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Simultaneous determination of morinidazole, its *N*-oxide, sulfate, and diastereoisomeric N^+-glucuronides in human plasma by liquid chromatography–tandem mass spectrometry

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ABSTRACT

Morinidazole is a new third-generation 5-nitroimidazole antimicrobial drug. To investigate the pharmacokinetic profiles of morinidazole and its major metabolites in humans, a liquid chromatography–tandem mass spectrometry method was developed and validated for simultaneous determination of morinidazole, its *N*-oxide metabolite (M4-1), a sulfate conjugate (M7), and two diastereoisomeric N^+-glucuronides (M8-1 and M8-2) in human plasma. A simple acetonitrile-induced protein precipitation was employed to extract five analytes and internal standard metronidazole from 50 μL human plasma. To avoid the interference from the in-source dissociation of the sulfate and achieve the baseline-separation of diastereoisomeric N^+-glucuronides, all the analytes were separated from each other with the mobile phase consisting of 10 mM ammonium formate and acetonitrile using gradient elution on a Hydro-RP C_{18} column (50 mm × 2 mm, 4 μm) with a total run time of 5 min. The API 4000 triple quadrupole mass spectrometer was operated under the multiple reaction-monitoring mode using the electrospray ionization technique. The developed method was linear in the concentration ranges of 10.0–12,000 ng/mL for morinidazole, 1.00–200 ng/mL for M4-1, 2.50–500 ng/mL for M7, 3.00–600 ng/mL for M8-1, and 10.0–3000 ng/mL for M8-2. The intra- and inter-day precisions for each analyte met the accepted value. Results of the stability of morinidazole and its metabolites in human plasma were also presented. The method was successfully applied to the clinical pharmacokinetic studies of morinidazole injection in healthy subjects, patients with moderate hepatic insufficiency, and patients with severe renal insufficiency, respectively.

1. Introduction

Morinidazole [*R*,*S*-1-(2-methyl-5-nitro-1*H*-imidazol-1-yl)-3-morpholinopropan-2-ol], a new third-generation 5-nitroimidazole antimicrobial drug, has recently completed phase III clinical trials in China for the treatment of amoebiasis, trichomoniasis, and anaerobic bacterial infections as a racemic mixture. Morinidazole is similar to metronidazole in structure. Preclinical studies demonstrated that morinidazole exhibited greater activity against trichomoniasis and amoebiasis, and had less toxicity than metronidazole in mice [1].

After an intravenous infusion administration, morinidazole undergoes extensive metabolism in humans [2]. The major metabolites have been identified as two diastereoisomeric N^+-glucuronides (M8-1 and M8-2), a sulfate conjugate (M7), and an *N*-oxide metabolite (M4-1) in human plasma and urine. *In vitro* studies demonstrated that the formation of M4-1 was catalyzed by CYP3A4 (unpublished data) and that morinidazole N^+-glucuronidation was mainly mediated by UGT1A9 [2]. To better understand disposition of morinidazole in patients and investigate the potential drug–drug interaction in humans, it is important to evaluate the pharmacokinetics of morinidazole and its major metabolites. Therefore, it is essential to establish a powerful method for determination of morinidazole and its major oxidative and conjugated metabolites in human plasma.

Until now, only an LC–MS/MS method for the evaluation of the effects of 24 bio-matrices on the quantification of morinidazole has been reported [3]. No bioanalytical method for the simultaneous determination of morinidazole and its main metabolites in plasma has been described. The major metabolites of morinidazole in human plasma included *N*-oxide and conjugated metabolites [2], which might be unstable and might be converted to the parent drug during sample storage and preparation [4,5]. Conjugated metabolites also might dissociate in MS ion source, thereby increasing the signal of the parent, which may cause its false over-estimation.

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Therefore, absolute separation of conjugates, not only from the biological matrix peak but also from the parent drug by suitable LC conditions in a short time, is crucial [6,7]. Additionally, the separation of two diastereoisomeric N^+-glucuronides is also necessary because they have identical parent ion and product ions. Gradient elution is generally used to obtain better resolution of achiral isomers and shorten the analysis time during LC–MS/MS bioanalytical method development. In the paper published previously by our lab, a gradient elution comprising a mobile phase of acetonitrile: 5 mmol/L ammonium acetate with 0.1% formic acid was employed to separate the *cis*/*trans* isomers of hydroxylated metabolites of apatinib in human plasma [8].

This study aims to develop a simple and rapid assay for the simultaneous determination of morinidazole and its four metabolites in human plasma through optimization of chromatographic separation and MS/MS conditions. The method was successfully applied to pharmacokinetic studies of morinidazole injection in healthy subjects, patients with moderate hepatic insufficiency, and patients with severe renal insufficiency.

