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Validated LC–MS–MS method for determination of *m*-nisoldipine polymorphs in rat plasma and its application to pharmacokinetic studies

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Abstract

A sensitive and specific liquid chromatography–tandem mass spectrometric (LC–MS–MS) method has been developed to determine *m*-nisoldipine in rat plasma. Sample was pretreated by a single-step protein precipitation with acetonitrile, in contrast to the liquid–liquid procedure frequently used for the extraction of 1,4-dihydropyridines from biologic samples. Separation of analyte and internal standard (I.S.) was performed on a Symmetry RP-C_{18} analytic column (50 mm × 4.6 mm, 3.5 μm) with a mobile phase consisting of acetonitrile–water (80:20, v/v) at a flow rate of 0.5 ml/min. The API 4000 triple quadrupole mass spectrometer was operated in multiple reaction monitoring (MRM) scan mode using TurboIonSpray ionization (ESI) source. The method was sensitive with a lower limit of quantification (LLOQ) of 0.2 ng/mL, with good linearity ($r \geq 0.9982$) over the linear range of 0.2–20 ng/mL. All the validation data, such as accuracy, precision, and inter-day repeatability, were within the required limits. The method was successfully applied to pharmacokinetic and relative bioavailability studies of *m*-nisoldipine polymorphs in rats.

Keywords: *m*-Nisoldipine; Polymorphs; Liquid chromatography–tandem mass spectrometry

1. Introduction

Among the various drugs currently available for the treatment of systematic hypertension, the calcium channel antagonists (CCAs) continue to receive much attention as a result of their benefits in the prevention of cardiovascular events and other complications. *m*-Nisoldipine (Fig. 1), as a new dihydropyridine calcium ion antagonist, was first composed in School of Pharmacy, Hebei Medical University [1]. It is stable in the form of solid when exposed to light, while nisoldipine almost lost activity under lighting condition for 30 min [2]. A comparison with nisoldipine showed that *m*-nisoldipine increases cardiac output and cardiac index significantly and equally effect on rabbits in decreasing mean arterial blood pressure and increasing regional blood flow [3]. The negative inotropic effect of *m*-nisoldipine on myocardium was dramatically less potent than that of nisoldipine. As a result, *m*-nisoldipine has relatively higher selectivity on the thoracic aorta than nisoldipine [4]. In the procedure of synthesis, A and B polymorphs of *m*-nisoldipine with different melting points were also discovered.

Analytic methods for determination of 1,4-DHP-CCAs in biologic samples were developed, mainly on the basis of liquid chromatography (LC) and gas chromatography (GC) coupled with different kinds of detection [5–9]. The concentrations of these compounds in human plasma were relatively low as a consequence of their high pharmacological potency. To our knowledge, LC–MS–MS method for determination of *m*-nisoldipine in rat plasma has not yet been reported. In the present study, a selective and sensitive LC–MS–MS method was first established using MRM mode for quantification of *m*-nisoldipine in plasma samples. Moreover, pharmacokinetic and relative bioavailability of A and B polymorphs of *m*-nisoldipine in rats were studied for the first time.

2. Experimental

2.1. Materials

m-Nisoldipine A (134.6 °C) and B (127.6 °C) polymorphs were supplied by School of Pharmacy, Hebei Medical University (Shijiazhuang, China), identified by X-ray (Table 1), IR

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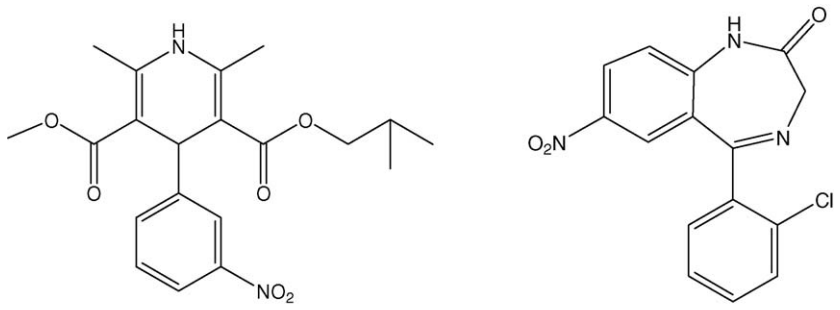

I II

Fig. 1. Chemical structures of *m*-nisoldipine (I) and clonazepam (II, internal standard).

