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Simultaneous determination of *m*-nisoldipine and its three metabolites in rat plasma by liquid chromatography–mass spectrometry

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ABSTRACT

A simple, sensitive and selective liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for the simultaneous determination of *m*-nisoldipine and its three metabolites in rat plasma has been developed using nitrendipine as an internal standard (IS). Following liquid–liquid extraction, the analytes were separated using an isocratic mobile phase on a reverse phase C_{18} column and analyzed by MS in the multiple reaction monitoring (MRM) mode. To avoid contamination by residual sample in the injection syringe, a special injection protocol was developed. We found that *m*-nisoldipine, metabolite M1 and IS could be ionized under positive or negative electrospray ionization conditions, whereas metabolite M and M2 could only be ionized in the positive mode. The mass spectrometry fragmentation pathways for these analytes are analyzed and discussed herein. The total analysis time required less than 5 min per sample. We employed this method successfully to study the metabolism of *m*-nisoldipine when it was orally administered to rats at a dose of 9 mg/kg. Three metabolites of *m*-nisoldipine and an unknown compound of molecular weight 386 were found for the first time in rat plasma. The concentration of the potentially active metabolite was approximately equal to its parent compound concentration.

1. Introduction

m-Nisoldipine (Fig. 1) is a new dihydropyridine calcium channel antagonist that was first created in the School of Pharmacy at Hebei Medical University [1]. This compound is stable in its solid form when exposed to light, whereas nisoldipine loses most of its activity after 30 min of light exposure [2]. This new compound has many advantages including an increased cardiac output and cardiac index when studied in rabbits and a less negative inotropic effect on myocardium [3,4]. According to the study, *m*-nisoldipine was not found in bile and urine and was found to be of an altered form [5]. In other words, *m*-nisoldipine was excreted mainly as metabolites. Therefore, it is essential to study the metabolites of *m*-nisoldipine to better understand its metabolic pathways.

To our knowledge, the metabolism of *m*-nisoldipine has not yet been studied. However, the metabolism of other 1,4-dihydropyridines (DHPs) has also been described [6–9]. The common metabolic pathway of these compounds in humans and animals involves the oxidation of the dihydropyridinic ring followed by the hydrolysis of the molecules' ester moieties. However, the metabolites produced by these biotransformations have also been discovered in the degradation process of DHPs [10]. Therefore, to reflect the real metabolic level of DHPs, analysis the metabolites of DHPs require complete degradation prevention during the analytical process or analysis the metabolites which are not the degradation products. In this study, two metabolites of *m*-nisoldipine (M1 and M2, Fig. 1) that were transformed by *Rhizopus arrhizus* AS 3.2896 [11], were found in rat plasma. According to the literature, M1 is likely the only active metabolite [6]. These identified metabolites (especially M1) that were not the degradation products, reflect the real metabolic level of *m*-nisoldipine.

Various analytical approaches to prepare biomatrices and determine the concentrations of DHPs in biological samples have been published [12–16]. However, the methods of HPLC-UV and HPLC-PDA are neither selective nor sensitive because it is difficult to separate DHPs and dehydro-DHPs. Thus, these methods are not suitable for the simultaneous determination of low concentrations of DHPs and their metabolites. The sensitive and specific method of liquid chromatography–tandem mass spectrometry (LC–MS/MS) is a preferred tool for determining DHP levels and analyzing their

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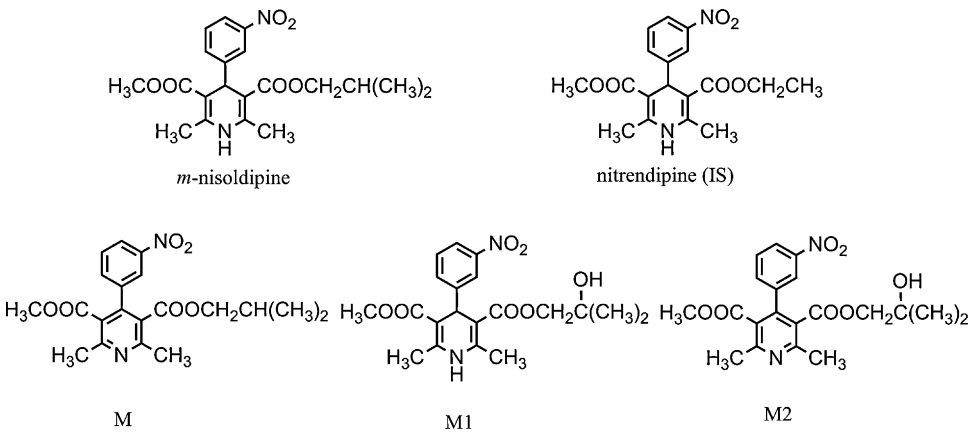

Fig. 1. Chemical structures of analytes and nitrendipine (IS).

metabolites in biological samples. We have successfully used this latter method to analyze *m*-nisoldipine in rat plasma [17]. DHPs could be ionized under positive or negative conditions with the signal response of $[M+H]^+$ or $[M-H]^-$ when ESI (Electrospray Ionization), a soft ionization technique of LC–MS/MS, was employed. The basic fragmentation of DHPs was followed by cleavage of the C–O bond of $[M+H]^+$, neutral loss of O–R on the side chain. Usually, in the negative product-ion mass spectrum of DHPs, the most abundant ion was at $m/z = 122.0$. However, the fragmentation of the metabolites of *m*-nisoldipine was different, and it will be discussed in this paper.