2. Experimental

2.1. Materials

Reference standards of racemic morinidazole (99.8% purity) and M4-1 (*N*-oxide metabolite, 99.9% purity) were provided by Jiangsu Hansoh Pharmaceutical Co. Ltd. (Lianyungang, China). M8-1 (N^+-glucuronide of *S*-morinidazole, 99.6% purity), M8-2 (N^+-glucuronide of *R*-morinidazole, 98.5% purity), and M7 (sulfate conjugate, 95.4%) were isolated and purified from human urine in our laboratory [2]. Metronidazole (internal standard, IS) was obtained from the National Institute for Food and Drug Control (Beijing, China). HPLC-grade acetonitrile and ammonium formate were purchased from Sigma (St. Louis, MO, USA). Millipore Milli-Q gradient purified water (Molsheim, France) was used throughout the study. Blank human plasma samples were supplied by the Tongji Hospital (Wuhan, China).

2.2. Chromatographic conditions

The HPLC system consisted of an LC-20AD pump and a SILHT$_A$ autosampler (Shimadzu, Kyoto, Japan). Separation of the analytes was achieved on a Synergi^{TM} 4 μm Hydro-RP C18 column (50 mm × 2 mm i.d., 4 μm; Phenomenex, Torrance, CA, USA) equipped with a Security-Guard C_{18} column (4 mm × 3.0 mm i.d.; Phenomenex). The mobile phase was a mixture of 10 mM ammonium formate in water (A) and acetonitrile (B). The gradient elution used was started from 5% B, linearly increased to 15% B in 3 min, held for 0.5 min, and finally decreased to 5% B to re-equilibrate the column for 1.4 min. The flow rate was 0.6 mL/min, and the column was maintained at room temperature. The total chromatographic run time was 5 min, including the re-equilibration time of the column.

2.3. Mass spectrometric conditions

An API 4000 triple quadrupole mass spectrometer (Applied Biosystems, Concord, Ontario, Canada) was used for mass spectrometric analysis and detection. Data processing was carried out on Analyst 1.4.1 software. Mass spectrometer with a TurboIon Spray (ESI) source was performed in positive mode. The source temperature was maintained at 400 °C, and the spray voltage was set at 3800 V. The nebulizer gas (Gas 1), heater gas (Gas 2), curtain gas and collision activated dissociation gas were set to 40, 50, 20 and 6 psi, respectively. The dwell time was kept at 80 ms for each analyte. The compound-dependent MS parameters are shown in Table 1.

Table 1
Mass spectrometric conditions for the analytes and internal standard.

Analyte	MRM transitions for quantification	CE (eV)	Response ratio
Morinidazole	*m/z* 271.0 → *m/z* 144.0	11	100%
M4-1	*m/z* 287.0 → *m/z* 100.0	42	100%
	m/z 287.0 → *m/z* 160.0	20	44%
M7	*m/z* 351.3 → *m/z* 271.0	16	100%
	m/z 351.3 → *m/z* 144.0	32	98%
M8-1 and M8-2	*m/z* 447.3 → *m/z* 320.0	30	100%
	m/z 447.3 → *m/z* 100.0	70	82%
	m/z 447.3 → *m/z* 144.0	42	74%
Internal standard	*m/z* 172.0 → *m/z* 82.0	20	100%

2.4. Preparation of calibration standards, quality control samples, and internal standard solutions

Calibration standard solutions and quality control samples were prepared from independently weighed standards. Standard stock solutions of morinidazole, M4-1, M7, M8-1, and M8-2 were prepared by dissolving accurately weighed reference substances in methanol at concentrations of 1000, 400, 231, 1000, and 545 μg/mL, respectively. Combined calibration solutions of morinidazole, M4-1, M7, M8-1, and M8-2 were diluted with methanol–water (50:50, v/v) to produce the following concentrations: 10.0/1.00/2.50/3.00/10.0, 30.0/3.00/7.50/9.00/30.0, 80.0/8.00/20.0/24.0/80.0, 400/20.0/50.0/60.0/200, 1000/50.0/125/150/500, 5000/100/250/300/1000, and 12,000/200/500/600/3000 ng/mL. The lower limit of quantification (LLOQ) and three levels of quality controls (QCs) were prepared from the stock solutions by serial dilutions with blank plasma at the following levels: 10.0/1.00/2.50/3.00/10.0, 20.0/2.00/5.00/6.00/20.0, 400/20.0/50.0/60.0/200, and 9600/160/400/480/2400 for morinidazole, M4-1, M7, M8-1, and M8-2, respectively.

An IS solution (500 ng/mL) was prepared by diluting 1.00 mg/mL stock solution of metronidazole with acetonitrile. LLOQ and QC samples were aliquoted into 1.5 mL polypropylene vials and stored at −20 °C until analysis. All the solutions were stored at 4 °C and were brought to room temperature before use.

2.5. Sample preparation

Frozen plasma samples from the human subjects were thawed to room temperature prior to preparation. After vortexing, a 50 μL aliquot of the IS solution (metronidazole, 500 ng/mL), 50 μL methanol–water (50:50, v/v), and 150 μL acetonitrile were added to 50 μL plasma sample. The mixture was vortexed for 1 min and centrifuged at 11,000 × *g* for 5 min. The supernatant was evaporated to dryness at 40 °C under a gentle stream of nitrogen, and the residue was reconstituted by addition of 100 μL acetonitrile–10 mM ammonium formate (5:95, v/v) and vortex-mixed for 1 min. A 5 μL aliquot of the resulting solution was injected onto the LC–MS/MS system for analysis.