Table 1
X-ray powder diffraction data for polymorphs of *m*-nisoldipine

Polymorph	Parameters	Peak number								
		1	2	3	4	5	6	7	8	9
A	2-Theta	9.40	23.22	24.44	12.58	10.58	26.04	19.80	22.40	13.48
	I/I_0 (%)	100.0	61.9	41.2	33.2	31.6	31.2	30.3	27.0	23.9
B	2-Theta	21.48	14.30	23.00	8.32	24.82	20.56	25.26	29.30	20.14
	I/I_0 (%)	100.0	66.3	55.1	50.0	33.1	32.1	31.6	28.8	27.8

Table 2
Differences of infra-red spectral data between two polymorphs of *m*-nisoldipine

Polymorph	Wave number (cm^{-1})									
A	3335	3244	3089	2965	–	2877	1699	1653	1530	1488
B	3350	3250	3093	2967	2948	2873	1677	1634	1528	1483
A	1433	–	1380	1348	1327	1305	1267	1252	1212	1186
B	1439	1399	1380	1343	–	–	–	–	1266	–
A	1147	1120	1097	1052	1020	983	954	925	911	826
B	–	1129	1093	1048	1018	–	–	933	898	826
A	806	783	750	705	678	636	587			
B	802	783	752	693	672	628	–			

(Table 2), and DSC (Fig. 2), and were of pharmaceutical purity. Clonazepam (Fig. 1), used as I.S., was obtained from Chinese Drug and Biological Products Quality Control Institute. Acetonitrile (Fair Lawn, NJ, USA) was of HPLC grade. Ultra pure water was used for all analysis.

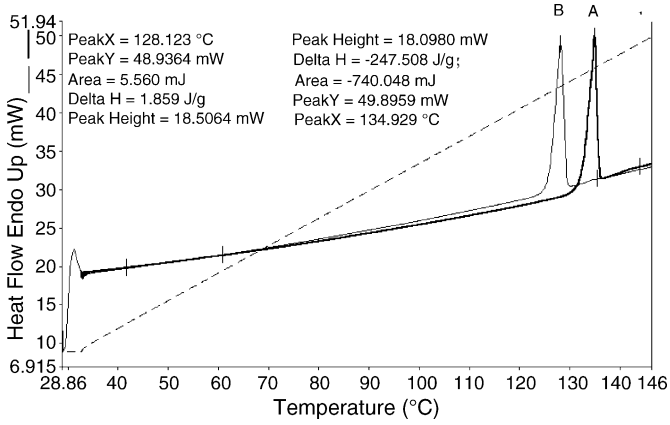

Fig. 2. DSC curves of A and B *m*-nisoldipine polymorphs.

2.2. Apparatus and operating conditions

The chromatographic separation system consisted of a quaternary pump (Agilent 1100), an online solvent degasser and an autosampler. An Applied Biosystems/MSD Sciex API 4000 triple quadrupole mass spectrometer (Foster City, CA, USA) equipped with ESI source was used for mass spectrometric analysis and detection. Instrument control and data acquisition were carried out with Applied Biosystems/MSD Sciex Analyst software (version1.4).

The LC analysis was performed at room temperature using a Symmetry RP-C_{18} analytic column (50 mm × 4.6 mm, 3.5 μm, Waters, PN: WAT 200625) with a mobile phase of acetonitrile–water (80:20, v/v) at a flow rate of 0.5 ml/min. The mass spectrometer was operated using a triple quadrupole mass spectrometer with ESI source in the negative ion detection. Turbo spray voltage (IS) was set at −4000 V. Source temperature was maintained at 450 °C. Nitrogen was used as nebulizing gas (30 psi g), auxiliary gas (30 psi g), and curtain gas (25 psi g). For collision-induced dissociation (CID), helium was employed as the collision gas at a pressure of 6 psi g. Quantification was

performed using MRM mode with the following transitions: m/z 387 → 122 for *m*-nisoldipine and m/z 314 → 278 for clonazepam, respectively, with a dwell time of 200 ms.