In this study, we developed a rapid and sensitive LC–MS/MS method for simultaneous analysis *m*-nisoldipine and its three metabolites in rat plasma using liquid–liquid extraction. Moreover, this method was successfully employed in the study of the metabolism of *m*-nisoldipine.

2. Experimental

2.1. Chemicals, reagents and materials

The standard samples of *m*-nisoldipine and M were kindly provided by Professor Du (School of Pharmacy, Hebei Medical University, Shijiazhuang, China). M1 and M2 were obtained by microbial biotransformation [11] (HPLC purity >97%). Nitrendipine (IS) was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). HPLC grade acetonitrile (Fisher, USA) was used for HPLC analysis and biological sample preparation. Deionized water was produced by a Heal Force-PWVF purification system (Shanghai CanRex Analyse Instrument Co., China). Analytical grade diethyl ether was purchased from Tianjin Chemical Corporation, China. Formic acid, acetic acid and ammonium acetate (HPLC grade) were purchased from the Dikma Technologies Inc.

2.2. Equipment and chromatographic conditions

2.2.1. Liquid chromatography

An Agilent 1200 liquid chromatography system (Agilent, USA) equipped with a quaternary solvent delivery system, an autosampler and a column compartment was used for all analyses. The chromatographic system consisted of a Symmetry RP-C18 analytical column (150 mm × 4.6 mm i.d., 5 μm, Waters, PN: WAT045905), and an isocratic mobile phase of acetonitrile:water (72:28, v/v) was used at a flow rate of 0.80 ml/min. The column was operated at room temperature, and a divert valve was used to divert the eluent to waste from 0 min to 2.5 min. A special injection program was used in which the sample syringe was first washed twice in an acetonitrile:water (90:10, v/v) solution. Then, the syringe drew 10 μl from the sample, washed the syringe again, and injected the sample. This protocol was performed to avoid contamination from the residual sample. The total analysis time required only 5 min for each run.

2.2.2. Mass spectrometer

Detection was performed using a 3200 QTRAP™ system, a hybrid triple quadrupole linear ion trap mass spectrometer equipped with Turbo V sources, and a TurboIonspray interface from Applied Biosystems/MDS Sciex (Applied Biosystems, Foster City, CA, USA). For all compounds, the MS instrument was operated in the electrospray ionization (ESI) source and the data were acquired in the multiple reaction monitoring (MRM) mode. The turbo spray temperature was maintained at 650 °C. Nebulizer gas (gas 1) and heater gas (gas 2) were set at 65 psi and 60 psi, respectively. The curtain gas was kept at 30 psi, and the interface heater was kept on. Nitrogen was used as the collision gas. The precursor-to-product ion pairs, declustering potential (DP), collision energy (CE), entrance potential (EP), ion spray voltage (IS) and cell exit potential (CXP) for each analyte are shown in Table 1. Instrument control and data acquisition were carried out using the Applied Biosystems/MSD Sciex Analyst software (version 1.4.2).

2.3. Preparation of the standard solution and quality control samples

The appropriate amounts of *m*-nisoldipine, M, M1, and M2 were separately weighed and dissolved in acetonitrile to make stock solutions. These four stock solutions were then mixed and diluted with the same diluent to prepare final mixed standard solutions containing 0.4 μg/ml of M, 1.6 μg/ml of *m*-nisoldipine, M1 and M2. A series of working solutions of these analytes were freshly prepared by diluting the mixed standard solution at appropriate ratios to yield concentrations of 0.5, 1, 2, 4, 8, 40 ng/ml for M, 2, 4, 8, 16, 32, 160 ng/ml for *m*-nisoldipine, M1 and M2. Nitrendipine was diluted in an acetonitrile:water (80:20, v/v) solution to obtain an IS solution of 100 ng/ml. To validate this method, three concentration levels of standard solutions containing M (1, 4, 40 ng/ml), *m*-nisoldipine, M1 (4, 16, 160 ng/ml), and M2 (4, 16, 160 ng/ml) were used to prepare the quality control (QC) plasma samples. All solutions were protected from light to prevent any possible light degradation.

2.4. Preparation of plasma samples

Plasma samples (200 μl) were used in the liquid–liquid extraction (LLE) after addition of the appropriate IS solutions (20 μl). The

Table 1
MS/MS transitions and optimal conditions used for MS/MS analysis.

Compound	Precursor ion (*m*/*z*)	Daughter ion (*m*/*z*)	DP (V)	CE (eV)	EP (V)	IS V	CXP (V)	RT window (min)
M1	403.20	208.00	−45.00	−26.00	−10.00	−4500.00	−5.00	3.06–3.21
M2	403.30	331.30	50.00	33.00	10.00	5500.00	3.00	3.20–3.39
Nitrendipine (positive mode)	361.30	315.20	23.00	16.00	10.00	5500.00	3.00	3.85–3.98
Nitrendipine (negative mode)	359.20	121.90	−41.00	−25.00	−10.00	−4500.00	−5.00	3.85–3.98
m-Nisoldipine	387.20	121.90	−38.00	−27.00	−10.00	−4500.00	−5.00	4.46–4.61
M	387.30	331.30	47.00	20.99	10.00	5500.00	3.00	4.64–4.82

mixture was vortexed for 20 s and allowed to stand at room temperature for 2 min. The quantity of 800 μl of diethyl ether was added, and the samples were vortexed for 90 s. Next, the upper layer was transferred to a clean tube, and the solvent was evaporated using N_2 (40 °C). The dry residue was redissolved with 200 μl of mobile phase acetonitrile:water (72:28, v/v). The samples were transferred into amber microvials, capped and placed in the autosampler.