2.6. Method validation

A full validation of the assay was performed according to the FDA guidelines [9].

The selectivity of the method was evaluated by analyzing six different sources of blank plasma samples and spiked plasma samples at the LLOQ level. By comparing the MRM chromatograms of the blank plasma samples with those of the corresponding spiked plasma samples at the LLOQ level, the response of coeluting interferences should be less than 20% of that of the analytes and less than 5% of the peak area of IS.

The linearity was assessed by analyzing calibration curves with seven levels in duplicate for morinidazole, M4-1, M7, M8-1, and M8-2 in human plasma on three separate days. In addition, two blank and two zero plasma samples were run to ensure the absence of interferences. The standard curves were calculated by a weighted ($1/x^2$) least squares linear regression method through the measurement of the peak-area ratio of each analyte to IS. The acceptance criteria for each back-calculated standard concentration were within ±15% deviation from the nominal value, except for the LLOQ level, at which a deviation of ±20% is permitted. The LLOQ, taken as the lowest concentration on the calibration curve that could be measured with acceptable accuracy and precision, was determined in six replicates on three consecutive validation days. The intra-day and inter-day precisions were required to be below 20%, and the accuracy to be within ±20%.

Precision and accuracy of the method were based on the QC samples at three concentration levels (20.0/2.00/5.00/6.00/20.0, 400/20.0/50.0/60.0/200, and 9600/160/400/480/2400 for morinidazole, M4-1, M7, M8-1, and M8-2, respectively) in six replicates on three validation days. The precision was expressed by relative standard deviation (RSD). The accuracy of the assay was expressed as relative error = (observed concentration − nominal concentration)/(nominal concentration) × 100%. The intra- and inter-day precisions were required to be below 15%, and the accuracy to be within ±15%.

To evaluate the precision and accuracy of the method in the sample dilution process, QC samples (at 100/2.00/5.00/6.00/20.0 μg/mL for morinidazole, M4-1, M7, M8-1, and M8-2) were diluted 20-fold using blank plasma before being analyzed in six replicates. The dilution procedure was considered valid if the accuracy and precision of diluted QC samples met the acceptance criteria of within ±15% of the nominal concentration and less than 15% of RSD.

The matrix effect was investigated by measuring the matrix factor, as defined by the ratio (analyte peak area in presence of plasma matrix from six different sources)/(analyte peak area in absence of plasma matrix), and was expressed as percent response relative to the neat solution (absolute matrix effect). In this experiment, the matrix effect was determined at two concentration levels (100/2.00/5.00/6.00/20.0 and 9600/160/400/480/2400 ng/mL for morinidazole, M4-1, M7, M8-1, and M8-2). The inter-subject variability of the matrix effect (relative matrix effect) at each concentration level should be less than 15%.

The recovery of morinidazole, M4-1, M7, M8-1, and M8-2 during sample preparation was estimated at three QC levels ($n = 6$) by comparing the peak area of each analyte. Samples that were spiked with the analytes prior to the extraction were compared with samples to which the analytes were added post-extraction. The extraction recovery of the IS was determined in a similar way, using the QC samples as a reference.

The stability of morinidazole and its main metabolites in human plasma was assessed by analyzing replicates ($n = 3$) of plasma samples exposed to different conditions (time and temperature) at concentrations of 100/2.00/5.00/6.00/20.0 and 9600/160/400/480/2400 ng/mL for morinidazole, M4-1, M7, M8-1, and M8-2, respectively. Short-term stability was determined after exposure of the spiked samples to room temperature for 6 h or after exposure of the ready-to-inject samples (after extraction, in the mobile phase) to the autosampler rack (room temperature) for 24 h. Long-term stability was evaluated after storage of the standard spiked plasma samples at −20 °C for 48 d. Freeze/thaw cycle stability was assessed after three complete freeze/thaw cycles on consecutive days. The analytes were considered stable in plasma when 85–115% of the nominal concentrations were found. In addition, the stability of the conjugated metabolites (M7, M8-1, and M8-2) was assayed separately during the sample preparation.

2.7. Application to pharmacokinetic studies

The validated LC–MS/MS method described above was applied to the determination of plasma concentrations of morinidazole, M4-1, M7, M8-1, and M8-2 from clinical pharmacokinetic studies in 12 healthy Chinese volunteers [2]. This method was also used in pharmacokinetic studies of 500 mg morinidazole by continuous intravenous infusion for 45 min to 12 patients with moderate hepatic insufficiency and to patients with severe renal insufficiency. The pharmacokinetic studies were approved by the ethics committees of the Third Xiangya Hospital of Central South University and Huashan Hospital affiliated to Fudan University, and conducted in accordance with the Declaration of Helsinki and the principles of Good Clinical Practice. Informed consent was obtained from all subjects after explaining the aims and risks of the study. Venous blood (4 mL) was collected and placed in heparinized tubes before the infusion, at 0.375 h after the start of the infusion, and at 0, 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24, 36, and 48 h after the end of the infusion. Plasma samples were separated and stored at −20 °C until analysis.

