Available online at www.sciencedirect.com
ScienceDirect
Journal of Pharmaceutical and Biomedical Analysis 43 (2007) 575–579
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Determination of azelnidipine in human plasma by liquid chromatography–electrospray ionization-mass spectrometry

Li Ding [a,*], Li Li [a], Pengcheng Ma [b]

[a] *Department of Pharmaceutical Analysis, China Pharmaceutical University, 24 Tongjiaxiang, Nanjing 210009, China*
[b] *Organization for State Drug Clinical Trial, Dermatology Hospital Affiliated to Chinese Academy of Medical Sciences, Nanjing 210042, China*

Received 24 May 2006; received in revised form 30 June 2006; accepted 4 July 2006
Available online 22 August 2006

Abstract

A liquid chromatography–electrospray ionization-mass spectrometry (LC–ESI-MS) method for the determination of azelnidipine in human plasma was established. Nicardipine was used as the internal standard (IS). After adjustment to a basic pH with sodium hydroxide solution (0.1 M), plasma samples were extracted with cyclohexane–diethyl ether (1:1, v/v) and separated on a C_{18} column with a mobile phase of 20 mM ammonium acetate solution–methanol–formic acid (25:75:0.5, v/v). The electrospray ionization was employed in a single quadrupole mass spectrometer for the determination. The method was linear over the concentration range of 0.05–40 ng/ml. The lower limit of quantification (LLOQ) was 0.05 ng/ml. The intra- and inter-run standard deviations were less than 9.5% and 11.0%, respectively. The method was successfully applied to study the pharmacokinetics of azelnidipine in healthy Chinese volunteers.

Keywords: Azelnidipine; LC–ESI-MS; Pharmacokinetics

1. Introduction

Azelnidipine [(±)-3-(1-diphenylmethylazetidin-3-yl)-5-isopropyl-2-amino-1,4-dihydro-6-methyl-4-(3-nitrophenyl)-3, 5-pyridinedicarboxylate] is a new 1,4-dihydropyridine (DHP) derivative whose chemical structure is shown in Fig. 1. It is a new calcium channel blocker (CCB) with selectivity for L-type calcium channels [1], and has recently been approved in Japan for the treatment of patients with hypertension. Besides azelnidipine, there are several other DHP derivatives in clinical use, such as nimodipine, nitrendipine, nicardipine, amlodipine and nifedipine. Although those DHP-type CCBs are proven to be very effective in lowering blood pressure in hypertensive patients, their use may be limited because of several drawbacks: most of them induced reflex tachycardia, facial flush and headache, and some of them had to be administered twice daily to achieve a 24-h control of blood pressure. But azelnidipine is a long-lasting CCB that did not induce reflex tachycardia. Once daily administration of azelnidipine achieved stable, 24-h control of blood pressure with a minimum change in heart rate. Azelnidipine has pharmacological features that are shared with no other CCBs: cardiac slowing action and high affinity to vascular tissues. These characteristics make azelnidipine a new generation CCB that can be used for the treatment of hypertensive patients with or without potential ischemic heart diseases [1,2].

Several articles described the pharmacokinetics of azelnidipine in Japanese volunteers [1–3]. But the pharmacokinetic profile of the drug in Chinese volunteers has not been reported. Recently, a new formulation of azelnidipine tablet was developed by Jiangsu Kanion Pharmaceutical Co., Ltd. (Lianyungang, China) and approved by State Food and Drug Administration of China to be put into phase I clinical trial. As entrusted by Jiangsu Kanion Pharmaceutical Co., Ltd., the investigation of the pharmacokinetics of the drug in Chinese volunteers was carried out. After an oral dose of 8 mg the maximum plasma concentration of azelnidipine in adult humans is about 5 ng/ml. To evaluate the pharmacokinetics of azelnidipine tablet

* Corresponding author. Tel.: +86 25 8327 1289; fax: +86 25 8327 1289.
E-mail address: dinglidl@hotmail.com (L. Ding).

0731-7085/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2006.07.011

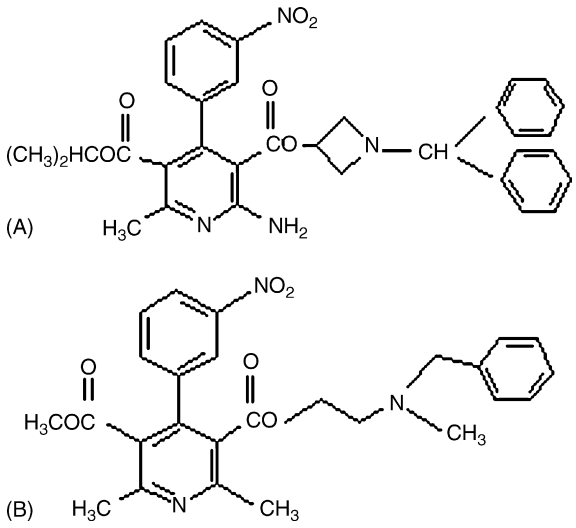

Fig. 1. Chemical structures of azelnidipine (A) and nicardipine (B).

in humans, a sensitive method for the determination of azelnidipine in human plasma is required. However the assay of azelnidipine in human plasma has not been reported. This paper describes the development and validation of a sensitive LC–ESI-MS method with an LLOQ of 0.05 ng/ml for the quantification of azelnidipine in human plasma. The assay has been successfully applied to study the pharmacokinetics of azelnidipine in Chinese volunteers.