2.5. Method validation

The calibration plasma samples were prepared by adding 20 μl of a series of working solutions and 20 μl of the IS solutions to 200 μl of blank plasma. These samples were then extracted as described in Section 2.4. The calibration curve was then generated and consisted of six concentration levels. Each sample was prepared and assayed in duplicate on separate days. Calibrations curves were constructed using the analyte and the IS peak-area ratios via a weighted ($1/x^2$) least-squares linear regression of the form of $y = bx + a$. Unknown sample peak-area ratios were then interpolated from the calibration curve to provide the analyte concentrations.

LLOQ was determined as the lowest concentration point of the standard curve. This is the point on the curve at which the concentration can be reliably and reproducibly measured over at least five replicates. The limit of detection (LOD) was defined as the amount of analyte that could be detected with a signal-to-noise ratio of three.

Three validation batches, each containing five replicates of QC samples at low, medium and high concentration levels, were assayed to assess the precision and accuracy of this method on three different days. The precision is expressed as the relative standard deviation (RSD) between the replicate measurements. Accuracy is defined as the relative error (RE) and is calculated using the formula RE % = [(measured value − theoretical value)/theoretical value] × 100.

The extraction recovery of the analytes of three QC samples was conducted by calculating the ratio of the peak areas of the extracted blank plasma spike before extraction relative to the peak areas of the equivalent blank plasma sample spike after extraction.

Short-term stability, long-term stability, autosampler stability and freeze–thaw cycle stability were assessed by analyzing three QC levels over five replicates. The QC samples were analyzed after storage at room temperature for 2 h, after storage at −20 °C for 1 month, after being placed in the autosampler at room temperature for 8 h post liquid–liquid extraction, and after three freeze–thaw cycles consisting of storage at −20 °C for a minimum of 12 h followed by thawing at room temperature. Deterioration of each analyte was defined as a greater than 15% difference in the tested sample versus the control at the nominal sample concentration.

The matrix effect (ME) was studied by analyzing three QC levels using five different drug-free plasma pools. Two sets of samples were prepared. Set A consisted of a set of neat quality control standards injected directly into the LC–MS. Set B consisted of a set of drug-free plasma samples extracted by LLE and then spiked with the quality control standards and finally injected into the LC–MS. The matrix effect was obtained by comparing the concentrations of the analytes found in Set B with those in Set A. The ME was calculated using ME % = Set B/Set A × 100 [18].

2.6. Animals and sampling

Male Sprague–Dawley (SD) rats (Certificate No. DK 0908083, weighting 250 ± 50 g) were obtained from the Experimental Animals Center of Hebei Province (Shijiazhuang, China). They were kept in an environmentally controlled breeding room for 3 days before starting the experiments, and they were fed with standard laboratory food and water ad libitum. For 12 h prior to dosing, the rats were starved to reduce the impact of food in the stomach and small intestine on absorption. Suspensions of *m*-nisoldipine (4 mg/ml) were prepared in 0.5% CMC-Na (sodium carboxymethylcellulose) by shaking for 5 min until a uniform solution formed. For pharmacokinetic studies, the prepared suspension was orally administrated to the five rats at a dose of 9 mg/kg. Blood samples (0.5 ml) were obtained from each rat at 15, 30, 60, 90, 120, 180, 300, 480, 720 and 1440 min after drug administration and collected in heparinized centrifuge tubes. Then, the samples were centrifuged at 10,000 rpm for 5 min, and the separated plasma was frozen at −20 °C prior to analysis.

3. Results and discussion

3.1. LC–MS–MS optimization

To increase the selectivity and sensitivity of the liquid–liquid extraction procedure, the mass spectrometry and the chromatography conditions were optimized. According to references [19–21], diethyl ether, ethyl acetate and hexane are the common reagents used for the extraction of 1,4-DHPs from plasma samples. In our study, although the mixture of diethyl ether or ethyl acetate with hexane gave cleaner extracts than the use of diethyl ether or ethyl acetate in their pure forms, diethyl ether was chosen as the solvent for the extraction of *m*-nisoldipine and its metabolites due to the high recoveries obtained for all compounds when it was used as the solvent. In addition, the use of diethyl ether resulted in cleaner extracts when compared to ethyl acetate extraction. Following this optimization, the volume of organic solvent and the vortex time were examined. The LLE method used in the present study is described in Section 2.4.

To identify the major species formed in the sequential fragmentation of the MS/MS analysis, a mass characterization study was first performed for the direct syringe pump infusion (flow rate 10.0 μl/min) of solutions of each compound (10.0 μg/ml in methanol). The parameters in the collision cell were optimized in both positive and negative ion mode. It was found that *m*-nisoldipine, M1 and IS could be ionized under positive or negative electrospray ionization conditions, whereas M and M2 could only be ionized in the positive mode. The product ion scan spectra of each compound in positive or negative mode are shown in Fig. 2. The positive electrospray mass spectra for both metabolites, M and M2, showed similar fragmentation (Fig. 3) and had the same base peak ions at *m*/*z* = 331.2. Capillary voltages, cone voltages and col-

