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Short communication

Determination of nifedipine in human plasma by ultra performance liquid chromatography–tandem mass spectrometry and its application in a pharmacokinetic study

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ABSTRACT

A fast, sensitive and selective ultra performance liquid chromatography–tandem mass spectrometric (UPLC–MS/MS) method was developed for the determination of nifedipine in human plasma. Nitrendipine was used as the internal standard. The sample preparation employed liquid–liquid extraction with a mixture of *n*-hexane–diethyl ether (1:3, v/v). Chromatographic separation was performed on an ACQUITY UPLC™ BEH C_{18} column. The mobile phase was composed of acetonitrile–10 mmol/L ammonium acetate (75:25, v/v) with a flow rate of 0.20 mL/min. The detection was performed on a triple quadrupole tandem mass spectrometer by multiple reaction monitoring (MRM) mode via electrospray ionization (ESI) source. A high throughput was achieved with a run time of 1.4 min per sample. The linear calibration curves were obtained in the concentration range of 0.104–52.0 ng/mL ($r^2 \geq 0.99$) with a lower limit of quantification (LLOQ) of 0.104 ng/mL. The intra- and inter-day precision (relative standard deviation, RSD) values were below 15% and the accuracy (relative error, RE) was −4.0% to 6.2% at three quality control levels. The method was fully validated and successfully applied to a clinical pharmacokinetic study of nifedipine sustained-release tablet in healthy male volunteers.

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1. Introduction

Nifedipine, 1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridine dicarboxylic acid dimethyl ester (Fig. 1A), is a dihydropyridine calcium channel blocker used widely in the treatment of cardiovascular disorders, such as hypertension, angina and atherosclerosis [1]. Nifedipine acts by inhibiting the transmembrane influx of calcium into cardiac and vascular smooth muscle cells, thus reducing muscle contraction and has predominantly vasodilatory effects on arteries with minimal effects on the myocardium and cardiac conduction [1,2]. It is reported that nifedipine has a very low bioavailability mainly due to presystemic metabolism, which may result in very low plasma concentration and substantial intersubject pharmacokinetic variability [3,4]. Moreover, the most undesirable property of nifedipine is its high photochemical sensitivity. A 96% degradation has been observed when the methanolic solution of nifedipine was exposed to laboratory light for 2 h [5]. These unfavorable pharmacokinetics and physical characteristic make the determination of nifedipine in plasma difficult. Therefore, a sensitive and specific analytical method is needed for the determination of nifedipine in human plasma.

Several analytical methods based on gas chromatography (GC) [6–8] and high performance liquid chromatography (HPLC) [9–15] have been reported for the determination of nifedipine in biological samples. Although some GC methods could provide high sensitivity for the pharmacokinetic study, thermal decomposition of nifedipine under GC condition was the major problem. HPLC coupled with UV detection [9–11] or electrochemical detection [12] offered another possibility for the determination of nifedipine in biological samples, but these methods were limited by low sensitivity, long analysis time or large volume of plasma samples required. An HPLC–MS method developed by Guo et al. [13] provided an LLOQ of 1.0 ng/mL, but needed a long chromatography run time (8 min) and 1.0 mL plasma per sample, which may not meet the requirement of sensitivity and desired sample throughput in pharmacokinetic and clinical studies of nifedipine. In a published HPLC–MS/MS method [14], although the LLOQ was improved to 0.5 ng/mL, the run time was about 15 min and a solid-phase extraction procedure was adopted. Wang et al. [15] developed an HPLC–MS/MS method employing liquid–liquid extraction achieving a short run time of 2.5 min with a sensitivity of 0.5 ng/mL.

