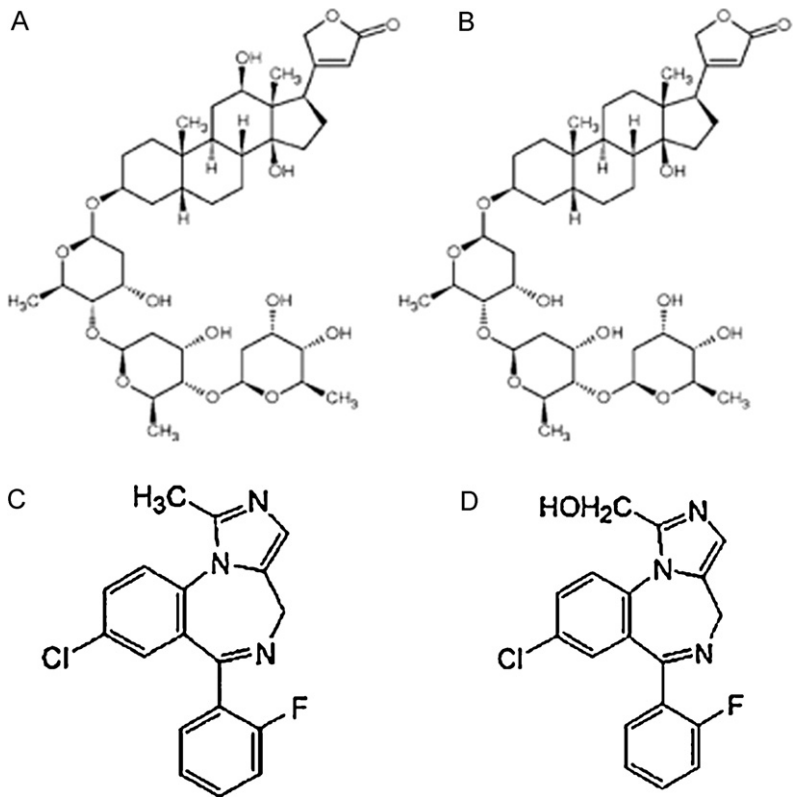

Fig. 1. Chemical structures of DG (A), digitoxin (B), MDZ (C) and 1′-OHMDZ (D).

in the mobile phase. As for different concentration of ammonium formate, 5 mM ammonium formate was selected as the mobile phase to get a maximum sensitivity of ammonium adduct ions $[M+NH_4]^+$. The MS/MS detection was conducted by monitoring the fragmentation of 798.33 → 651.36 (*m*/*z*) for DG and 782.67 → 635.24 (*m*/*z*) for IS. The fragmentation pattern of DG and IS was consistent with previous report [22].

Under ESI^+ condition, MDZ and 1′-OHMDZ yielded protonated molecule $[M+H]^+$ as the predominant ion for each compound. The ions in the product ion mass spectrum were at *m*/*z* of 291.11, 244.00, 102.10 for MDZ and *m*/*z* of 324.02, 203.05, 168.01 for 1′-OHMDZ. The most abundant ion in the product ion mass spectrum was at 291.11 (*m*/*z*) for MDZ and 324.02 (*m*/*z*) for 1′-OHMDZ. However, under the current LC–MS/MS conditions, there were interferences from endogenous plasma components at the retention times of the target analytes when 291.11 (*m*/*z*) and 324.02 (*m*/*z*) were selected as monitoring fragmentation of MDZ and 1′-OHMDZ. After optimization, the MS/MS detection was conducted by monitoring the fragmentation of 326.05 → 244.00 (*m*/*z*) for MDZ, 342.02 → 168.01 (*m*/*z*) for 1′-OHMDZ with good sensitivity and without interfering peaks observed in the blank plasma.

The mobile phase was optimized using different proportions of organic solvent–water to achieve good resolution and symmetric peak shapes for the analysis. To enhance the sensitivity of protonated molecule $[M+H]^+$ for MDZ and 1′-OHMDZ, formic acid was added in the mobile phase. Methanol used as organic solvent showed better sensitivity compared with acetonitrile. The water content was decreased to obtain better peak shape, higher sensitivity, and shorter running time for the analytes. At equal conditions, the analytes and the IS showed good symmetric peak shapes, as well as shorter running time of 3 min. Thus, a mobile phase consisted of methanol–water (containing 5 mM ammonium formate and 0.1% formic acid) and the ratio of 80:20 (v/v) was finally selected as the mobile phase.