3. Results and discussion

3.1. Optimization of mass spectrometric conditions

The positive ionization mode was selected for the quantification of morinidazole and its metabolites by virtue of the presence of a basic tertiary amine group or quatemary ammonium in their structures. To decrease the extent of the in-source dissociation of N-oxide metabolite M4-1 and sulfate conjugate M7 and to obtain higher response of the analytes, electrospray ionization (ESI) source was selected in preference to atmospheric pressure chemical ionization (APCI) in this experiment.

In the Q1 full scan mode, protonated molecules at m/z 271, m/z 287, m/z 351, and m/z 172 were observed for morinidazole, M4-1, M7, and IS, respectively. M8-1 and M8-2 both showed molecular ion peak (M^+) at m/z 447. Fig. 1 presents the product ion spectra of $[M+H]^+$ or M^+ ions from the analytes and IS, as well as their proposed fragmentation patterns [2]. Compared with the parent drug, the metabolites had low plasma concentrations and poor MS responses. To maximize the signal response/sensitivity of the metabolites, summation of two or three MRM transitions were used in the detection: m/z 287 → (160 + 100) for M4-1, m/z 447 → (144 + 320 + 100) for M8-1 and M8-2, and m/z 351 → (144 + 271) for M7, respectively. The most suitable CEs for each transition were determined by observing the response of the fragment ion peaks obtained. Multiple MRM transitions resulted in 1.5- to 2-fold increase in the signal-to-noise ratios at the LLOQ level, than the individual MRM transition. For IS, the product ion spectrum of $[M+H]^+$ ion showed a major fragment ion at m/z 82, which was selected as quantitative MRM transition.

Furthermore, the additional parameters including the declustering potential (DP), source temperature, were also optimized carefully to maximize the MS responses. It was observed that the source temperatures of ESI (ranged from 300 to 600 °C) had little effect on the ion-source dissociation of M7.

3.2. Optimization of chromatographic conditions

During the LC–MS/MS analysis, each analyte could be quantified without the need to completely resolve the chromatographic peaks because specific MRM transitions were utilized. However, in this study, chromatographic resolution was necessary based on the fact that M8-1 and M8-2 are diastereoisomeric N^+-glucuronides of morinidazole, with the same m/z values of parent ions and

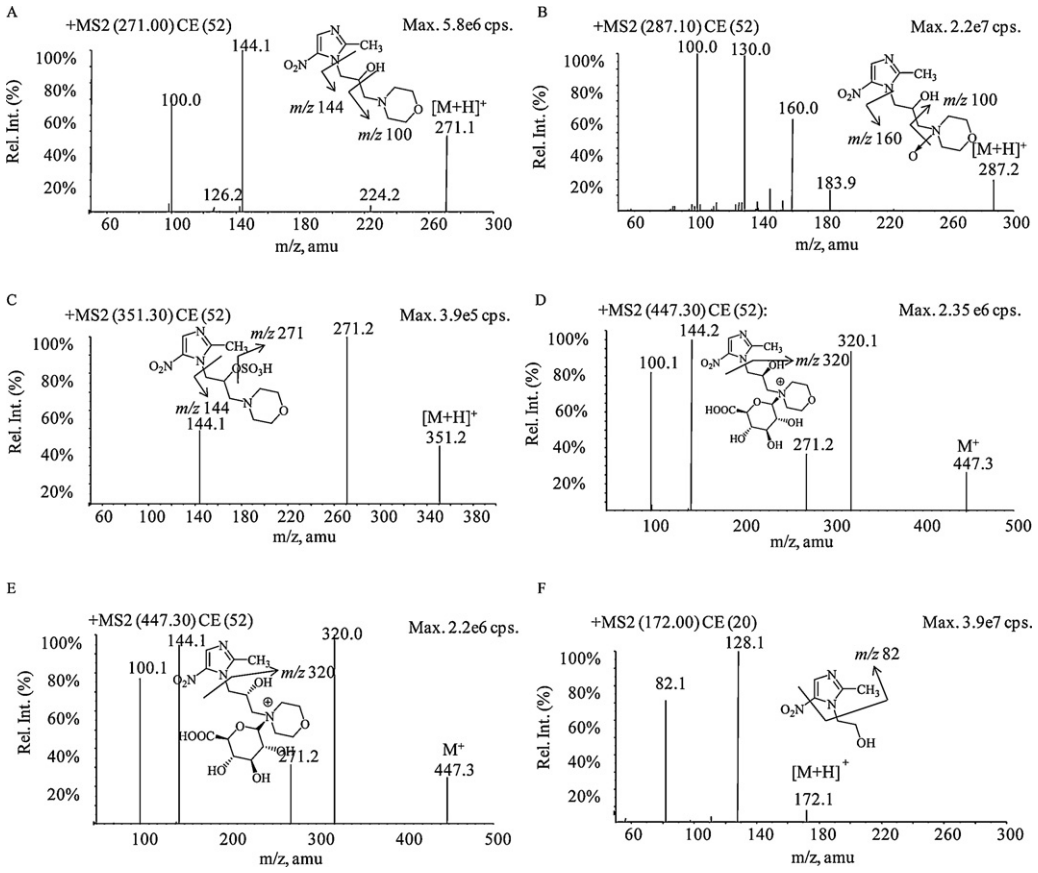

Fig. 1. Product ion mass spectra of morinidazole (A), M4-1 (B), M7 (C), M8-1 (D), M8-2 (E), and metronidazole (F).

