Journal of Pharmaceutical and Biomedical Analysis 47 (2008) 923–928

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Ultra-performance liquid chromatography–tandem mass spectrometry for the determination of lacidipine in human plasma and its application in a pharmacokinetic study

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ARTICLE INFO

Article history:
Received 16 March 2008
Received in revised form 8 April 2008
Accepted 10 April 2008
Available online 25 April 2008

Keywords:
Lacidipine
Determination
UPLC–ESI-MS/MS
Human plasma
Pharmacokinetics

ABSTRACT

A selective, rapid and sensitive ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–ESI-MS/MS) method was developed and validated for the quantification of lacidipine in human plasma. With nifedipine as an internal standard, sample pretreatment involved a simple liquid–liquid extraction with *tert*-butyl methyl ether of 1 ml plasma. The analysis was carried out on an Acquity™ UPLC BEH C_{18} column (50 mm × 2.1 mm, 1.7 μm) with flow rate of 0.28 ml/min. The mobile phase was 30 mM ammonium acetate buffer–acetonitrile (18:82, v/v, pH 5.5). The detection was performed on a triple-quadrupole tandem mass spectrometer by multiple reaction monitoring (MRM) mode via electrospray ionization (ESI). Linear calibration curves were obtained in the concentration range of 0.025–10.000 ng/ml, with a lower limit of quantification of 0.025 ng/ml. The intra- and inter-day precision (R.S.D.) values were below 15% and accuracy (RE) was −12.7% to 11.9% at all QC levels. The method was successfully applied to a clinical pharmacokinetic study of lacidipine in healthy volunteers following oral administration.

1. Introduction

Lacipidine, chemically designated as (E-4-{2-[3-(1,1-dimethyl-ethoxy)-3-oxo-1-propenyl]phenyl}-1,4-dihydro-2,6-di-methyl-3,5-pyridine-dicarboxylic acid diethyl ester), is a calcium channel blocker developed for oral administration. Besides antihypertensive effect, lacidipine has also shown anti-atherosclerotic and antioxidant effects [1–5], antibacterial activity with respect to 389 Gram-positive and Gram-negative bacterial strains [6], a beneficial effect on bone metabolism, and an antihypertensive effect [7]. It has long duration of action because of its high degree of lipophilicity [8,9]. The suggested therapeutic dosage is one 4 mg tablet daily and therapeutic plasma levels are very low (under 5 ng/ml at the peak time) [10]. Lacidipine undergoes extensive first-pass hepatic metabolism and has a mean absolute bioavailability of ∼10% (range 3–59%). It is completely metabolized in the liver by cytochrome P450 3A4 (CYP3A4) to pharmacologically inactive metabolites. Therefore, the determination of the level of unchanged drug in plasma requires an analytical method with high sensitivity.

To date, some assays for the determination of lacidipine in human plasma and urine have been reported, including HPLC with amperometric detection and ultraviolet detection [11–13], SPE-HPTLC and SPE-HPLC with RIA detection [14,15]. Baranda et al developed a HPLC method with diode array detection for the separation five calcium channel blockers including lacidipine [16]. Due to the low systemic levels, conventional analytical methods, such as HPLC with ultraviolet detection were of limited use for bioanalytical determinations of lacidipine in plasma. The failure of radioimmunoassay method was due to the reacting substances. The SPE-HPTLC and SPE-HPLC with RIA detection were not ideal for large numbers of sample determination, because of the time consuming sample preparation, the relative instability of the analyte in mobile phase and the complexity of the whole procedure. Therefore, attempts were made to improve the productivity. Ramakrishna et al. [17] described a liquid chromatography with tandem mass spectrometry (LC–MS/MS) method which achieved better sensitivity. The assay was found to be linear in the range 0.1–25 ng/ml with a LLOQ of 0.1 ng/ml. Baranda et al. [18] developed a LC–MS/MS method for the quantification of five 1,4-dihydropyridine calcium channel antagonists amodipine, lercanidipine, nitrendipine, felodipine and lacidipine in human plasma. The method was sensitive with a limit of detection about 1 ng/ml for each drug in plasma.