Sample preparation is a critical step for accurate and reliable LC–MS/MS assay. Till now, methods for simultaneous extraction of MDZ, 1′-OHMDZ and DG from plasma have not been reported yet. In this study, 50 μL ammonium hydroxide was added to alkalinize plasma samples before extraction. And then analytes were extracted using a single step liquid–liquid extraction. The extraction efficiency of different solvents was compared during the method development, including diethyl ether, ethyl acetate, tert-butyl methyl ether alone or in combination with dichloromethane. As a result, tert-butyl methyl ether/dichloromethane (75:25, v/v) was chosen as the extraction solvent in the study because of the highest extraction recovery (>81.7%).

We first report here an extraction method to simultaneously extract the two most commonly used probe substrates and its major metabolites from100 μL plasma sample instead of using two separate extraction procedures from a larger volume of 200 μL plasma as reported [6,21]. This extraction procedure greatly shortened the duration of a study to simultaneously measure MDZ and DG.

3.2. Method validation

3.2.1. Selectivity and matrix effects

Selectivity was tested by comparing the chromatograms of six different batches of blank rat plasma with samples at LLOQ levels. Representative SRM chromatograms of blank plasma and the plasma with added MDZ, 1′-OHMDZ, DG at LLOQ levels are shown in Fig. 2. There was no significant interference in the SRM channel for the analytes at the expected retention time. The retention times for MDZ, 1′-OHMDZ, DG and IS were 1.56, 1.62, 1.53 and 1.89 min, respectively.

Absolute and relative matrix effect (ME) on the spectral response of MDZ, 1′-OHMDZ and DG was assessed using the procedure described by Matuszewski et al. [23] and Bi et al. [24]. The variability was acceptable with RSD values <7.4%, <5.9% and <11.5%