product ions. Although the extent of in-source dissociation of M7 under ESI mode was much lower than that under APCI (with a ratio of 1:10), the risk of interference with the parent drug still existed. The chromatographic conditions were optimized to achieve adequate chromatographic separation and good peak shape for all the analytes and IS. Log P values of morinidazole, M4-1, M8-1, M8-2, and M7 were calculated to be 0.46, −1.39, −3.68, −3.68, and 0.067, respectively, using Advanced Chemistry Development ACD/Labs software, ADME Suite, Version 5.0. Based on the results, we systematically tested different column materials and mobile phases before deciding on the current Synergi^{TM} 4 μm Hydro-RP C_{18} column and the combination of 10 mM ammonium formate solution (A) and acetonitrile (B) as the mobile phase. During the early stage of method development, a small amount of acidic modifiers (0.2% formic acid) was added to the mobile phase to improve the MS response of the analytes of interest and IS. However, the retention time of M8-2 was much shorter, whereas that of M8-1 remained unchanged, resulting in the difficulty for the separation of M8-1 and M8-2. Hence, the mobile phase without formic acid was further investigated. The analytical run time under optimized chromatographic condition was 5.0 min, and the retention times of morinidazole, M4-1, M7, M8-1, M8-2, and IS were 3.2, 1.0, 2.8, 1.3, 2.1, and 1.9 min, respectively (Fig. 2).

3.3. Method validation

3.3.1. Assay selectivity and LLOQ

Fig. 2 shows typical chromatograms of a blank human plasma (A), blank plasma sample spiked with morinidazole, M4-1, M7, M8-1, and M8-2 at LLOQ concentrations and IS (B), a plasma sample from a healthy volunteer at 8 h (C), and a plasma sample (diluted before analysis) from a patient with severe renal insufficiency at 8 h (D) after the start of the intravenous infusion of morinidazole and sodium chloride injection. As shown in the figure, an adequate sensitivity was achieved, and the background was interference-free.

The LLOQ was established as 10.0 ng/mL for morinidazole, 1.00 ng/mL for M4-1, 2.50 ng/mL for M7, 3.00 ng/mL for M8-1, and 10.0 ng/mL for M8-2, respectively. The precision at LLOQ of the five analytes was between 3.1% and 13.3%, and the accuracy was between −4.6% and 3.5% (Table 2).

Based on signal-to-noise ratio of 3:1, the LOD (limit of detection) values of morinidazole, M4-1, M7, M8-1 and M8-2 were estimated as 0.7, 0.3, 1.0, 1.0 and 1.0 ng/mL, respectively.

3.3.2. Linearity of calibration curves

Calibration curves were established in the ranges of 10.0–12,000 ng/mL for morinidazole, 1.00–200 ng/mL for M4-1, 2.50–500 ng/mL for M7, 3.00–600 ng/mL for M8-1, and 10.0–3000 ng/mL for M8-2 in plasma, and were complied with the predefined acceptance criteria.

Typical equations of the calibration curves for morinidazole, M4-1, M7, M8-1, and M8-2 were $y = 3.04 \times 10^{-3}\ x + 3.66 \times 10^{-3}$ ($r = 0.9985$), $y = 2.84 \times 10^{-2}\ x + 2.54 \times 10^{-4}$ ($r = 0.9986$), $y = 5.00 \times 10^{-3}\ x + 3.87 \times 10^{-4}$ ($r = 0.9989$), $y = 2.24 \times 10^{-3}\ x + 3.02 \times 10^{-4}$ ($r = 0.9976$), and $y = 2.78 \times 10^{-3}\ x + 9.73 \times 10^{-4}$ ($r = 0.9989$), respectively, where y represents the peak area ratio of analyte to IS and x represents the plasma concentrations of each analyte.

3.3.3. Precision and accuracy

Table 2 shows the intra- and inter-batch precision and accuracy values for all the five analytes from QCs. The intra- and inter-assay precision for all compounds were less than 12.2% and 13.6%, respectively. The inter-assay mean accuracy was between −4.0%