Compared with HPLC, UPLC is recently developed technology and provides a higher peak capacity, greater resolution, increased sensitivity and higher speed of analysis. This paper presents, for the first time, the development and validation of a simple, selective and specific UPLC–ESI-MS/MS method in the multiple reaction monitoring (MRM) mode for the quantification of lacidipine in human

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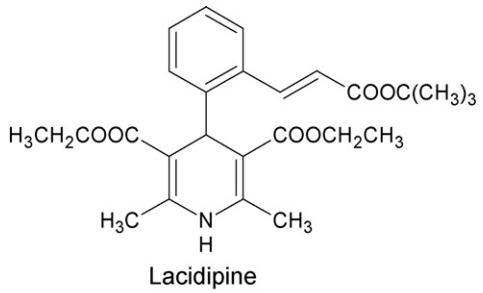
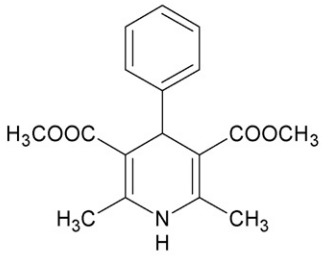
doi:10.1016/j.jpba.2008.04.018



Lacidipine



Nifedipine

Fig. 1. Chemical structures of lacidipine and nifedipine (I.S.).

plasma using its structural analogue, nifedipine, as the internal standard (I.S.). The method was validated over the concentration range 0.025–10.000 ng/ml. The sensitive method was successfully applied to a pharmacokinetic study of lacidipine following oral administration. It can also be easily extendible to other biological matrices in preclinical trials.

2. Experimental

Light exposure of all lacidipine samples was kept to a minimum.

2.1. Reagents and chemicals

Lacidipine (99.9% of purity) and nifedipine (I.S., 99.9% of purity) (Fig. 1) were purchased from National institute for the control of pharmaceutical and biological products (Beijing, China). The primary stock solutions were prepared separately in methanol (100.0 μg/ml for lacidipine and 140.0 μg/ml for I.S.). Methanol and acetonitrile (HPLC grade) were purchased from Caledon Laboratories Ltd. (Georgetown, Canada). Water was redistilled and filtered through 0.22 μm membrane filter before use.

2.2. Apparatus and operation conditions

2.2.1. Liquid chromatography

The chromatography was performed on Acquity™ UPLC system (Waters Corp., Milford, MA, USA) with cooling autosampler and column oven enabling temperature control of the analytical column. An Acquity™ UPLC BEH C_{18} column (50 mm × 2.1 mm, 1.7 μm; Waters Corp., Milford, MA, USA) was employed. The column temperature was maintained at 40 °C. The mobile phase contained 18% 30 mM ammonium acetate buffer and 82% acetonitrile (v/v, pH 5.5). The flow rate was set at 0.28 ml/min. The auto-sampler was conditioned at 4 °C and the sample volume injected was 2.0 μl. The total run time was 2.0 min.

2.2.2. Mass spectrometry

Triple-quadrupole tandem mass spectrometric detection was carried out on a Micromass® Quattro micro™ API mass spectrometer (Waters Corp., Milford, MA, USA) with an electrospray ionization (ESI) interface. The ESI source was set in positive ionization mode. Quantification was performed using MRM of the transitions of m/z 473.3 → 354.0 for lacidipine, m/z 347.20 → 314.9 for I.S., respectively, with scan time of 0.10 s per transition. The optimal MS parameters were as follows: capillary 3.20 kV, cone 15.0 V, extractor 2.00 V, source temperature 120 °C, desolvation temperature 400 °C cone gas flow 60 l/h, desolvation gas flow 850 l/h, dwell time 0.05 s. Nitrogen was used as the desolvation and cone gas. The optimized collision energy of lacidipine and I.S. were 14.0 and 8.0 eV. All data collected in centroid mode were acquired and processed using MassLynx™ NT 4.1 software with QuanLynx™ program (Waters Corp., Milford, MA, USA).

2.3. Preparation of standards and quality control samples

Standard stock solutions of lacidipine and I.S. were both prepared in methanol at the concentration of 100.0 and 140.0 μg/ml, respectively. The internal standard working solution was diluted with 50% methanol to 140.0 ng/ml. The lacidipine solution was then serially diluted with 50% methanol to provide working standard solutions of desired concentrations. All the solutions were stored at 4 °C.