Ultra performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) possesses advantages of high sensitivity and sample throughput over conventional LC–MS/MS system and is rapidly applied to the analysis of drugs in biological samples [16–18]. We had published some UPLC–MS/MS methods to determine amlodipine [16] and nimodipine [17], which are structural

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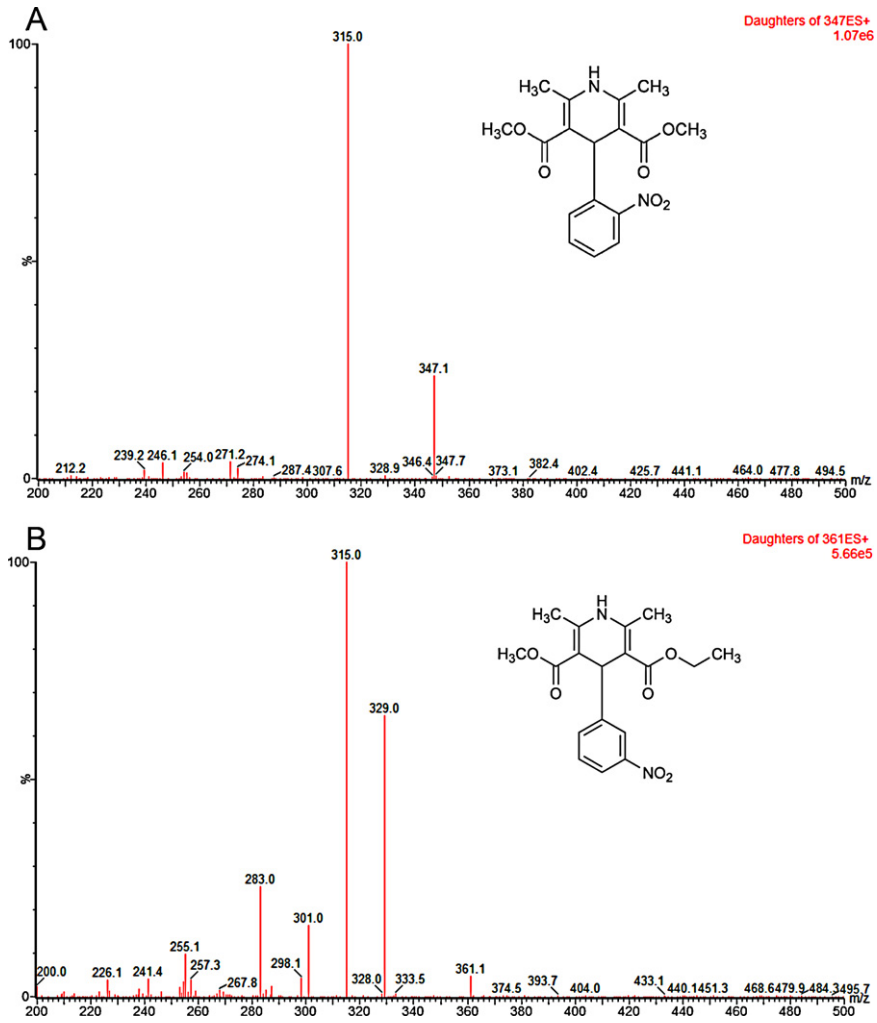

Fig. 1. Full scan product ion mass spectra of $[M+H]^+$ of nifedipine (A) and nitrendipine (B).

analogue of nifedipine. The high sensitivity and fast analysis of UPLC–MS/MS may also benefit the pharmacokinetic and clinical studies of nifedipine.

This paper describes a fast, sensitive and simple UPLC–MS/MS approach which enables a rapid determination of nifedipine with good accuracy in human plasma. The LLOQ was 0.104 ng/mL, which was lower than those reported in literatures [13–15]. The total run time of the method per sample was 1.4 min, which was shorter than those reported [13–15]. This method was fully validated and applied to a pharmacokinetic study in 20 healthy volunteers after oral administration of 10 mg nifedipine sustained-release tablet.

2. Experimental

2.1. Reagents and chemicals

Reference standards of nifedipine (99.5% purity, Fig. 1A) and nitrendipine (internal standard, IS, 99.4% purity, Fig. 1B) were purchased from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, PR China). Acetonitrile of HPLC grade was obtained from Tedia (Fairfield, OH, USA). Ammonium acetate of HPLC grade was supplied by Dikma (Richmond Hill, NY, USA). Sodium hydroxide, diethyl ether and *n*-hexane were of analytical grade and provided by Yuwang Chemical Reagent Plant (Shandong, China). Water was purified by redistillation and filtered through a 0.22 μm membrane filter before use.