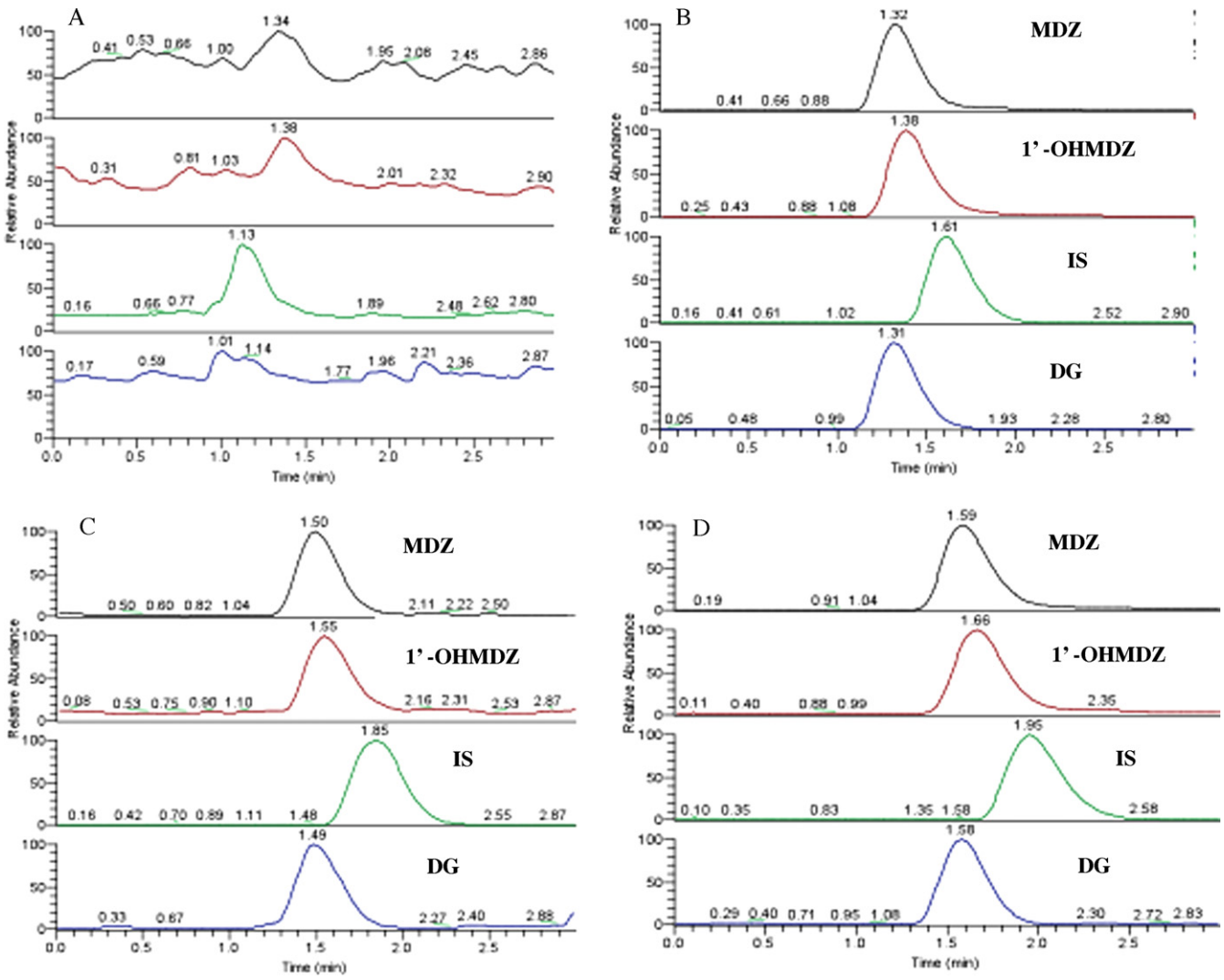

Fig. 2. Representative selectivereaction monitoring (SRM) chromatograms of MDZ, 1′-OHMDZ, digitoxin and DG in rat plasma. (A) A blank plasma sample; (B) a blank mobile phase sample spiked with MDZ, 1′-OHMDZ, digitoxin and DG;(C) a blank plasma sample spiked with MDZ, 1′-OHMDZ, digitoxin and DG at LLOQ; (D) a plasma sample from a rat after a single oral administration of MDZ (15 mg/kg) and DG (0.25 mg/kg).

at QC concentrations of MDZ, 1′-OHMDZ and DG respectively. The values of absolute ME at QC concentrations of analytes in five different batches of rat plasma were ranging from 54.6 to 55.5%, 62.8 to 71.4% and 81.9 to 83.7% for MDZ, 1′-OHMDZ and DG, respectively. These values of absolute ME indicated that there was an ionization suppression effect for MDZ, 1′-OHMDZ and DG under the present LC–MS/MS conditions. However, the linearity of the established calibration curves as well as precision and accuracy data were not affected by such ionization suppression. Thus, the present analytical method was considered reliable for analytes determination in rat plasma, in spite of the matrix effects existed.

3.2.2. Linearity and lower limit of quantification

Linearity was assessed by analyzing MDZ, 1′-OHMDZ and DG samples over 2–400 (MDZ, 1′-OHMDZ) and 0.5–200 ng/mL (DG) concentration ranges in rat plasma. The calibration curves could be achieved by weighting ($1/X^2$) linear regression of peak area of MDZ, 1′-OHMDZ or DG over that of IS. The slope, the intercept and the correlation coefficient (r^2) for each standard curve from each analytical run were determined automatically by Finnigan Lcquan software program.

The representative standard curves, correlation coefficient values and linear ranges of MDZ, 1′-OHMDZ and DG are as summarized below: $Y = -0.0375201 + 0.385302X$ ($r^2 = 0.992$), $Y = -0.0163335 + 0.234246X$ ($r^2 = 0.995$), $Y = -0.0232809 + 0.071602X$ ($r^2 = 0.995$) over concentrations range of 2.0–400 ng/mL for MDZ and 1′-OHMDZ, 0.5–100 ng/mL for DG. The lowest concentrations of MDZ, 1′-OHMDZ and DG on the calibration curve were 2, 2 and 0.5 ng/mL, respectively. The analytes' response at these concentration levels was >5 times of the baseline noise. The precision and accuracy at these concentration levels were acceptable, with <7.2% of the CVs and range between −3.3 and 16.0% of the relative errors for MDZ, <6.5% of the CVs and range between −9.9 and 5.4% of the relative errors for 1′-OHMDZ, <8.6% of the CVs and range between −2.7 and 14.1% of the relative errors for DG. Thus, the lowest concentration on the calibration curve was accepted as the LLOQ.