Calibration standards were prepared by spiking 1.0 ml of blank human plasma with working standard solutions of lacidipine. The effective concentrations in standard plasma samples were 0.025, 0.050, 0.100, 0.250, 0.500, 1.000, 2.500, 5.000, 10.000 ng/ml. One calibration curve was constructed on each analysis day using freshly prepared calibration standards. The quality control samples (QCs) were prepared with blank plasma at low, middle and high concentrations of 0.050, 0.500, 8.000 ng/ml. The standards and quality controls were extracted on each analysis day with the same procedure for plasma samples as described below.

2.4. Plasma sample preparation

1.0 ml plasma specimens were transferred into 10.0 ml conical glass tubes and spiked with 50 μl internal standard working solution (140.0 ng/ml). Samples then were alkalinized to pH 10 with 100 μl of 15 M aqueous ammonia and vortex mixed for 30 s prior to the addition of the extraction solvent. Then 4 ml volume of *tert*-butyl methyl ether was added and the mixture was vortex mixed for another 3 min. After centrifugation at 3000 × *g* for 7 min, the upper organic layer was carefully transferred into a vacuum concentration equipment and evaporated. The dry residue was then reconstituted with 100 μl mobile phase and 2 μl solution was injected into the UPLC–MS/MS system.

2.5. Method validation

Validation runs were conducted on six separate occasions over a period of 2 months. Each validation run consisted of a minimum of one set of calibration standards and five replicates of QC plasma samples at three concentrations. The results from QC plasma samples in three runs were used to evaluate the precision and accuracy of the method developed.

2.5.1. Selectivity

Selectivity was studied by comparing chromatograms of six different batches of blank plasma obtained from six subjects with those of corresponding standard plasma samples spiked with lacidipine and I.S. and plasma sample after oral doses of lacidipine tablets.

2.5.2. Linearity and lower limit of quantification (LLOQ)

Calibration curves were prepared by making serial dilution of the working stock and assaying standard plasma samples at nine concentrations of lacidipine ranging 0.025–10.000 ng/ml. The linearity of each calibration curve was determined by plotting the peak area ratio (y) of lacidipine to I.S. versus the nominal concentration (x) of lacidipine. The calibration curves were constructed by weighted ($1/X$) least square linear regression. The lower limit of quantification is defined as the concentration which should be at least 5 times the response compared to blank response [19]. The validation of LLOQ was conducted in at least six different batches of blank plasma. It was validated using an LLOQ sample for which an acceptable accuracy (RE) within ±20% and a precision (R.S.D.) below 20% were obtained.

2.5.3. Precision and accuracy

For determining the intra-day accuracy and precision, a replicate analysis of QC plasma samples of lacidipine was performed on the same day. The run consisted of a calibration curve and five replicates of each low, mid, and high concentration quality control samples. The inter-day accuracy and precision were assessed by analysis of three batches on different days. The precision was expressed as the relative standard deviation (R.S.D.) and the accuracy as the relative error (RE).

2.5.4. Extraction recovery and matrix effect

The extraction recovery of lacidipine was determined by calculating the peak areas obtained from blank plasma samples spiked with analyte before extraction with those from blank plasma samples, to which analyte was added after extraction. According to the guidance of USFDA [20], recovery experiments should be performed at three concentrations (low, mid, and high). So this procedure was repeated for five replicates at three concentrations of 0.050, 0.500, 8.000 ng/ml.

In order to evaluate the matrix effect on the ionization of analyte, i.e. the potential ion suppression or enhancement, lacidipine at three concentration levels were added to the extract of 1.0 ml of blank plasma, evaporated and reconstituted with 100 μl of mobile phase. The corresponding peak areas (A) were compared with those of the lacidipine standard solutions evaporated directly and reconstituted with the same mobile phase (B). The ratio ($A/B \times 100$)% was used to evaluate the matrix effect. The matrix effect of internal standard was also evaluated using the same method.