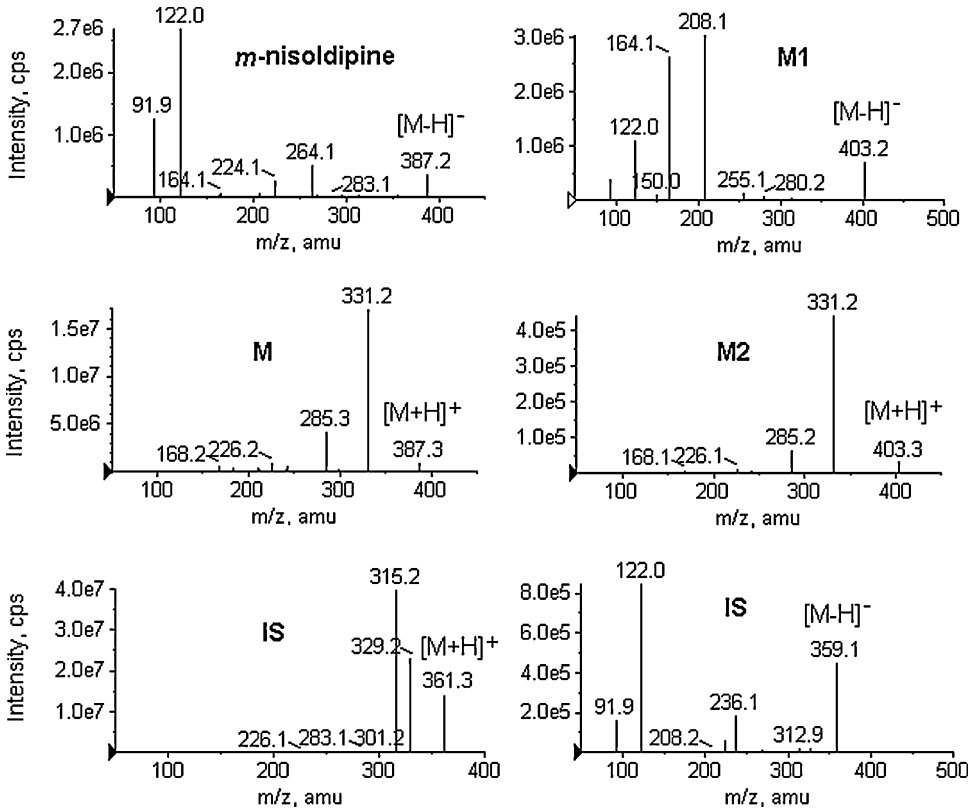

Fig. 2. The product ion scan spectra of each compound in the positive and negative modes.

lision energies were optimized to obtain the greatest intensity of the most abundant product ion. These parameters were used for further MS/MS experiments.

In the negative electrospray mass spectra, the three 3,5-pyridinedicarboxylate compounds (*m*-nisoldipine, M1 and IS) were found to utilize the same degradation pathway to eliminate nitrobenzene. Fig. 4 shows the fragmentation patterns of the three compounds. However, it seems that the existence of a hydroxyl in the alcohol moiety of the side chain ester group complicated the degradation pattern of the whole molecule. Although there is a little difference in the structures of *m*-nisoldipine and M1, their degradation patterns were dissimilar. Specifically, the product ion of $m/z = 122.0$ dominated the *m*-nisoldipine spectrum, while $m/z = 208.1$ was dominant in the spectrum of M1 after optimization. Using positive ESI, we observed that these samples predominantly formed protonated quasimolecular ions $[M+H]^+$ in the full scan spectra, with a $m/z = 389.4$ for *m*-nisoldipine, a $m/z = 405.4$ for M1 and a $m/z = 361.3$ for IS. The most abundant ion in the product-ion

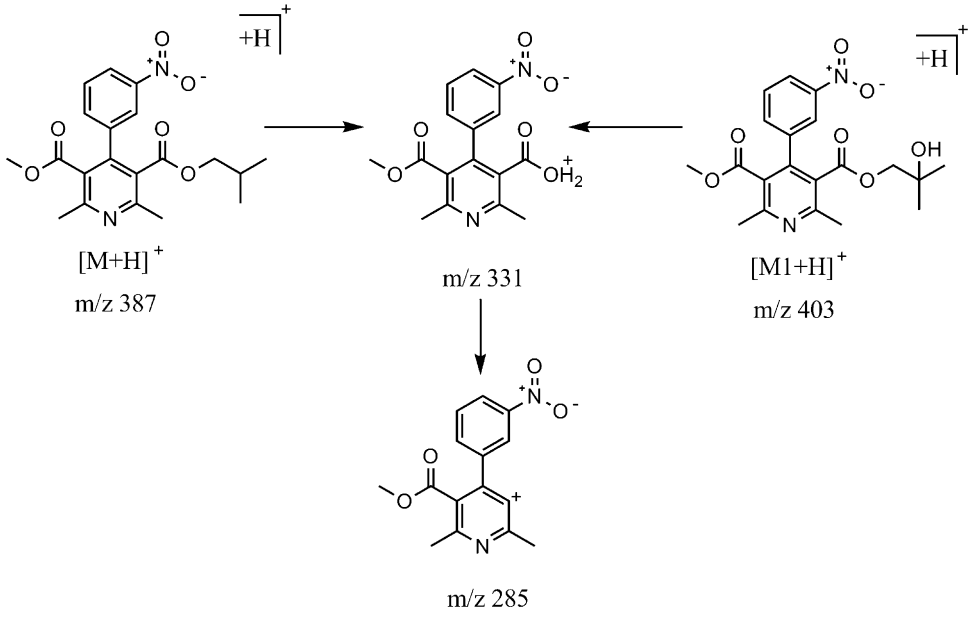

Fig. 3. Proposed mass fragmentation pathways for M and M2 in the positive mode.