2.3. Standard solutions

Standard stock solutions of *m*-nisoldipine were prepared by dissolving the standards in acetonitrile–water (80:20, v/v) at the final concentration of 1 mg/mL. This solution was further diluted in the same diluent to obtain the concentration: 200 ng/mL. And the solution of 200 ng/mL was appropriately diluted in the mixture of acetonitrile–water (80:20, v/v) so as to obtain working solutions for calibration standards and quality control samples as follows: 20 and 16 ng/mL (HQC, high quality control), 10 and 4 ng/mL (MQC, medium quality control), 1.5 and 0.5 ng/mL (LQC, low quality control) and 0.2 ng/mL. Clonazepam was diluted in acetonitrile–water (80:20, v/v) to obtain an I.S. working solution at the concentration of 20 ng/mL. Standard solutions were stored at 4 °C and protected from light until use.

2.4. Methods of disposal

A 100 μL plasma sample was added with 100 μL of I.S. (20 ng/mL) and 200 μL acetonitrile. After vortex mixing for 30 s and centrifuged at 8000 r/min for 5 min, the supernatant were transferred into the brown glass autosampler vials. A 10 μL of aliquots was injected into the chromatographic system.

All the above procedures were carried out in a short period of time in order to protect the solutions and spiked plasma sample from light as 1,4-DHP derivatives are unstable, especially in solutions, as a result of photo-degradation in day light or laboratory light.

2.5. Method validation

Plasma samples were quantified by comparison of the peak area ratios of *m*-nisoldipine and clonazepam in sample.

Calibration curves were calculated using weighted ($1/c^2$) linear regression of internal ratios (analyte/I.S. peak areas) versus analyte concentrations. Spiked quality control (QC) samples ($n = 6$) at three concentration levels (0.5, 4 and 16 ng/mL) were assayed to determine precision and accuracy on the three different validation days by comparing calculated values against nominal concentration. The precision was expressed by the relative standard deviation (R.S.D.), whereas the accuracy was expressed by the relative error (RE).

The recovery of *m*-nisoldipine was tested at three concentration levels (0.5, 4 and 16 ng/mL) by adding known amounts of the studied compound to the drug-free plasma samples ($n = 3$). A 100 μL plasma sample was added with 100 μL of I.S. (20 ng/mL), 100 μL of *m*-nisoldipine (0.5, 4 and 16 ng/mL), and 100 μL acetonitrile. The peak area ratios of analyte and I.S. were calculated. Recoveries were evaluated by comparing the calculated concentration with the assay value for a known amount of *m*-nisoldipine prepared and assayed in mobile phase.

The stability of *m*-nisoldipine was assessed at low and high concentration (0.5 and 16 ng/mL, respectively) after storage of working solutions at ambient temperature for 8 h, after storage of samples in the autosampler for 4 h and after three freeze (−20 °C)/thaw (room temperature) cycles.

2.6. Animals and sampling

Male Spraque–Dawley (SD) rats (Certificate No. DK 0508083, weighting 300 ± 50 g) were obtained from Experimental Animals Center of Hebei Province (Shijiazhuang, China). They were kept in environmentally controlled breeding room for 3 days before starting the experiments, and fed with standard laboratory food and water ad libitum, and fasted for 12 h before dosing.

Suspension of *m*-nisoldipine A and B polymorphs (4 mg/mL) was, respectively, prepared by 0.5% CMC-Na (sodium carboxymethylcellulose) shaking for 5 min for uniformity. For pharmacokinetic studies, 10 rats were randomly assigned to two groups: group 1 and group 2. Each group contained five rats. Then suspensions of *m*-nisoldipine A and B were orally administrated to group 1 and group 2 at a dose of 9 mg/kg, respectively. Blood samples (0.3 ml) were obtained from each rat at 15, 30, 45, 60, 75, 90, 120, 180, 240, 330, 420, 540, and 660 min after the administration and collected in heparinized centrifuge tube, respectively. Then the samples were centrifuged at 8000 r/min for 5 min and separated plasma was frozen at −20 °C prior to analysis.

2.7. Pharmacokinetic analysis

Pharmacokinetic parameters such as time point of maximum plasma concentration (T_{max}), area under the plasma concentration–time curve from 0 h to the last measurable concentration (AUC_{0-t}), area under the plasma concentration–time curve from 0 h to infinity ($AUC_{0-\infty}$), elimination rate constant (k_e), elimination half-life ($t_{1/2}$), apparent volume of distribution (V), and clearance (CL) were calculated with one-compartmental model ($1/c^2$) of 3P97 program (the Chinese Society of Mathematical Pharmacology) on a personal computer. Maximum plasma concentration (C_{max}) used the actual measured values. The relative bioavailability was calculated as $(AUC_{0-t})_B/(AUC_{0-t})_A$. Bioequivalence was evaluated by comparing the pharmacokinetics parameters of two different polymorphs. The comparison of the pharmacokinetic parameters and analysis of variance (ANOVA) was carried out using SAS Version 6.12 (SAS Institute Inc., Cary, NC, USA). Differences in T_{max} were evaluated using a non-parametric test.