2.5.5. Stability [21]

2.5.5.1. Freeze and thaw stability. The effect of freeze and thaw cycles on the stability of plasma samples containing lacidipine was determined by subjecting five aliquots of QC samples at low, mid and high concentration unextracted quality control samples to four freeze–thaw cycles. After completion of every cycle, the samples were analyzed and the experimental concentrations were compared with the nominal values. The accuracy values of three concentrations in four freeze–thaw cycles were calculated.

2.5.5.2. Long-term stability. Five aliquots of QC samples at low, mid and high concentration unextracted QC samples were stored at −70 °C for 30 days. Then, the samples were processed and analyzed and the concentrations obtained were compared with the nominal values.

2.5.5.3. Short-term stability. Five aliquots of QC samples at low, mid and high concentration unextracted QC samples were kept at ambient temperature (25 °C) for 12 h in order to determine the short-term stability of lacidipine in human plasma. Then the samples were processed and analyzed and the concentrations obtained were compared with the nominal values.

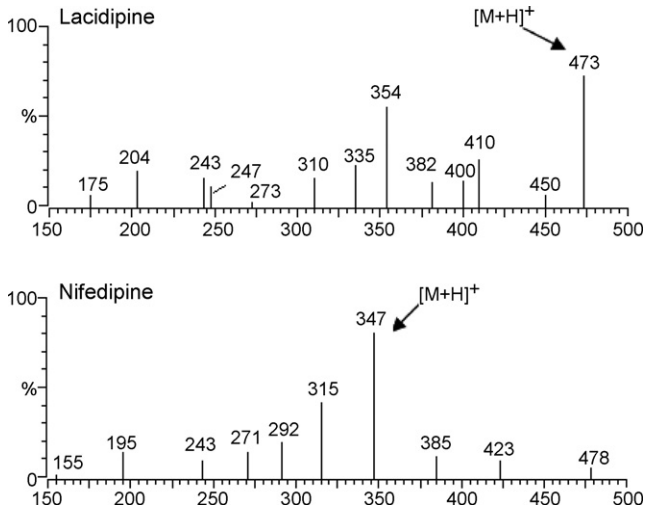


Fig. 2. Representative full-scan ESI+-MS/MS spectra for lacidipine and nifedipine.

2.5.5.4. Post-preparation stability. In order to estimate the stability of lacidipine in the prepared sample, five aliquots of QC samples at low, mid and high concentration were kept in an autosampler maintained at 4 °C for about 4 h. Then, the samples were analyzed and the concentrations obtained were compared with the nominal values.

2.5.5.5. Stock solution stability. To test the stock solution stability of lacidipine and the I.S., five aliquots of stock standard (100.0 μg/ml for lacidipine) and the I.S. (140.0 μg/ml) solution were left at 4 °C for 30 days. Then, the concentrations were analyzed and compared with the fresh stock solution.

2.6. Application to pharmacokinetic study

The method was applied to determine the plasma concentrations of lacidipine from a clinical trial in which 18 healthy male volunteers received a lacidipine tablet 4 mg oral. The pharmacokinetic study was approved by the Ethical Committee of XiangYa Second Hospital of Central South University and 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 before and 0.33, 0.67, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0, 12 and 24 h post-dosing. Samples were promptly centrifuged and plasma was separated and stored at −70 °C until analysis.

The maximum plasma concentration (C_{max}) and their time were noted directly. The elimination rate constant (k_e) was calculated by linear regression of the terminal points of the semi-log plot of plasma concentration against time. Elimination half-life ($t_{1/2}$) was calculated using the formula $t_{1/2} = 0.693/k_e$. The area under the plasma concentration–time curve (AUC_{0-t}) to the last measurable plasma concentration (C_t) was calculated by the linear trapezoidal rule. The area under the plasma concentration–time curve to time infinity ($AUC_{0-\infty}$) was calculated as: $AUC_{0-\infty} = AUC_{0-t} + C_t/k_e$.