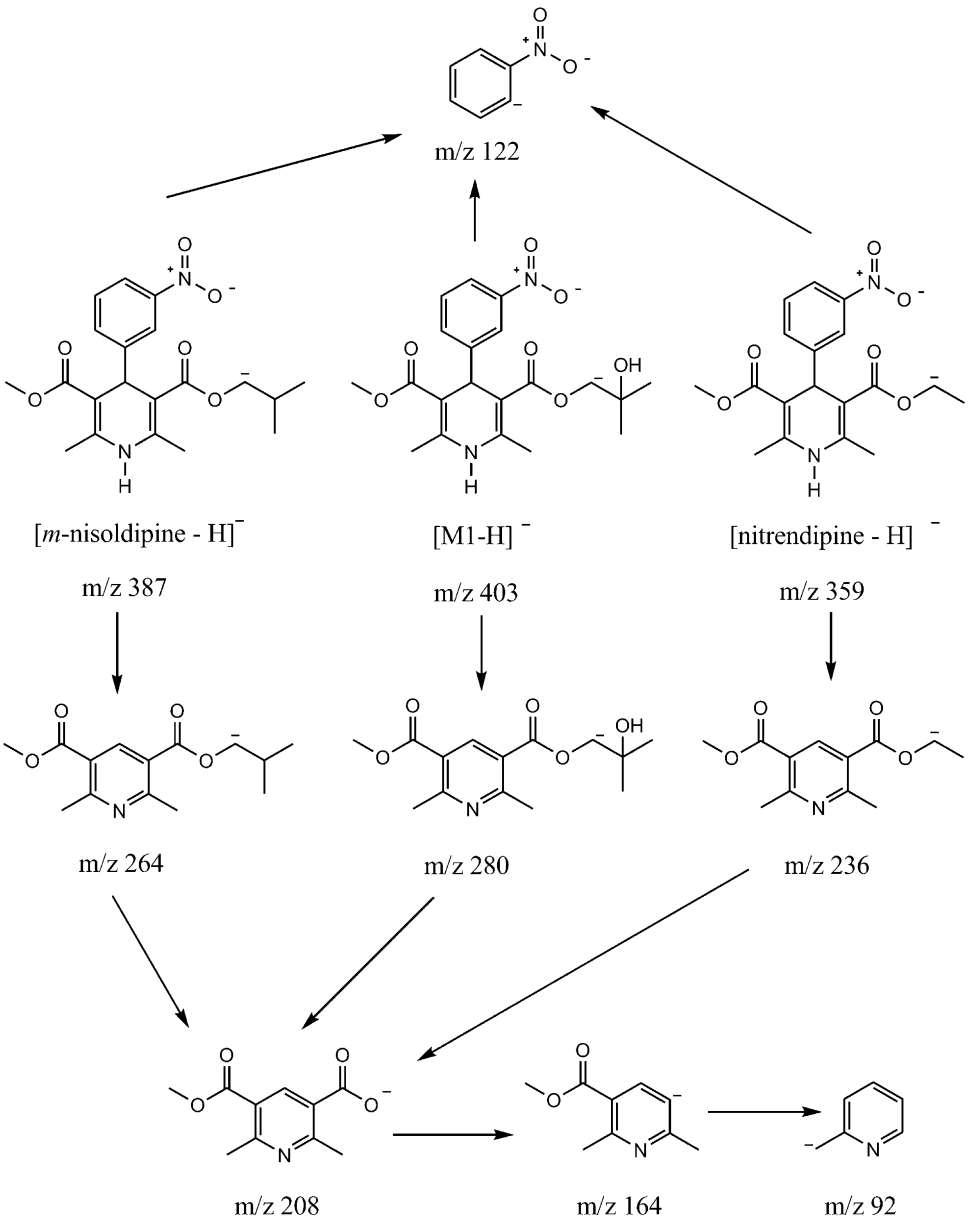

Fig. 4. Proposed mass fragmentation pathways for *m*-nisoldipine, M1 and nitrendipine (IS) in the negative mode.

mass spectrum was at $m/z = 315.2$ in all cases. However, the MRM response under the optimized conditions of the negative response was higher than the positive response. Another advantage of negative MRM was its low background noise, and signal-to-noise (S/N) ratio was 6-fold higher compared to positive ion mode. Therefore, we chose to use the negative ion mode to analyze *m*-nisoldipine and M1. The precursor transitions to product ions were as set as follows: $m/z = 387.2–121.9$ for *m*-nisoldipine and $m/z = 403.2–208.0$ for M1.

In our earlier study, we found that a mobile phase consisting of acetonitrile and water can reduce the chromatographic separating time and promote the formation of a good peak shape [17]. When the percentage of acetonitrile in the mobile phase reached 72%, we observed that the retention time for all compounds was between 3.06 min and 4.82 min. Further, the endogenous compounds that may cause matrix effects eluted within 3 min. Several mobile phase additives were investigated including formic acid, ammonium formate, acetic acid, and ammonium acetate. However, these additives did not cause a better response than pure water. Therefore, a mobile phase consisting of a acetonitrile:water (72:28, v/v) solution was selected, and the LC effluent was sprayed into the mass spectrometer interface between 2.5 min and 5.5 min with a ten-way valve. The LC effluent was discarded as waste between 0 min and 2.5 min to reduce any potential endogenous interference. Chromatograms from a drug-free rat plasma sample, a rat plasma sample spiked with 0.05 ng/ml of M and a rat plasma sample spiked with 0.2 ng/ml of the other three analytes are shown in Figs. 5 and 6.