3.2.3. Precision and accuracy

Intra- and inter-batch precision and accuracy were assessed from the results of QC samples. The intra- and inter-batch precision and accuracy data for MDZ, 1′-OHMDZ and DG are summarized in Table 1. The reproducibility of the method was defined by examining both intra- and inter-batch variance. All values of precision and accuracy were with in the acceptable range and the method was accurate and precise. For MDZ, 1′-OHMDZ and DG, the intra-batch precision ranged over 8.5–13.5%, 9.1–12.3% and 7.5–9.9%, and the

Table 1
Intra- and inter-batch precision and accuracy for data for assays of MDZ, 1′-OHMDZ and DG in rat plasma ($n = 5$).

Compound	Nominal conc. (ng/mL)	Precision		Accuracy mean relative error (%)
		Mean ± SD	RSD[a] (%)	
Intra-batch				
MDZ	5.0	5.6 ± 0.8	13.5	12.0
	50.0	56.7 ± 4.8	8.5	13.4
	320.0	315.1 ± 34.9	11.1	−1.5
1′-OHMDZ	5.0	5.4 ± 0.7	12.3	8.8
	50.0	54.9 ± 5.0	9.1	9.8
	320.0	313.7 ± 29.6	9.4	−2.0
DG	1.0	0.9 ± 0.1	9.4	−7.4
	10.0	10.8 ± 1.1	9.9	7.9
	80.0	82.3 ± 6.2	7.5	2.8
Inter-batch				
MDZ	5.0	5.1 ± 0.7	13.1	1.6
	50.0	53.4 ± 4.2	7.9	6.8
	320.0	296.3 ± 28.2	9.5	−7.4
1′-OHMDZ	5.0	5.4 ± 0.5	8.8	8.0
	50.0	52.7 ± 3.7	7.1	5.4
	320.0	296.4 ± 23.1	7.8	−7.4
DG	1.0	1.0 ± 0.1	12.1	−4.4
	10.0	10.4 ± 1.0	9.4	−4.4
	80.0	78.5 ± 5.4	6.9	−1.9

[a] RSD = relative standard deviation.

inter-batch precision was 7.9–13.1%, 7.1–8.8% and 6.9–12.1%. The mean intra-batch relative errors were −1.5 to 13.4%, −2.0 to 9.8% and −7.4 to 7.9%, and the mean inter-batch relative errors were −7.4 to 6.8%, −7.4 to 8.0% and −4.4 to −1.9% for MDZ, 1′-OHMDZ and DG, respectively.

3.2.4. Recovery and stability

The recovery of MDZ, 1′-OHMDZ and DG from rat plasma following liquid–liquid extraction procedure ranged over 86.8–93.3%, 84.6–86.4% and 81.7–85.1% respectively. The recoveries were similar at all analyte concentrations without concentration dependence.

The stability of MDZ, 1′-OHMDZ and DG was investigated under various storage conditions and all stability results are shown in Table 2. MDZ, 1′-OHMDZ and DG were stable (relative errors within ±15%) without significant degradation under various storage conditions. MDZ, 1′-OHMDZ and DG in rat plasma can therefore be stored at room temperature for 4 h, under −80 °C storage condition for 1 month. MDZ, 1′-OHMDZ and DG were stable in the reconstituted solution for 12 h in the autosampler.

3.3. In vivo pharmacokinetics study to simultaneously phenotype CYP3A and P-gp activity

MDZ is the most widely accepted probe for determination of in vivo CYP3A activity and DG is the most commonly used probe for measurement of in vivo P-gp activity. The pharmacokinetic param-

Table 2
Stability of MDZ, 1′-OHMDZ and DG in rat plasma under various storage conditions ($n = 5$).