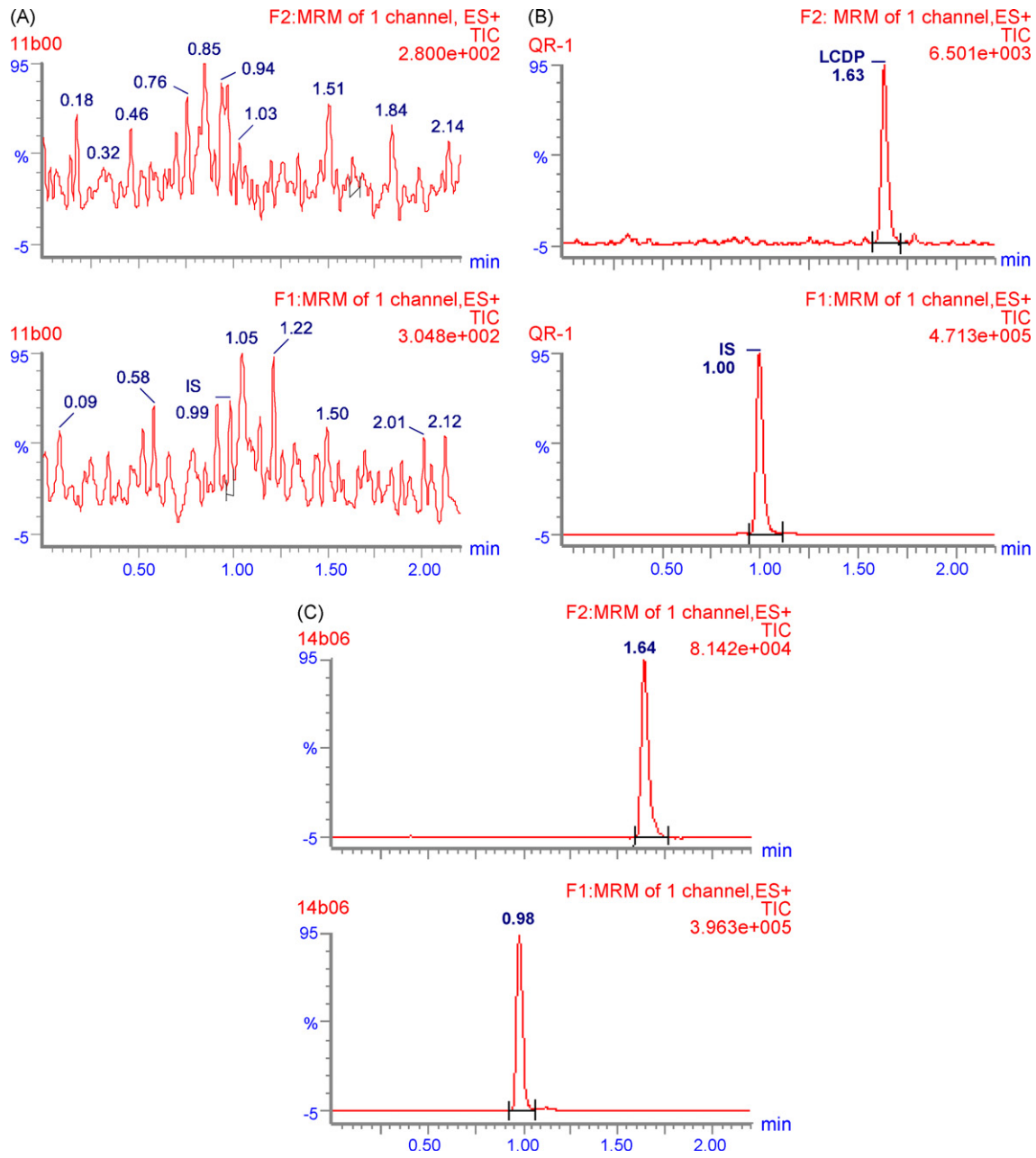


Fig. 3. Representative MRM chromatograms for lacidipine (LCDP) and nifedipine (I.S.) from (A) a blank plasma samples; (B) a plasma sample with added lacidipine and nifedipine at an LLOQ level (0.025 ng/ml); (C) a plasma sample from a volunteer 1.5 h after oral administration of lacidipine (4 mg). The retention times of lacidipine and I.S. were 1.64 and 1.00 min, respectively.