3.2. Method validation

3.2.1. Selectivity

The selectivity of the method was tested by comparing the chromatograms of five different batches of blank plasma samples and the spiked plasma samples. Figs. 5 and 6 demonstrate typical chromatograms of a blank plasma sample, a blank plasma sample spiked at LLOQ with IS, and a test plasma sample obtained 3 h after administration of *m*-nisoldipine to the rat. All blank plasma samples were found to be free of interferences originating from the compounds of interest.

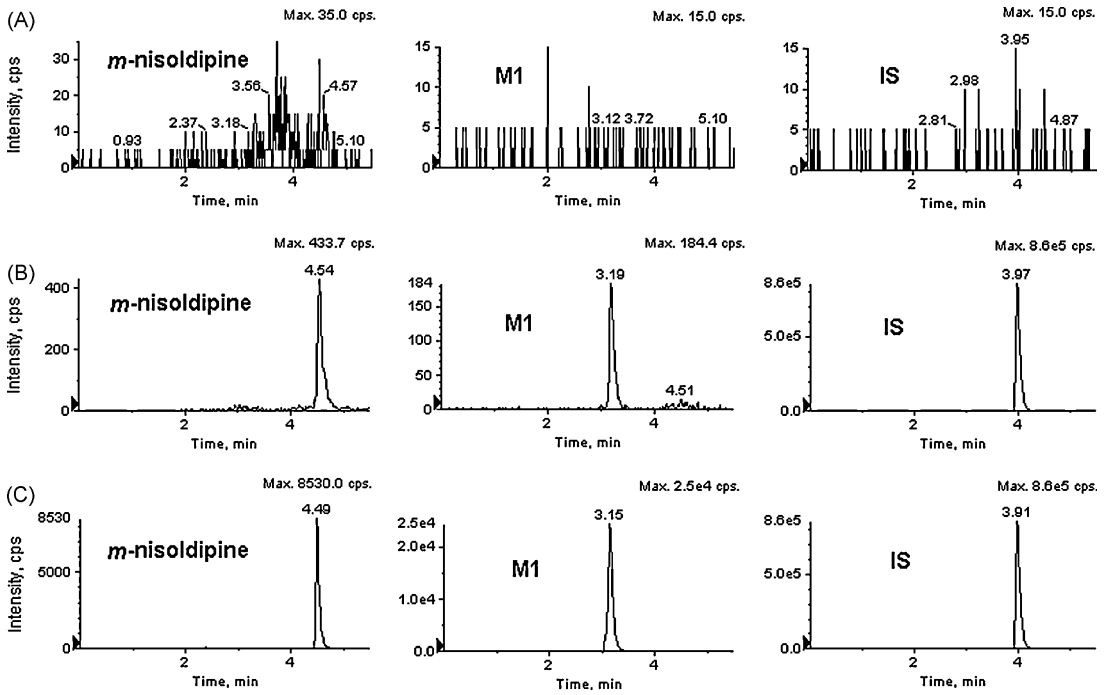

Fig. 5. Chromatograms of *m*-nisoldipine and M1 in negative multiple reaction monitoring (MRM) scan mode. (A) A blank plasma sample, (B) a blank plasma sample spiked with 0.2 ng/ml *m*-nisoldipine, 0.2 ng/ml M1 and 10 ng/ml IS, and (C) a rat plasma sample collected 3 h after the oral administration of a racemic mixture of *m*-nisoldipine.

3.2.2. Calibration curve and LOD

Under the optimized conditions, the calibration curves were obtained. The regression equation of the calibration curves and their correlation coefficients (r^2) are shown in Table 2. The difference between the nominal standard concentration and the back-calculated concentrations determined from the weighted ($1/x^2$) linear regression line varied from −18.1% to +13.2% for each point on the five calibration curves of *m*-nisoldipine, −11.6% to +10.8% for those of M, −10.7% to +12.9% for those of M1, and −14.3% to +17.8% for those of M2. All calibration curves were found to be suitable for the analysis of rat serum.

3.2.3. Precision, accuracy and stability

For *m*-nisoldipine and its metabolites, intra-day and inter-day precisions were evaluated using three levels of QC samples on three sequential runs with five replicates. All QC levels for the four analytes had intra-day and inter-day RSDs that did not exceed 9.3%. The deviations from the expected concentrations (as a measurement of