2.2. Apparatus and operation conditions

2.2.1. Liquid chromatography

The chromatography was performed on an ACQUITY^{TM} UPLC system (Waters Corp., Milford, MA, USA) with cooling autosampler and column oven. An ACQUITY UPLC^{TM} BEH C_{18} column (50 mm × 2.1 mm, 1.7 μm; Waters Corp., Milford, MA, USA) was employed for the separation and the column temperature was maintained at 40 °C. The mobile phase was composed of acetonitrile–10 mmol/L ammonium acetate (75:25, v/v) and delivered at a flow rate of 0.20 mL/min. The autosampler was conditioned at 4 °C and 10 μL of sample solution was injected.

2.2.2. Mass spectrometry

A triple-quadrupole tandem mass spectrometer (Micromass® Quattro micro^{TM} API mass spectrometer, Waters Corp., Milford, MA, USA) equipped with electrospray ionization (ESI) interface was used for analytical detection. The ESI source was operated in positive ionization mode. Quantification was performed using multiple reaction monitoring (MRM) of the transitions of m/z 347.1 → 315.0 for nifedipine and m/z 361.1 → 315.0 for IS, with the scan time of 0.10 s per transition. The optimal MS parameters were as follows: capillary voltage of 3.0 kV, cone voltage of 20 V for nifedipine and 15 V for IS, source temperature of 110 °C and desolvation temperature of 350 °C. Nitrogen was used as the desolvation and cone gas with a flow rate of 500 and 50 L/h, respectively. Argon was

used as the collision gas at a pressure of approximately 0.276 Pa. The optimized collision energy for nifedipine and IS was 8 and 15 eV, respectively. All data was collected in centroid mode and processed using MassLynx™ NT 4.1 software with QuanLynx™ program (Waters Corp., Milford, MA, USA).

2.3. Preparation of standard and quality control samples

Stock standard solutions of nifedipine and IS were prepared in methanol at the concentrations of 104 and 98.0 μg/mL, respectively. The stock solution of nifedipine was then serially diluted with methanol–water (50:50, v/v) to provide working standard solutions at desired concentrations for preparing calibration standard samples. For the preparation of quality control (QC) samples, appropriate amount of nifedipine was dissolved in methanol to give a concentration of 107 μg/mL. An IS working solution of 0.980 μg/mL was obtained by diluting the stock solution of IS with methanol–water (50:50, v/v). All the solutions were protected from light with aluminum foil and stored at 4 °C.

Calibration standards were prepared daily by spiking 500 μL of blank plasma with 50 μL of nifedipine working standard solutions to yield final concentrations of 0.104, 0.260, 0.520, 1.56, 5.20, 15.6, 52.0 ng/mL. The QC samples were prepared in bulk with blank plasma at low, mid and high concentrations of 0.214, 2.14, and 42.8 ng/mL and stored at −70 °C after preparation. The standards and quality controls were extracted on each analysis day with the same procedures for plasma samples as described below.

2.4. Plasma sample preparation

To a 500 μL aliquot of plasma sample in a 10 mL clean glass tube, 50 μL of IS solution and 500 μL of 1 mol/L sodium hydroxide were added. The mixture was vortexed for 30 s and extracted with 3 mL of *n*-hexane–diethyl ether (1:3, v/v) by vortexing for 1 min. After centrifugation at 3500 rpm for 10 min, the upper organic layer was transferred into another set of clean glass tube and evaporated to dryness at 40 °C under a gentle stream of nitrogen. The residue was reconstituted in 100 μL of acetonitrile–water (75:25, v/v), and transferred to an autosampler vial. An aliquot of 10 μL was injected onto the UPLC–MS/MS system for analysis. All the development and validation work was carried out under sodium lamp illumination.

2.5. Method validation

The method was validated for selectivity, linearity, precision, accuracy, extraction recovery, matrix effect and stability according to FDA guidance for validation of bioanalytical methods [19].