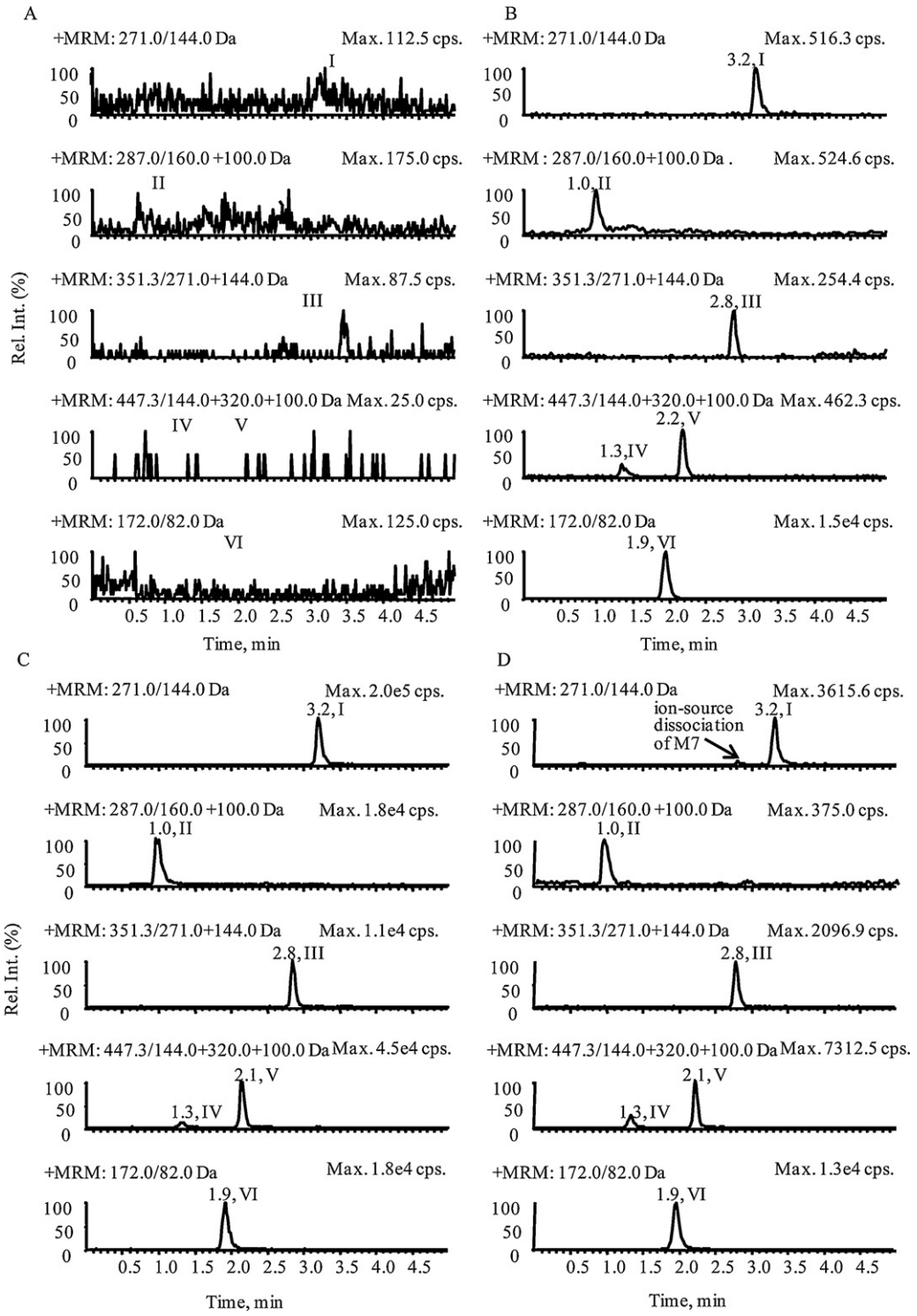

Fig. 2. Typical MRM chromatograms of morinidazole (I), M4-1 (II), M7 (III), M8-1 (IV), M8-2 (V), and metronidazole (IS, VI) in human plasma. (A) Blank plasma sample; (B) plasma spiked with 10.0 ng/mL morinidazole, 1.00 ng/mL M4-1, 2.50 ng/mL M7, 3.00 ng/mL M8-1, 10.0 ng/mL M8-2, and 500 ng/mL metronidazole (IS); (C) plasma sample from a healthy volunteer at 8 h and (D) a plasma sample (diluted before analysis) from a patient with severe renal insufficiency at 8 h after an intravenous infusion administration of 500 mg morinidazole.

and 4.4%. The precision and accuracy of dilution QC samples for all analytes were acceptable.

3.3.4. Extraction recovery and matrix effects

The mean recoveries of all analytes extracted from human plasma at three QC concentrations were almost 100% in all cases. The detail results are shown in Table 3. The mean recovery for the IS was 94.0% ± 3.9% (n = 6). The simple protein precipitation procedure showed satisfactory recovery for morinidazole and its metabolites in human plasma.

The matrix effects of morinidazole and metabolites were evaluated at two concentrations (100/2.00/5.00/6.00/20.0 and 9600/160/400/480/2400 ng/mL for morinidazole, M4-1, M7, M8-1, and M8-2, respectively). The analyte peak area ratio for the

Table 2
Accuracy and precision of the LC–MS/MS method to determine morinidazole and four metabolites in human plasma (in three consecutive days, six replicates for each day).