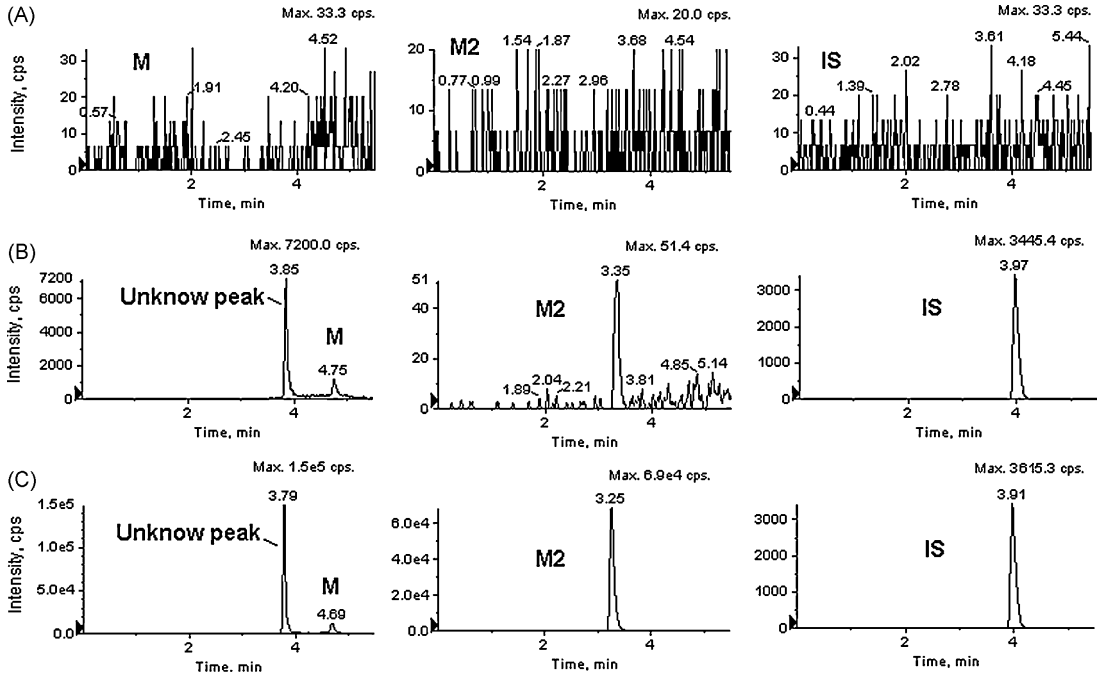

Fig. 6. Chromatograms of M and M2 in positive multiple reaction monitoring (MRM) scan mode. (A) A blank plasma sample, (B) a blank plasma sample spiked with 0.05 ng/ml M, 0.2 ng/ml M2 and 10 ng/ml IS, and (C) a rat plasma sample collected 3 h after the oral administration of *m*-nisoldipine.

Table 2
The regression equations and limits of detection of the measured compounds.

Measured compound	Regression equation	Linear range (ng/ml)	r^2	LOD (ng/ml)
m-Nisoldipine	$y = 0.0164x + 0.0006$	0.2–16	0.9968	0.06
M	$y = 0.6970x + 0.0263$	0.05–4	0.9923	0.002
M1	$y = 0.0122x - 0.0016$	0.2–16	0.9987	0.06
M2	$y = 0.5855x - 0.0765$	0.2–16	0.9979	0.005

Table 3
Stability results of the analytes in spiked plasma samples ($n = 5$).

Analytes		Short-term stability (2 h at room temperature)			Long-term stability (1 month at −20 °C)			Autosampler stability (8 h at room temperature)			Freeze–thaw stability (three cycles)		
		Mean ± SD (ng/ml)	RSD (%)	RE (%)	Mean ± SD (ng/ml)	RSD (%)	RE (%)	Mean ± SD (ng/ml)	RSD (%)	RE (%)	Mean ± SD (ng/ml)	RSD (%)	RE (%)
m-Nisoldipine	LQC	0.429 ± 0.013	6.6	−3.3	0.376 ± 0.078	5.6	−3.5	0.412 ± 0.016	7.0	5.6	0.389 ± 0.021	7.5	6.3
	MQC	1.58 ± 0.11	5.4	−2.0	1.53 ± 0.14	4.9	−1.0	1.64 ± 0.13	6.2	5.4	1.59 ± 0.23	7.2	4.1
	HQC	16.45 ± 1.76	4.8	−3.9	15.65 ± 2.24	3.6	−4.3	16.45 ± 1.76	4.7	2.6	15.85 ± 1.79	5.3	0.8
M	LQC	0.129 ± 0.012	5.8	3.4	0.091 ± 0.010	5.0	−3.9	0.118 ± 0.015	5.0	8.1	0.132 ± 0.017	8.2	2.7
	MQC	0.417 ± 0.008	5.6	−2.0	0.415 ± 0.019	4.4	7.6	0.408 ± 0.017	5.7	3.8	0.389 ± 0.014	7.1	−0.9
	HQC	4.42 ± 0.69	4.2	3.7	4.56 ± 0. 96	3.9	−0.5	4.22 ± 0.47	4.3	0.7	3.92 ± 0.80	5.3	−5.4
M1	LQC	0.412 ± 0.010	4.9	8.4	0.415 ± 0.014	8.1	3.6	0.381 ± 0.021	4.0	−5.2	0.397 ± 0.012	4.9	0.9
	MQC	1.61 ± 0.09	3.5	−2.2	1.59 ± 0.06	3.3	−0.4	1.74 ± 0.17	6.2	5.7	1.58 ± 0.26	6.0	−1.5
	HQC	16.32 ± 2.43	7.1	10.5	18.26 ± 2.01	6.1	8.4	16.92 ± 2.34	5.8	2.7	14.85 ± 1.40	4.1	5.9
M2	LQC	0.411 ± 0.019	5.1	7.1	0.404 ± 0.016	3.7	−0.7	0.477 ± 0.030	8.1	4.8	0.406 ± 0.043	3.8	−0.6
	MQC	1.64 ± 0.07	4.7	7.6	1.57 ± 0.05	7.9	−2.4	1.69 ± 0.26	6.3	6.9	1.54 ± 0.32	8.4	−3.8
	HQC	15.73 ± 3.06	8.1	−5.4	15.78 ± 2.12	5.4	−4.6	17.76 ± 4.57	6.7	−7.8	15.28 ± 4.11	6.0	−3.5

accuracy) ranged from −9.1% to +3.9%, −4.7% to +6.2%, −7.3% to +2.4% and −6.9% to +5.5% for *m*-nisoldipine, M, M1 and M2, respectively. These results are within our acceptance criteria and indicate that the method is accurate, reliable and reproducible.