2.5.1. Selectivity

The selectivity was evaluated by comparing chromatograms of six different batches of blank plasma obtained from six subjects with those of corresponding standard plasma samples spiked with nifedipine and IS, and those of plasma samples obtained after oral dose of 10 mg nifedipine sustained-release tablet.

2.5.2. Linearity and LLOQ

Calibration curves were constructed by assaying standard plasma samples at seven concentrations in the range of 0.104–52.0 ng/mL with weighted ($1/x^2$) least squares linear regression. The LLOQ is defined as the lowest concentration on the calibration curve, at which an acceptable accuracy (relative error, RE) within ±20% and a precision (relative standard deviation, RSD) below 20% can be obtained.

2.5.3. Precision and accuracy

The intra-day precision and accuracy were evaluated by determining replicate QC samples of nifedipine on the same day. The validation run consisted of two sets of calibration standards and six replicates of LLOQ and QC samples at three concentrations. For determining the inter-day accuracy and precision, analysis of three batches of QC samples was performed on three consecutive days.

2.5.4. Extraction recovery and matrix effect

The extraction efficiency of nifedipine was determined by analyzing six replicates of plasma samples at three QC concentration levels of 0.214, 2.14, and 42.8 ng/mL. The recovery was calculated by comparing the peak areas obtained from extracted spiked samples with those of samples spiked post-extraction at corresponding concentrations. To evaluate the matrix effect, nifedipine at three concentration levels was added to the extract of 500 μL of blank plasma, dried and reconstituted with 100 μL of acetonitrile–water (75:25, v/v). The peak areas (A) were compared with those of standard solutions at equivalent concentrations (B). The ratio (A/B × 100)% was used to evaluate the matrix effect. The extraction recovery and matrix effect of IS at a single concentration of 0.980 μg/mL were also evaluated using the same procedure.

2.5.5. Stability

The stability of nifedipine in human plasma was assessed by analyzing three replicates of low, mid and high QC samples under different temperature and time conditions. The freeze–thaw stability was performed by subjecting unextracted QC samples to three freeze (−70 °C)–thaw (room temperature) cycles. QC samples were stored at −70 °C for 50 days and at ambient temperature for 4 h to determine the long-term and short-term stability, respectively. The post-preparation stability was studied by analyzing the extracted QC samples kept in the autosampler at 4 °C for 12 h.

2.6. Pharmacokinetic study

The method was applied to determine the plasma concentrations of nifedipine from a clinical trial in which 20 healthy male volunteers were involved and each received one nifedipine sustained-release tablet (containing 10 mg nifedipine). The pharmacokinetic study was approved by the local Ethics Committee and carried out in the hospital. All volunteers gave their signed informed consent to participate in the study according to the principles of the Declaration of Helsinki. Blood samples were collected pre-dosing (0) and at 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, 10.0, 12.0, 24.0 and 36.0 h post-dosing. The plasma was immediately separated by centrifugation and stored at −70 °C until analysis. All activities of the clinical phase were conducted under sodium lamp illumination.

3. Results and discussion

3.1. Optimization of mass spectrometry

UPLC–MS/MS operation parameters were carefully optimized for the determination of nifedipine. The mass spectrometer was tuned in both positive and negative ionization modes with ESI for optimum response of nifedipine. It was found that the signal intensity of positive ion was higher than that of negative ion. In the precursor ion full-scan spectra, the most abundant ions were protonated molecules $[M+H]^+$ at *m/z* 347.1 and 361.1 for nifedipine and IS, respectively. Parameters such as desolvation temperature, ESI source temperature, capillary and cone voltage, flow rate of desolvation gas and cone gas were optimized to obtain the highest intensity of protonated molecule of nifedipine. The product ion scan spectra (Fig. 1) showed high abundance fragment ions

at m/z 315.0 for both nifedipine and IS. The collision gas pressure and collision energy of collision-induced decomposition (CID) were optimized for maximum response of the fragmentation of m/z 315.0 for nifedipine. The precursor → product ion transitions of m/z 347.1 → 315.0 for nifedipine and m/z 361.1 → 315.0 for IS were chosen for MRM. No cross-talk was observed between the MRMs of the analytes.