Analyte	Concentration (ng/mL)		R.S.D. (%)		RE (%)
	Added	Found	Intra-day	Inter-day	
Morinidazole	10.0	9.54	7.4	8.5	−4.6
	20.0	19.6	6.8	11.7	−2.0
	400	408	4.3	11.6	2
	9600	9683	3.4	13.6	0.9
M4-1	1.00	1.04	7.0	13.3	3.5
	2.00	2.08	8.5	8.1	4.0
	20.0	21.1	4.9	8.5	3.7
	160	164	4.4	9.5	2.6
M7	2.50	2.55	6.9	10.5	1.9
	5.00	5.22	9.9	8.8	4.4
	50.0	51.2	5.4	7.4	2.4
	400	400	4.0	8.3	0.1
M8-1	3.00	2.89	6.7	10.3	−3.7
	6.00	5.83	12.2	4.0	−4.0
	60.0	59.2	6.4	9.3	−1.3
	480	464	3.0	13.2	−3.3
M8-2	10.0	9.70	7.0	3.1	−3.0
	20.0	19.8	8.5	12.1	−0.8
	200	197	5.4	4.6	−1.6
	2400	2329	3.0	13.4	−3.0

extracted blank matrix spiked with solution ranged from 93.9% to 102% compared with the same nominal solution prepared in mobile phase for all analytes, and the RSD values from six lots of plasma were less than 11%. The absolute and relative matrix effects for IS were 105% and 5.1%, respectively (Table 3). The results showed that the matrix effects of morinidazole, M4-1, M7, M8-1, M8-2, and IS could be ignored under the present LC–MS/MS conditions.

3.3.5. Stability

The stability tests of morinidazole and its four metabolites were designed to cover anticipated conditions of handling typical clinical samples. As shown in Table 4, the analytes were stable under the typical plasma storage and processing conditions used throughout the current study. The conjugated metabolites were stable, and no conversions to morinidazole were observed during the sample preparation. These stability results were considered sufficient for the validation.

Table 3
Recovery and matrix effect of morinidazole, M4-1, M7, M8-1, M8-2 and IS ($n = 6$) in human plasma.

Analyte	Concentration (ng/mL)	Recovery (%)	Matrix factor (%)
Morinidazole	20.0	92.3 ± 3.8	95.0 ± 5.5
	400	92.9 ± 2.6	
	9600	101 ± 3	94.4 ± 2.4
M4-1	2.00	85.4 ± 7.2	94.9 ± 8.2
	20.0	87.0 ± 3.4	
	160	95.7 ± 3.7	102 ± 3
M7	6.00	95.8 ± 11.0	94.3 ± 5.1
	60.0	92.0 ± 4.4	
	480	100 ± 3	96.8 ± 2.6
M8-1	20.0	90.1 ± 10.6	93.9 ± 7.0
	200	95.8 ± 3.1	
	2400	98.4 ± 2.4	95.3 ± 2.6
M8-2	5.00	92.9 ± 10.7	99.4 ± 3.6
	50.0	92.9 ± 2.7	
	400	101 ± 2	96.3 ± 3.1
IS	500	94.0 ± 3.9	105 ± 5

Table 4
Summary of stability of morinidazole and four metabolites in human plasma under various storage conditions ($n = 3$).

Analyte	Added (ng/mL)	Short-term (6 h at 25 °C)		Freezing for 48 days (−20 °C)		Autosampler for 24 h (25 °C)		Three freeze–thaw cycles	
		Found (ng/mL)	RE (%)	Found (ng/mL)	RE (%)	Found (ng/mL)	RE (%)	Found (ng/mL)	RE (%)
Morinidazole	20.0	20.3 ± 0.9	1.4	19.6 ± 1.0	−2.0	19.5 ± 1.7	−2.7	22.0 ± 1.2	10.2
	9600	8404 ± 266	−12.5	9750 ± 299	1.6	8864 ± 321	−7.7	10,405 ± 694	8.4
M4-1	2.00	2.15 ± 0.16	7.7	2.00 ± 0.23	0.1	2.12 ± 0.17	5.9	1.92 ± 0.25	−3.9
	160	161 ± 5	0.9	159 ± 8	−0.7	164 ± 9	2.3	155 ± 5	−2.9
M7	5.00	4.97 ± 0.21	−7.1	5.17 ± 0.45	3.3	4.87 ± 0.23	−2.6	4.65 ± 0.23	−7.0
	400	371 ± 15	−7.3	386 ± 7	−3.4	380 ± 15	−5.0	389 ± 36	−2.8
M8-1	6.00	5.13 ± 0.43	1.4	5.52 ± 0.53	−8.0	6.12 ± 0.64	2.0	6.06 ± 0.31	1.1
	480	441 ± 15	−12.5	425 ± 17	−11.4	462 ± 20	−3.7	421 ± 14	−12.3
M8-2	20.0	19.0 ± 0.4	−4.8	20.0 ± 1.1	0.1	19.0 ± 1.4	−4.8	18.8 ± 0.6	−6.0
	2400	2338 ± 34	−2.6	2334 ± 40	−2.7	2227 ± 62	−7.2	2270 ± 79	−5.4