Although LC–MS/MS methods generally reduce the need for sample clean up procedures because of their inherent selectivity and sensitivity, the duration of time required for sample processing was not short enough to avoid instability issues. Therefore, analyte stability during sample transport, storage and preparation is of concern in the interpretation of concentration analyses of drugs and their metabolites. In our study, QC plasma samples subject to short-term storage, long-term storage, storage in the autosampler, and three freeze–thaw cycles were examined for their stability, and the results are summarized in Table 3. As can be seen from Table 3, there were no significant differences (RSDs between 3.3% and 8.4%, REs between −7.8% and +10.5%) in the assay concentrations at any QC level following the above conditions. This indicates that *m*-nisoldipine and its three metabolites in our plasma samples were stable.

3.2.4. Matrix effects and extraction recovery

The matrix effects are generally caused by coeluting, i.e., the process of undetected matrix components reducing or enhancing the ion intensity of the analytes and thus affecting the reproducibility and accuracy of the assay. In this paper, the mean absolute matrix effect values obtained for *m*-nisoldipine were 96.1 ± 2.3%, 94.3 ± 4.6% and 90.8 ± 4.5% at concentrations of 0.4, 1.6, and 16 ng/ml, respectively ($n = 5$). The values for M were 81.2 ± 4.1%, 85.7 ± 3.2% and 79.2 ± 3.9% at levels of 0.1, 0.4 and 4 ng/ml, respectively ($n = 5$). The values for M1 were 98.5 ± 6.1%, 97.1 ± 6.8% and 92.2 ± 4.9% at levels of 0.4, 1.6 and 16 ng/ml, respectively ($n = 5$). The absolute matrix effect values for M2 were 79.7 ± 7.1%, 82.4 ± 5.5% and 83.2 ± 2.9% at levels of 0.4, 1.6 and 16 ng/ml, respectively ($n = 5$). These results suggested that the effect of ionization suppression from M and M2 was larger than the effects from *m*-nisoldipine and M1. The reason for this difference may be that M and M2 were detected in positive mode, whereas *m*-nisoldipine and M1 were detected in the negative mode. The extraction recovery of M was 87.4 ± 5.9% at a concentration of 0.4 ng/ml ($n = 5$). The extraction recoveries of *m*-nisoldipine, M1 and M2 were 85.3 ± 6.1%, 80.7 ± 5.4% and 86.4 ± 4.1% at a constant concentration of 1.6 ng/ml ($n = 5$).

3.3. Application of the method

The method described above has successfully been applied to analyze plasma samples obtained from rats that were orally administered single doses of *m*-nisoldipine (9 mg/kg). *m*-Nisoldipine and its three metabolites were detected at all time points over the duration of 0.25–24 h. The mean plasma concentration time profiles for *m*-nisoldipine and its metabolites after oral administration of *m*-nisoldipine are presented in Fig. 7. The metabolite M1 (the only potential active metabolite) was found in plasma at concentrations that were approximately equal to the concentration of its parent compound after *m*-nisoldipine administration. An unknown peak of molecular weight 386 was observed when we were attempting to detect the precursor product ion transitions between $m/z = 387.3$ and $m/z = 331.3$ using MRM in the positive ion mode. This indicated that the drug may have been degraded or biotransformed into another compound. This other compound could be an isomer of M. However, the chemical structure of the unknown peak warrants further investigation.

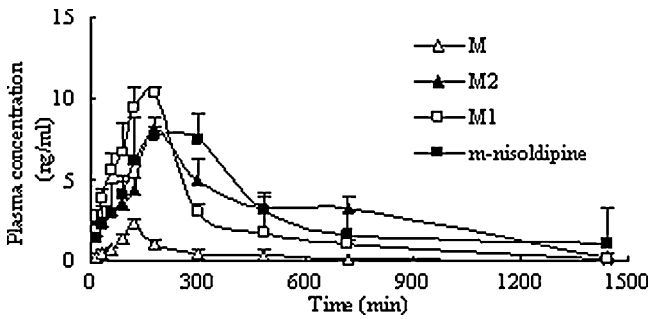

Fig. 7. Mean plasma concentration time curves of *m*-nisoldipine in rats after oral administration of *m*-nisoldipine. Each point represents the mean ± SD ($n = 5$).

4. Conclusion

A sensitive and selective LC–MS/MS method has been developed and validated for the simultaneous determination of the concentrations of *m*-nisoldipine and its three metabolites in rat plasma. Plasma samples were pretreated with one-step liquid–liquid extraction and analyzed using an isocratic LC condition. The time needed for individual sample analysis was only 5 min, which makes this an attractive procedure for the high-throughput bioanalysis of *m*-nisoldipine and its metabolites. To avoid contamination by residual sample, a modified injection protocol was designed. We found that *m*-nisoldipine, M1 and IS could be ionized under positive or negative electrospray ionization conditions, whereas M and M2 could only be ionized in the positive ion mode. This method was successfully employed to study the metabolism of *m*-nisoldipine administered to rats at a dose of 9 mg/kg. Three metabolites of *m*-nisoldipine and an unknown compound (molecular weight 386) were found for the first time in rat plasma, and the concentration of the potential active metabolite M1 was approximately equal to its parent compound. This study provides information that is relevant for the study of the metabolism of other 1,4-dihydropyridines.

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