In the methods developed by Guo et al. [13] and Streel et al. [14], the atmospheric pressure chemical ionization (APCI) was used. In our study, however, ESI provided better sensitivity for the analyte. This could be due to a low flow rate (0.2 mL/min) used with the UPLC column of small particles and narrow internal diameter, which suits the ESI source better.

3.2. Optimization of chromatography

Chromatographic conditions were optimized to obtain high sensitivity, good peak shape and short retention time. The separation and ionization of nifedipine and IS were affected by the composition of mobile phase. Acetonitrile–water and methanol–water in various proportions were tested. In view of the response of nifedipine, retention times and peak shapes of both nifedipine and IS, 75% acetonitrile was the best.

The ionization of nifedipine and IS was increased by adding additive in the mobile phase. Therefore, formic acid and ammonium acetate were attempted to improve the response. When formic acid was added in the mobile phase, the peak shape of nifedipine was unacceptable. The response of nifedipine was distinctly increased by adding ammonium acetate. Both nifedipine and IS were found to have higher response and better peak shapes in the mobile phase containing 10 mmol/L ammonium acetate. Finally, acetonitrile–water containing 10 mmol/L ammonium acetate (75:25, v/v) was adopted as the mobile phase.

Two channels were used for recording the response, channel 1 for nifedipine at retention time of 0.90 min, and channel 2 for IS at retention time of 1.03 min. As shown in Fig. 2, no interference was observed for either nifedipine or IS. Both nifedipine and IS were rapidly eluted with a total run time of 1.4 min per sample. This is the shortest analysis time reported so far for the determination of nifedipine [13–15], which is contributed by the combination of fast UPLC and selective MRM.

3.3. Selection of extraction method

Several extraction solvents including diethyl ether, *n*-hexane, *n*-hexane–diethyl ether (1:3, v/v) and *n*-hexane–dichloromethane (7:3, v/v) were investigated for the liquid–liquid extraction. *N*-Hexane–diethyl ether (1:3, v/v) was found to be more efficient with extraction recovery of nifedipine about 60%, while the recoveries of other solvents were below 45%. To further improve the recovery of the analyte, sodium hydroxide as the alkaline reagent at different concentrations was considered. The maximum response of nifedipine was achieved with sodium hydroxide at 1 mol/L and *n*-hexane–diethyl ether (1:3, v/v) as the extraction solvent. The mean recovery of nifedipine in our method was 87.0% which was higher than 70.5% in the literature [13].

3.4. Method validation

3.4.1. Selectivity

Selectivity was determined by comparing the chromatograms of six different batches of blank human plasma with those of the corresponding spiked plasma. As shown in Fig. 2, no interference from endogenous substance was observed at the retention time of nifedipine and IS.