3. Results and discussion

3.1. LC–MS–MS optimization

Parameters of MSD were optimized in order to obtain more abundant deprotonated molecular ions of analytes [10]. By negative ESI mode, the analyte and I.S. formed predominately deprotonated molecular ions $[M - H]^-$ (m/z 387 and 314) in full scan mass spectra. In the product ion spectra, several fragment ions were obtained, but the ions at m/z 122 and 278 were chosen

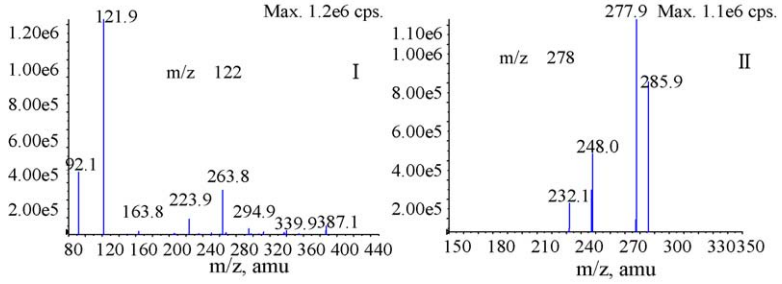

Fig. 3. Product ion mass spectrum of *m*-nisoldipine (I) and clonazepam (II).

because they displayed much greater intensity than the others in the acquisition of *m*-nisoldipine and I.S., respectively. The product ion spectra of the analyte and I.S. are shown in Fig. 3. The most suitable collision energy was employed by observing the maximum response obtained for the fragment ion peak.

Ionization detection mode was evaluated during the early stage of method development. HPLC-ESI-MS method for determination of nisoldipine in human plasma has been reported in positive ion mode ($[M+Na]^+$, *m/z* 411) [11]. In this study, it was found that positive ion detection mode could not offer high sensitivity for the analyte using either ESI or atmospheric pressure chemical ionization (APCI) sources. In the other hand the strong and stable response of ion was obtained in negative ion detection using ESI source, which was helpful for the sensitivity and reproducibility of the measurement. Thus, negative ion detection mode was employed.

The chromatographic conditions were also optimized. The addition of acidic modifiers to the mobile phase was found not to improve the sensitivity. In order to reduce chromatographic separating time, acetonitrile was chosen as the organic solvent. Therefore, a mobile phase consisting of acetonitrile–water (80:20, v/v) was selected in the report, which resulted short chromatographic time (3 min) for each plasma sample.

Liquid–liquid extraction and solid phase extraction methods had been mainly used for the extraction of 1,4-DHPs from biologic samples. In the present study, a simple single-step protein