Storage conditions	Compound	Nominal conc. (ng/mL)	Calculated conc.	
			Mean ± SD	Era (%)
−20 °C/3 freeze–thaw cycles	MDZ	5.0	5.3 ± 0.2	5.7
		50.0	53.1 ± 2.7	6.2
		320.0	327.2 ± 20.0	2.2
	1′-OHMDZ	5.0	5.2 ± 0.4	3.0
		50.0	53.7 ± 3.1	7.4
		320.0	346.4 ± 27.6	8.3
	DG	1.0	1.0 ± 0.2	−1.4
		10.0	10.4 ± 0.5	4.4
		80.0	90.7 ± 3.7	13.4
−80 °C/30 days	MDZ	5.0	4.9 ± 0.8	−2.2
		50.0	52.3 ± 2.0	4.6
		320.0	308.3 ± 22.0	−3.7
	1′-OHMDZ	5.0	4.5 ± 0.4	−9.6
		50.0	51.6 ± 2.0	3.2
		320.0	315.0 ± 25.0	−1.6
	DG	1.0	0.9 ± 0.1	−5.9
		10.0	10.3 ± 0.4	3.3
		80.0	85.6 ± 4.8	7.0
Room temperature/24 h (extracted sample)	MDZ	5.0	5.2 ± 0.2	3.1
		50.0	55.0 ± 3.6	10.0
		320.0	299.8 ± 13.6	−6.3
	1′-OHMDZ	5.0	5.0 ± 0.2	0.8
		50.0	54.2 ± 3.0	8.4
		320.0	310.0 ± 9.4	−3.2
	DG	1.0	1.0 ± 0.1	−2.4
		10.0	10.5 ± 0.5	4.9
		80.0	79.6 ± 2.6	−0.5
Room temperature/4 h	MDZ	5.0	4.9 ± 0.8	−2.9
		50.0	49.3 ± 3.8	−1.4
		320.0	321.5 ± 16.1	0.5
	1′-OHMDZ	5.0	5.0 ± 0.6	−8.3
		50.0	51.3 ± 3.9	2.5
		320.0	338.9 ± 18.2	6.0
	DG	1.0	1.1 ± 0.2	4.9
		10.0	10.1 ± 0.9	0.5
		80.0	85.7 ± 3.8	7.2

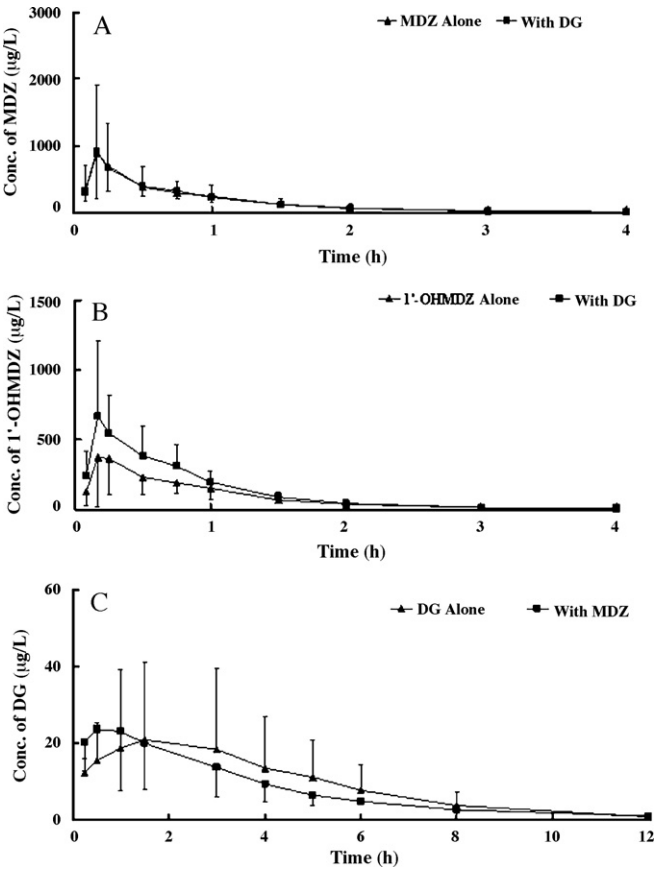

Fig. 3. Plasma concentration–time curves of MDZ (A), 1′-OHMDZ (B) and DG (C) when MDZ (15 mg/kg) and DG (0.25 mg/kg) were administered alone or in combination dosage (mean ± SD, $n = 5$).