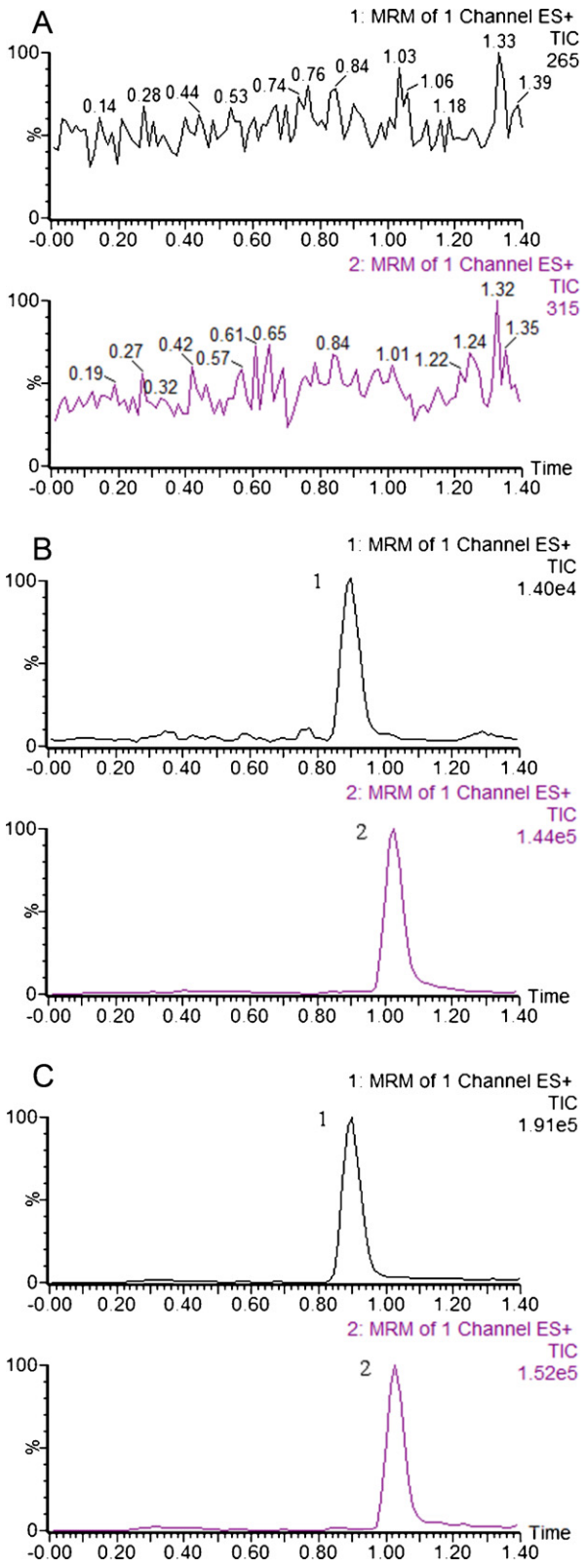

Fig. 2. Representative MRM chromatograms of nifedipine (peak 1, channel 1) and nitrendipine (peak 2, channel 2) in human plasma samples. (A) A blank plasma sample; (B) a blank plasma sample spiked with nifedipine at the LLOQ of 0.104 ng/mL and nitrendipine (0.980 μg/mL); (C) a plasma sample from a volunteer 1.5 h after oral administration of nifedipine. The retention times of nifedipine and nitrendipine were 0.90 min and 1.03 min, respectively.

Table 1
Precision and accuracy for the determination of nifedipine in human plasma (intra-day: $n = 6$; inter-day: $n = 6$ series per day, 3 days).

Nominal concentration (ng/mL)	Intra-day		Inter-day	
	RSD (%)	RE (%)	RSD (%)	RE (%)
0.104	9.2	1.8	17	−4.0
0.214	8.1	−2.8	14	−1.7
2.14	8.4	6.2	11	1.0
42.8	3.8	3.1	13	−1.4

3.4.2. Linearity and LLOQ

The standard calibration curves for nifedipine were linear over the concentration range of 0.104–52.0 ng/mL ($r^2 \geq 0.99$). A typical regression equation for the calibration curves was $y = 1.25 \times 10^{-1} x + 4.19 \times 10^{-3}$, $r = 0.9993$, where y is the peak area ratio of nifedipine to IS, and x is the concentration of nifedipine in plasma.

The LLOQ for nifedipine was 0.104 ng/mL in plasma, which was lower than those reported in the literatures [13–15]. The high sensitivity could be attributed to the peak sharpness produced by the UPLC chromatographic system and the improved ionization efficiency of the MS system. With the present LLOQ, nifedipine could be determined in plasma samples until 36 h after a single oral dose of 10 mg.

3.4.3. Precision and accuracy

The data of intra- and inter-day precision and accuracy for nifedipine from QC samples are presented in Table 1. The precision and accuracy of the present method conformed to the criteria for the analysis of biological samples according to the guidance of FDA where the RSD determined at each concentration level is required to be not exceeding 15% (20% for LLOQ) and RE within ±15% (±20% for LLOQ) of the actual value.