2. Experimental

2.1. Materials

Azelnidipine, nicardipine and azelnidipine tablet (containing 8 mg azelnidipine) were obtained from Jiangsu Kanion Pharmaceutical Co., Ltd. (Lianyungang, China). Methanol was of HPLC grade (Merck, Darmstadt, Germany). Formic acid, ammonium acetate, cyclohexane and diethyl ether were analytical grade purity and purchased from Nanjing Chemical Reagent Co., Ltd. (Nanjing, China).

2.2. Instrument and conditions

Azelnidipine was analyzed using an Agilent Technologies Series 1100 LC/MSD SL system (Agilent Technologies, Palo Alto, CA). A Hanbon Lichrospher 5-C_{18} column, 5 μm, 250 mm × 4.6 mm i.d. (Jiangsu Hanbon Science & Technology Co., Ltd., Jiangsu, China) was used for the separation. The mobile phase was 20 mM ammonium acetate solutiom–methanol–formic acid (25:75:0.5, v/v) at a flow rate of 1 ml/min. The column temperature was maintained at 25 °C. LC–ESI-MS was carried out using nitrogen to assist nebulization. A quadrupole mass spectrometer equipped with an electro-spray ionization source was set with a drying gas (N_2) flow of 10 L/min, nebulizer pressure of 40 psi, drying gas temperature of 350 °C, capillary voltage of 4 kV and the positive ion mode. The fragmentor voltage was 130 V. LC–ESI-MS was performed in selected-ion monitoring mode using target ions at $[M+H]^+$ *m/z* 583.2 for azelnidipine and $[M+H]^+$ *m/z* 480.2 for the IS.

2.3. Sample preparation

To a 1-ml aliquot plasma sample, 50 μl IS solution (100 ng/ml) and 100 μl 0.1 M sodium hydroxide solution were added. Vortex-mixed for 10 s. The mixture was extracted with 5 ml cyclohexane–diethyl ether (1:1, v/v) by vortex for 3 min. Following centrifugation and separation, the organic phase was evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 120 μl of mobile phase, and a 30 μl aliquot was injected for analysis by LC–ESI-MS. All the process was protected from light.

2.4. Preparation of standard solutions

The stock solution of azelnidipine was prepared in methanol at the concentration of 1 mg/ml. Working solutions of azelnidipine were prepared at concentrations of 10 μg/ml, 1 μg/ml, 100 ng/ml, 10 ng/ml and 1 ng/ml by serial diluting the stock solution with methanol. The stock solution of IS was prepared in methanol at the concentration of 1 mg/ml and diluted to 100 ng/ml with methanol before using. All the solutions were stored at −20 °C away from light.

2.5. Preparation of calibration curves and quality control samples

Calibration standards of azelnidipine were prepared at concentration levels of 0.05, 0.1, 0.3, 1, 3, 10, 20 and 40 ng/ml by spiking appropriate amount of the working solutions in 1 ml blank plasma. The calibration curve was prepared and assayed along with quality control (QC) samples and each batch of clinical plasma samples.

The quality control (QC) samples were prepared in blank plasma at the concentrations of 0.05, 0.2, 3 and 30 ng/ml, respectively. The spiked plasma samples (calibration standards and quality controls) were then treated following the sample preparation procedure on each analytical run along with the unknown samples. The results of the QC samples provided the basis of accepting or rejecting the run.

2.6. Matrix effects

The matrix effect was defined as the direct or indirect alteration or interference in response due to the presence of unintended analytes or other interfering substances in the sample [4]. The matrix effect of assay was evaluated by comparing the peak area of analytes resolved in blank plasma sample's reconstituted solution (the final solution of blank plasma after extracted and reconstitution) with that resolved in mobile phase. Four different concentration levels of azelnidipine (0.05, 0.2, 3 and 30 ng/ml) were evaluated by analyzing five samples at each level. The blank plasmas used in this study were from five different batches of healthy human blank plasma. If the ratio <85% or >115%, a matrix effect was implied. The results

showed there was no matrix effect of the analytes observed in this study.

2.7. Clinical study design and pharmacokinetic analysis

The assay was used to determine azelnidipine in plasma samples after administration a dose of 8 mg azelnidipine to 12 healthy Chinese volunteers in a clinical study. The clinical study protocol was reviewed and approved by the Ethics Committee of Dermatology Hospital Affiliated to Chinese Academy of Medical Sciences. All volunteers were given written informed consent to participate in the study according to the principles of the Declaration of Helsinki.