eters such as the total clearance or AUC of MDZ and the AUC of DG, or formation clearance of 1′-OHMDZ can be used as in vivo measures of CYP3A or P-gp activities. For this reason we used these two drug cocktails to phenotype in vivo CYP3A and P-gp activities in an efficient, time-saving manner, and thus an analytical method to simultaneously determine MDZ, 1′-OHMDZ, and DG was needed. The fully-validated method was successfully applied to determine the plasma concentrations of MDZ, 1′-OHMDZ and DG in rats following a single oral administration of MDZ (15 mg/kg) and DG (0.25 mg/kg). The SRM chromatograms of plasma obtained from pre- and post-dosing rats showed no significant interfering peak at the retention times of MDZ, 1′-OHMDZ, DG and the IS, indicating the method was specific enough for the pharmacokinetics study. The average plasma concentration–time profile of MDZ, 1′-OHMDZ and DG is depicted in Fig. 3, and the calculated pharmacokinetic parameters of MDZ, 1′-OHMDZ and DG when MDZ and DG were administered alone or in combination are presented in Table 3.

It was reported that because of the specificity of both MDZ and DG, these drugs were not expected to interact [6]. To avoid any other possible interactions, we also staggered the dosing of MDZ and DG by 1 h according to the previously reported method [6]. Moreover, by comparing the pharmacokinetic parameters when being administered alone or in combination, we can measure whether there were pharmacokinetic interactions including metabolism interaction between the two probe drugs. The average plasma concentration–time profiles and pharmacokinetic parameters of MDZ, 1′-OHMDZ, and DG were not significantly different ($P > 0.5$) when the drugs were administered alone versus those obtained in combination. All these results indicate that co-administration of DG and MDZ in the manner we have described here could simultaneously and efficiently determine P-gp and CYP3A activity in rat without a significant pharmacokinetic interaction happened.

Table 3
Pharmacokinetic parameters of MDZ, 1′-OHMDZ and DG when MDZ and DG were administered alone or in combination (mean ± SD, $n = 5$).

Parameters	Alone	Co-administration
MDZ		
C_{max} (ng/mL)	926.8 ± 983.5	950.6 ± 673.9
T_{max} (h)	0.3 ± 0.1	0.2 ± 0.1
$AUC_{0-4\,h}$ (ng h/mL)	614.5 ± 487.3	601.8 ± 201.1
$AUC_{0-\infty}$ (ng h/mL)	625.3 ± 485.6	606.7 ± 200.6
1′-OHMDZ		
C_{max} (ng/mL)	421.6 ± 327.6	723.9 ± 479.2
T_{max} (h)	0.3 ± 0.1	0.2 ± 0.1
$AUC_{0-4\,h}$ (ng h/mL)	351.2 ± 193.5	513.4 ± 245.8
$AUC_{0-\infty}$ (ng h/mL)	361.4 ± 192.8	519.0 ± 246.2
DG		
C_{max} (ng/mL)	21.6 ± 19.7	26.6 ± 13.9
T_{max} (h)	1.1 ± 0.6	0.6 ± 0.3
$AUC_{0-12\,h}$ (ng h/mL)	111.9 ± 105.6	96.2 ± 43.5
$AUC_{0-\infty}$ (ng h/mL)	118.5 ± 107.9	107.9 ± 43.7

Data are the mean ± SD. *Abbreviations*: C_{max}, maximum plasma concentration; T_{max}, maximum time to reach C_{max}; $AUC_{0-4\,h}$, area under the plasma concentration–time curve from time 0 to 4 h; $AUC_{0-12\,h}$, area under the plasma concentration–time curve from time 0 to 12 h; $AUC_{0-\infty}$, area under the plasma concentration–time curve from time zero to infinity.

4. Conclusions

A new, rapid and sensitive liquid chromatography/tandem mass spectrometry (LC–MS/MS) method had been developed and validated for simultaneous determination of MDZ, 1′-OHMDZ and DG in rat plasma using digitoxin as the internal standard. The method was validated according to FDA (2001) guidance and showed high sensitivity, reliability, specificity and excellent efficiency with a total running time of 2.0 min per sample. And this validated LC–MS/MS method was successfully applied to simultaneous measurement of in vivo P-gp and CYP 3A activity in rats. To our knowledge, this is the first report to develop and validate a LC–MS/MS method for the simultaneous determination of MDZ, 1′-OHMDZ and DG to simultaneously measure in vivo CYP3A and P-gp activity.

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