3.4.4. Extraction recovery and matrix effect

The extraction recoveries of nifedipine from human plasma were 87.7 ± 7.1%, 89.3 ± 6.2% and 83.9 ± 7.7% at concentrations of 0.214, 2.14, and 42.8 ng/mL, respectively. The mean extraction recovery of IS was 89.3 ± 7.0%. In terms of the matrix effect, all the ratios defined as in Section 2 were between 85% and 115%. No significant matrix effect for nifedipine was observed, indicating that the ionization competition between the analyte and the endogenous co-elutions was negligible.

3.4.5. Stability of samples

The stability data of nifedipine under various conditions is summarized in Table 2, which indicated a good stability of nifedipine over all steps of the determination. The method is therefore proved to be applicable for routine analysis.

3.5. Pharmacokinetic application

This validated UPLC–MS/MS method was successfully applied to a pharmacokinetic study of nifedipine sustained-release tablet in 20 healthy male volunteers. The mean plasma concentration–time curve of nifedipine in single dose study is shown in Fig. 3. After administration of 10 mg nifedipine, the C_{max} and T_{max} were 40.6 ± 8.0 ng/mL and 3.15 ± 0.88 h, respectively. Plasma concentration declined with a $t_{1/2}$ of 7.53 ± 2.10 h. The AUC_{0-t} and $AUC_{0-\infty}$ values obtained were 281.2 ± 99.4 ng.h/mL and 293.1 ± 103.9 ng.h/mL, respectively. The pharmacokinetic parameters were comparable to those reported in the literature [15].

Table 2
Stability of nifedipine in human plasma at three QC levels ($n = 3$).

Nominal concentration (ng/mL)	Found concentration (ng/mL; mean ± SD)	RSD (%)	RE (%)
Short-term stability			
0.214	0.230 ± 0.009	3.7	7.4
2.14	1.97 ± 0.17	8.7	−8.0
42.8	43.5 ± 2.6	6.0	1.5
Long-term stability			
0.214	0.218 ± 0.015	6.8	1.9
2.14	2.31 ± 0.17	7.2	8.1
42.8	43.1 ± 1.3	3.1	0.8
Freeze–thaw stability			
0.214	0.212 ± 0.017	2.9	−1.8
2.14	2.29 ± 0.18	7.6	7.0
42.8	42.8 ± 2.7	6.2	−0.1
Post-preparation stability			
0.214	0.226 ± 0.024	11	5.5
2.14	2.08 ± 0.16	7.8	−2.9
42.8	43.5 ± 1.3	3.0	1.7

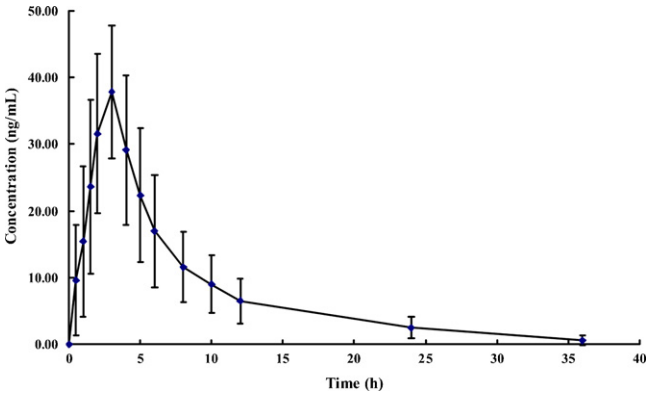

Fig. 3. Mean plasma concentration–time curve of nifedipine in 20 male volunteers after a single oral dose of 10 mg nifedipine sustained-release tablet.

4. Conclusion

A fast, selective and sensitive UPLC–MS/MS method for the determination of nifedipine in human plasma was developed. Compared with the published methods, this method provided superior sensitivity with an LLOQ as low as 0.104 ng/mL in plasma. A short analysis time of 1.4 min per sample made the method attractive particularly in high-throughput bioanalysis of nifedipine. The sharp peaks produced by UPLC were of particular advantage when coupled with electrospray tandem mass spectrometry. This method has been successfully applied to a pharmacokinetic study of nifedipine sustained-release tablet in healthy volunteers.

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