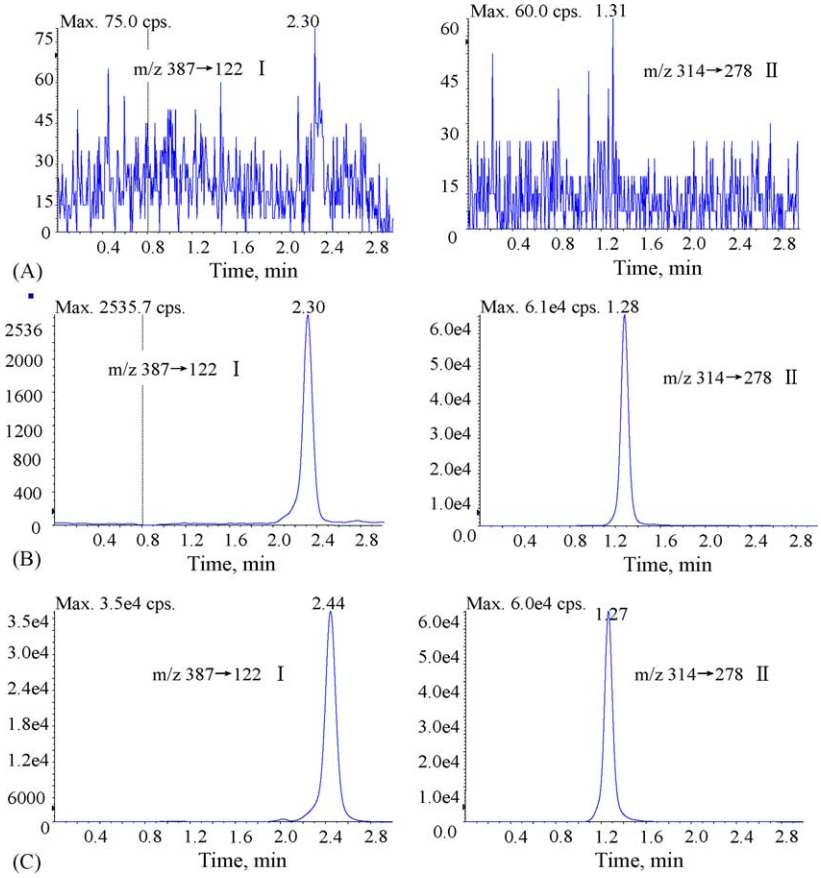

Fig. 4. Chromatograms of *m*-nisoldipine (I) and clonazepam (II) in multiple reaction monitoring (MRM) scan mode. (A) A blank plasma sample; (B) a blank plasma sample spiked with *m*-nisoldipine at the LLOQ of 0.2 ng/mL and clonazepam and (C) a rat plasma sample collected at 3 h after an oral administration of (A) polymorph of *m*-nisoldipine (9 mg/kg). Peaks (I) *m*-nisoldipine and (II) clonazepam.

precipitation was executed, and high recovery and good precision and accuracy were obtained.

3.2. Method validation

3.2.1. Specificity

The specificity of the method was tested by comparing the chromatograms of blank plasma and spiked plasma. Under the above conditions the retention times of I.S. and *m*-nisoldipine were about 1.3 and 2.4 min, respectively. The lack of matrix effects was tested with different batches of blank plasma samples ($n = 6$) and no matrix effects were detected. Fig. 4 demonstrates that no significant interferences were detected from endogenous substances with the analyte and I.S.

3.2.2. Linearity and lower limits of quantification

The calibration curves for the determination of *m*-nisoldipine had been prepared by analyzing spiked plasma samples in the range of 0.2 to 20 ng/mL in all cases based on duplicate assays of each concentration level. The linear regression analysis of *m*-nisoldipine was carried out by plotting the peak area ratio of the analyte and I.S. (*Y*-axis) versus the analyte concentrations (*X*-axis) in ng/mL. The regressive equation was as follows: $Y = 0.159X - 0.00919$ ($r \geq 0.9982$, $n = 6$), with good back-calculated precision (R.S.D. $\leq 4.6\%$) and accuracy (RE $\leq 9.8\%$).

The LLOQ was defined as the lowest concentration that can be measured with sufficient precision (R.S.D. $\leq 20\%$) and accuracy (RE $\leq 20\%$). The current assay offered a LLOQ of 0.2 ng/mL (Fig. 4), with a RE of less than 5.42% and R.S.D. of less than 12.12% ($n = 6$). The method was more sensitive than HPLC method with a limit of detection 3 ng/mL in plasma [5].

3.2.3. Precision, accuracy and recovery

0.5, 4, and 16 ng/mL of *m*-nisoldipine were spiked in blank plasma and analyzed at above conditions. Accuracy, intra- and inter-day precisions were calculated. Table 3 shows the result of precisions and accuracy. The recoveries of the method, determined at three different concentrations (0.5, 4, and 16 ng/mL) were 112.3, 106.7, and 104.8% ($n = 6$), respectively.

3.2.4. Stability

The stability of *m*-nisoldipine was investigated at low and high concentration (0.5 and 16 ng/mL) under a variety of storage and process conditions. It was found that *m*-nisoldipine was stable in the solution of acetonitrile–water (80:20, v/v) for 8 h and the reconstituted solution was stable for 4 h in the autosampler. RE of *m*-nisoldipine between the initial concentrations and the concentrations following three freeze–thaw cycles ranged from 3.10 to 6.74%. It was indicated that *m*-nisoldipine was stable during the three freeze–thaw cycles. Nevertheless, most of the 1,4-DHP derivatives are sensitive to the light as a result of photo-degradation, and the solution of *m*-nisoldipine is not stable under lighting condition. To prevent oxidation of the DHP ring and transmutation of the nitro group in nitrobenzene ring, sample preparation and instrumental analyses were performed under feeble yellow light [2].