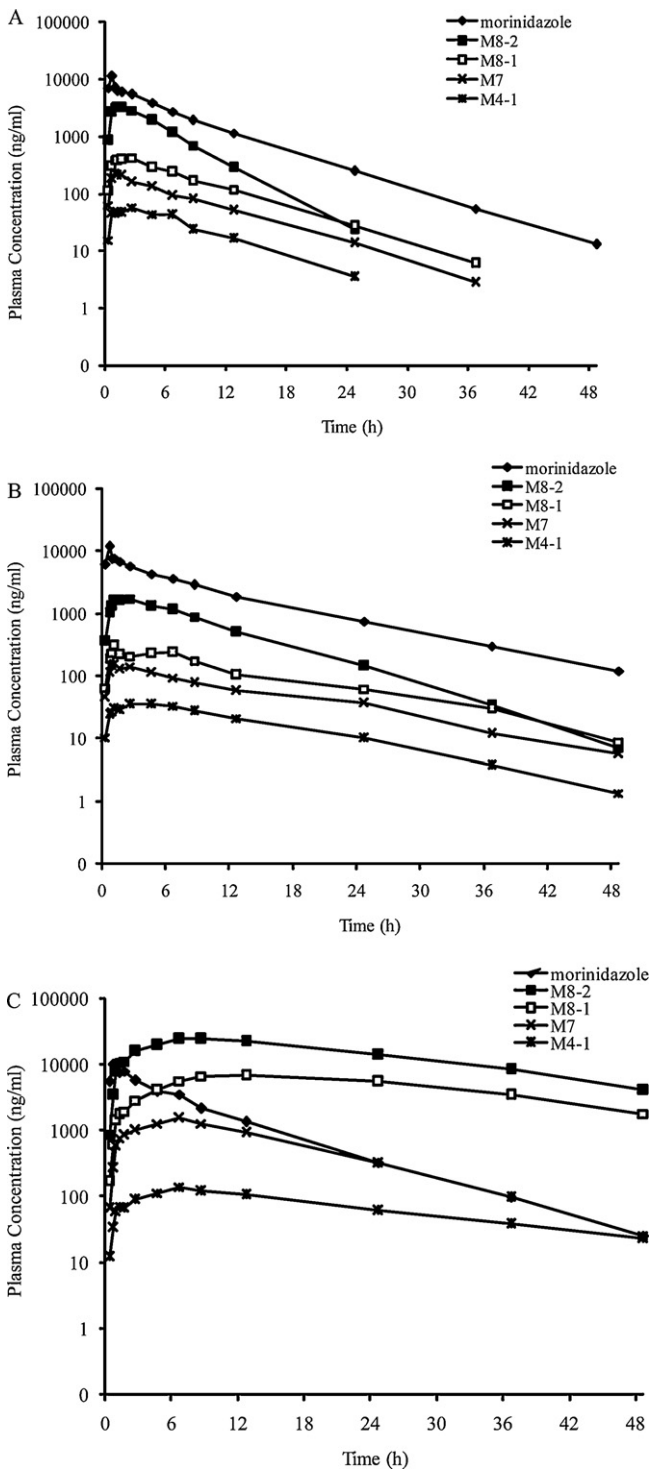

Fig. 3. Typical plasma concentration–time profiles of morinidazole, M4-1, M7, M8-1, and M8-2 after an intravenous infusion administration of 500 mg morinidazole to a healthy volunteer (A), a patient with moderate hepatic insufficiency (B), and a patient with severe renal insufficiency (C).

3.4. Application of the method to clinical pharmacokinetic studies

The validated LC–MS/MS method was successfully applied to the determination of morinidazole, M4-1, M7, M8-1, and M8-2 in human plasma after being administered with 500 mg of morinidazole by continuous intravenous infusion for 45 min to 12 healthy Chinese subjects, 12 patients with moderate hepatic insufficiency, and 12 patients with renal severe insufficiency. The method allowed the determination of morinidazole and its four metabolites up to 48 h after the termination of intravenous administration. To quantify the high concentrations of metabolites observed in plasma samples from patients with severe renal insufficiency, the samples were diluted before analysis. The plasma concentration–time curves of morinidazole and its four metabolites in a healthy subject, in a patient with moderate hepatic insufficiency, and in a patient with severe renal insufficiency are shown in Fig. 3A–C, respectively. The pharmacokinetic parameters of the parent drug and its metabolites in healthy subjects have been published previously [2].

4. Conclusion

A sensitive, accurate, and precise LC–MS/MS method was developed and validated for the simultaneous determination of morinidazole and its four metabolites, namely, *N*-oxide (M4-1), sulfate (M7), and N^+-glucuronides (M8-1 and M8-2) in human plasma. Gradient elution was used on a short column to avoid the interference from the in-source dissociation of the conjugated metabolites and to permit the separation of two diastereoisomeric N^+-glucuronides (M8-1 and M8-2). The present method has several benefits, including fast throughput (a total run of 5 min), small sample size requirement (50 μL plasma), ease of sample preparation (protein participation), and high sensitivity and selectivity (LLOQ at 10.0/1.00/2.50/3.00/10.0 ng/mL for morinidazole, M4-1, M7, M8-1, and M8-2). The method was successfully applied to characterize the pharmacokinetic profiles of morinidazole and its four metabolites after an intravenous infusion administration of 500 mg morinidazole in healthy subjects, patients with moderate hepatic insufficiency, and patients with severe renal insufficiency.

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