3. Results and discussion

3.1. Optimization of the chromatographic separation and MS/MS working conditions

The separation and ionization of lacidipine and I.S. were affected by the composition of mobile phase. Therefore, the selection of mobile phase components was critical. In experiment, different ratio (50:50, 40:60, 30:70 and 20:80) of water/acetonitrile was used to mobile phase and 18% water and 82% acetonitrile (v/v) in mobile phase was believed suitable in view of retention time and peak shape of drug. Ammonium acetate was employed to supply the ionic strength. It was found that a mixture of 30 mM ammonium acetate buffer–water/acetonitrile could preferably improve peak shape and was finally adopted as the mobile phase.

The selection of MRM transitions and associated acquisition parameters (collision energy and cone voltage) were evaluated for best response under positive mode ESI conditions by infusing a standard solution, via a syringe pump, into the mobile phase. The corresponding full-scan ESI^+-MS/MS spectra for lacidipine and nifedipine are shown in Fig. 2.

The very narrow chromatographic peaks with a peak width about 5 s, produced by UPLC™ indicated an increase in the chromatographic efficiency which produced a fast separation. Both lacidipine and I.S. were rapidly eluted with retention times less than 2.0 min (see Fig. 3). The shorter analysis time may meet the requirement for high sample throughput in bioanalysis.

Table 1
Precision and accuracy for the determination of lacidipine in human plasma (intra-day: $n = 5$; inter-day: $n = 5$ series per day, 6 days)

Added C (ng/ml)	Found C (ng/ml)	Intra-run R.S.D. (%)	Inter-run R.S.D. (%)	Accuracy RE (%)
0.050 (low)	0.048 ± 0.006	11.3	8.7	8.4
0.500 (mid)	0.510 ± 0.080	5.5	8.7	−7.6
8.000 (high)	8.245 ± 0.800	3.3	5.1	−7.2

3.2. Selection of IS

The best internal standard in LC–MS assay is a deuterated form of the analyte. In our laboratory, no deuterated lacidipine was available, therefore, a compound being structurally or chemically similar to the analyte was considered. In LC–MS/MS the I.S. should also have similar chromatographic and mass spectrometric behaviours to the analyte, and mimic the analyte in any sample preparation steps. Nifedipine was chosen as the internal standard for the assay because of its similarity of structure, retention time and ionization to lacidipine. The results showed it was suitable in retention time and ionization of lacidipine.

3.3. Selection of extraction method

As lacidipine is a lipophilic compound, liquid–liquid extraction was applied to extract the analyte. Several extraction solvents such as ethyl acetate, diethyl ether, *N*-hexane, hexane hexamethylene-dichloromethane and *tert*-butyl methyl ether were investigated, and it was found that *tert*-butyl methyl ether extracted the analyte more efficiently.

3.4. Method validation

3.4.1. Selectivity

Selectivity was assessed by comparing the chromatograms of six different batches of blank human plasma with the corresponding spiked plasma. As shown in Fig. 3, no interference from endogenous substance was observed at the retention time of lacidipine and I.S.

3.4.2. Linearity and LLOQ

The standard calibration curves for lacidipine were linear over the concentration range of 0.025–10.000 ng/ml ($r^2 > 0.99$) by using weighted least square linear regression analysis with a weigh factor of $1/x$. A typical equation for the calibration curves was: $y = 0.236459x + 0.000380327$, $r^2 = 0.9956$.

The lower limit of quantification for lacidipine was 0.025 ng/ml (S/N ≥ 5) with 2 μl injected into the UPLC column with RE within ±20% and R.S.D. lower than 20%. Compared with the previous method regarding the determination of lacidipine in human plasma, the present method gave a higher sensitivity with an LLOQ of 0.025 ng/ml. The high sensitivity could be attributed to the extra resolution and peak sharpness produced by the UPLC chromatographic system and the improved ionization efficiency under the mass spectrometric conditions.

3.4.3. Precision and accuracy

The data of intra-day and inter-day precision and accuracy for the method are listed in Table 1. The intra-day precision for low, mid and high QC levels of lacidipine were 11.3%, 5.5% and 3.3%, and that of inter-day analysis were 8.7%, 8.7%, 5.1%, respectively. The precision and accuracy of the present method conform to the criteria for the analysis of biological samples according to the guidance of USFDA, where the precision (R.S.D.) determined at each concentration level is required not exceeding 15%.