Table 3
Precision and accuracy of the method to determine *m*-nisoldipine in rat plasma ($n = 3$ days, six replicates per day)

Added concentration (ng/mL)	Found concentration (ng/mL)	Accuracy RE (%)	Precision (R.S.D.%)	
			Intra-day	Inter-day
0.5	0.52	7.1	5.79	8.40
4	4.33	8.6	3.01	3.21
16	15.97	2.0	4.54	5.02

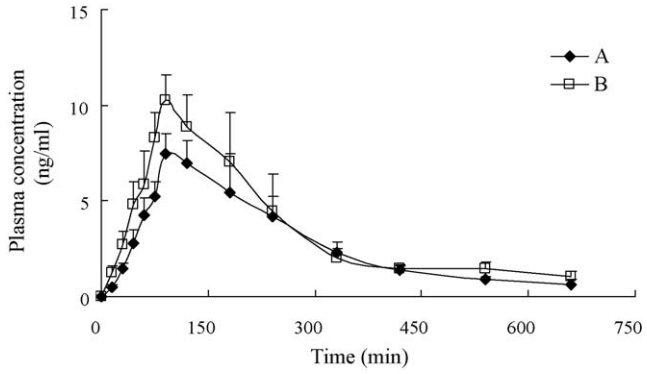

Fig. 5. Mean plasma concentration–time curves of *m*-nisoldipine in rats after oral administration of A and B polymorphs of *m*-nisoldipine. Each point represents the mean + S.D. ($n = 5$).

3.3. Pharmacokinetic studies

The described method was applied to analysis of plasma samples after oral administration of A and B polymorphs of *m*-nisoldipine. Fig. 5 shows the mean plasma concentration–time curves of *m*-nisoldipine after oral administration of polymorphs A and B to rats, and the relevant pharmacokinetic parameters are listed in Table 4. "Single-peak" was observed in both curves. The statistical results showed that 90.0% CIs (confidence intervals) for the geometric mean ratios of AUC and C_{max} were 0.95–1.45 and 1.17–1.63, respectively. In general, bioequivalence was considered to exit if the 90.0% CIs for these two parameters were between 0.80 and 1.25. Therefore, these two

Table 4
Mean pharmacokinetic parameters after oral administration of A and B polymorphs of *m*-nisoldipine to the rats ($n = 5$)

Parameters	Polymorphs	
	A ($\bar{X} \pm$ S.D.)	B ($\bar{X} \pm$ S.D.)
T_{max} (h)	1.91 ± 0.08	1.48 ± 0.30*
C_{max} (ng/mL)	7.46 ± 1.06	10.3 ± 1.33*
$t_{1/2}$ (h)	2.28 ± 0.52	2.55 ± 0.46
k_e (h^{-1})	0.32 ± 0.07	0.28 ± 0.06
$AUC_{0\sim t}$ (ng h/mL)	25.99 ± 4.81	30.39 ± 5.24*
$AUC_{0\sim\infty}$ (ng h/mL)	28.5 ± 4.18	34.3 ± 4.61*
CL (*F*/L/h/kg)	0.32 ± 0.05	0.27 ± 0.04
V (*F*/L/kg)	1.08 ± 0.37	1.01 ± 0.30

* Statistical difference with respect to A ($p < 0.05$).

polymorphs of *m*-nisoldipine were found not to be bioequivalent. The AUC of *m*-nisoldipine B was larger than that of A, and relative bioavailability was 116.95%. It was concluded that polymorph B could give higher bioavailability than polymorph A. Moreover, T_{max} and C_{max} values showed statistical significant difference between *m*-nisoldipine A and B, which indicated that the crystal form could influence the rate and extent of absorption of *m*-nisoldipine.

4. Conclusions

A sensitive and specific liquid chromatography–tandem mass spectrometric (LC–MS–MS) method has been developed to determine *m*-nisoldipine in rat plasma. The interference of matrix components was not observed using MS (ESI) as the detector in MRM negative ion mode. *m*-Nisoldipine and I.S. obtained good resolution. The fact and the reported sensitivity allow the application of this method in pharmacokinetic studies.

It was the first time that pharmacokinetics of *m*-nisoldipine polymorphs were investigated in rats. A and B polymorphs of *m*-nisoldipine were found not to be bioequivalent. This study provides evidences for the research and new drug development of *m*-nisoldipine.

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