Table 2
Stability of lacidipine in human plasma at three QC levels ($n = 5$)

Stability	Accuracy (mean ± R.S.D.) (%)		
	0.050 (ng/ml)	0.500 (ng/ml)	8.000 (ng/ml)
Short-term stability	108.8 ± 4.2	99.2 ± 8.0	93.7 ± 2.1
Long-term stability	107.2 ± 7.2	99.3 ± 9.1	92.5 ± 3.7
Freeze–thaw stability	105.6 ± 4.7	100.4 ± 6.5	92.1 ± 2.5
Post-preparation stability	98.4 ± 8.4	99.0 ± 7.3	99.4 ± 6.2

3.4.4. Extraction recovery and matrix effect

The extraction recoveries of lacidipine from human plasma were 76.7 ± 15.5%, 84.2 ± 5.0%, and 79.1 ± 11.0% at concentration levels of 0.050, 0.500 and 8.000 ng/ml, respectively, and the mean extraction recovery of I.S. was 76.1 ± 10.1%.

In terms of matrix effect, all the ratios $(A/B \times 100)$% defined as in Section 2 were between 85% and 115%, which means no matrix effect for lacidipine and I.S. in this method.

3.4.5. Stability

The stock solution of lacidipine in plasma was found to be stable at room temperature for 12 h, at 4 °C for 4 h, at the −70 °C for 30 days, at freeze and thaw stability (Table 2). The stock solutions of lacidipine and I.S. were stable for at least 1 month with there being less than 5% difference in the measured concentrations of the stored and the freshly prepared solutions. The results from all stability tests presented in demonstrated a good stability of lacidipine over all steps of the determination.

3.5. Pharmacokinetic application

The present method was successfully applied to the pharmacokinetic study of lacidipine after oral administration in healthy male volunteers. Mean plasma concentration–time curve of lacidipine in single dose study is shown in Fig. 4.

After administration of a single dose of 4 mg lacidipine, the C_{max}, T_{max}, $t_{1/2}$, AUC_{0-24} and $AUC_{0-\infty}$ were 2.358 ± 2.024 ng/ml, 1.59 ± 0.40 h, 8.49 ± 2.29 h, 9.449 ± 5.864 ng h/ml and 10.618 ± 6.485 ng h/ml, respectively. Wide individual differences in the phar-

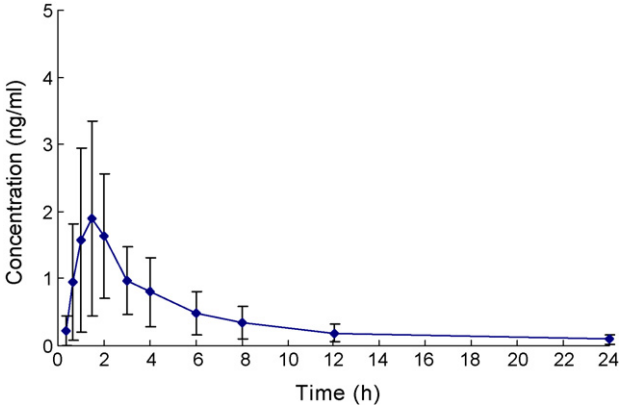


Fig. 4. Mean plasma concentration–time curve of lacidipine in 18 volunteers after a single oral dose (4 mg) of lacidipine.

macokinetic parameters were observed, suggesting that patients may benefit from individualized lacidipine treatment.

4. Conclusion

The recently developed ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–ESI-MS/MS) method for the determination of lacidipine in human plasma has been established. Compared with the published methods, the sharp peaks produced by UPLC are of particular advantage when coupled to electrospray mass spectrometry, reducing ion suppression and offering superior sensitivity with an LLOQ of 0.025 ng/ml, satisfactory resolution and selectivity. The method has been successfully applied to a pharmacokinetic study of lacidipine given in tablet form to healthy volunteers. Therefore, this analytical UPLC–ESI-MS/MS method can be considered as a promising technique that has obvious advantages compared with conventional analytic techniques in this field of application.

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