Twelve healthy young Chinese volunteers participated in the study. For these 12 volunteers, their mean age was 20 years (range: 18–22 years); mean body weight was 59.5 kg (range: 52–71 kg). Following an overnight fast, each volunteer received one tablet containing 8 mg azelnidipine. Standard meals were provided after 4 h post-dose. Blood samples were collected pre-dose and at 0.5, 1, 1.5, 2, 3, 4, 6, 9, 12, 24, 48, 72, and 96 h post-dose. The azelnidipine plasma concentrations of these samples were determined, and the pharmacokinetics of the drug in healthy young Chinese volunteers was evaluated. Model-independent pharmacokinetic parameters were calculated for azelnidipine. The maximum plasma concentration (C_{max}) and the time to it (t_{max}) were noted directly. The elimination rate constant (k_{el}) 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 $t_{1/2} = 0.693/k_{el}$. The area under the plasma concentration–time curve $AUC_{0–96}$ to the last measurable plasma concentration was calculated by the linear trapezoidal rule.

3. Results and discussion

3.1. Conditions for ESI-MS

Because azelnidipine is a weak basic compound, the polarity of monitoring ion was selected as positive. Fig. 2(A) shows a typical mass spectrum of the positive ions of azelnidipine at 130 V fragmentor voltage obtained by the scan monitoring. The base peak in the mass spectrum is the pseudo-molecular-ion $[M+H]^+$ of azelnidipine at m/z 583.2. Therefore, the $[M+H]^+$ ion at m/z 583.2 was chosen as the target ion for azelnidipine monitoring in the ESI-MS assay. In order to achieve the highest assay sensitivity for azelnidipine, the optimal fragmentor voltage of the ESI-MS was investigated. The intensities of azelnidipine $[M+H]^+$ ion at m/z 583.2 were compared at the fragmentor voltages of 70, 90, 110, 130, 150 and 180 V. The result showed that the highest sensitivity was obtained using a fragmentor voltage of 130 V. So, the fragmentor voltage was set at 130 V in the ESI-MS assay for azelnidipine. At this fragmentor voltage, the base peak in the mass spectrum of IS was at $[M+H]^+$ m/z 480.2, see Fig. 2(B). Therefore, the positive ion $[M+H]^+$ m/z 480.2 of IS was selected as the target ion in the SIM.

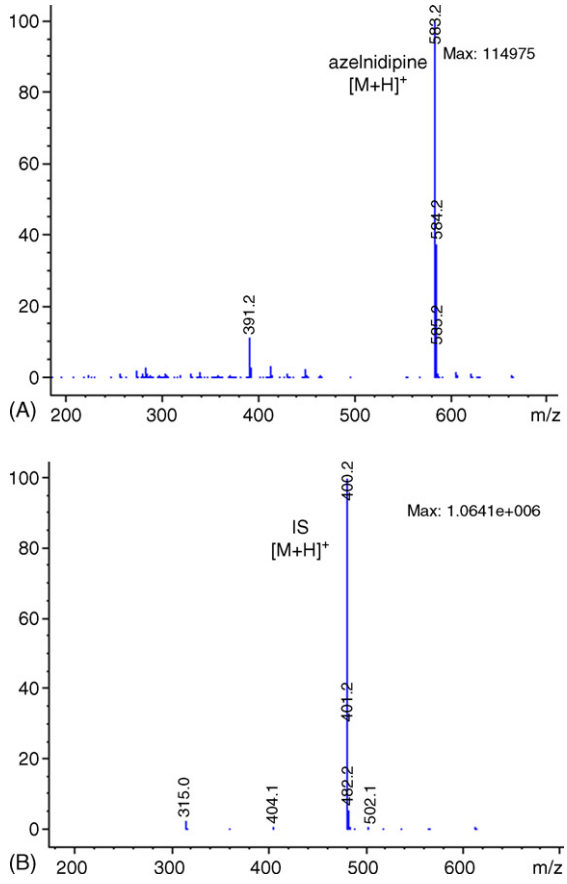

Fig. 2. Mass spectra of the positive ion of azelnidipine (A) and IS (B) at 130 V fragmentor voltage.

3.2. Chromatography

Methanol was used as the organic portion of the mobile phase in the assay. Using a higher percentage of methanol in the mobile phase could achieve shorter retention times of the analytes in HPLC and improve the ionization efficiency of the analytes in the ESI-MS. However, while the percentage of methanol in the mobile phase increased more than 75%, the determination of the analytes was interfered by endogenous substances in the plasma. The experiment results showed that formic acid could improve the separation and increase the MS sensitivity. When the formic acid ratio reached to 0.5%, the analytes could be separated completely from the endogenous substances in the plasma. At the same time, the retention times of the analytes were obviously shortened. Further experiment results showed that an ammonium acetate solution could not only improve peak shapes of azelnidipine and IS, but also increase the MS sensitivity to azelnidipine and IS. So a 20 mM ammonium acetate solution was adopted in mobile phase. Finally, high sensitivity, good separation of azelnidipine and short run time were obtained by using an elution system of 20 mM ammonium acetate solutiom–methanol–formic acid (25:75:0.5, v/v) as the mobile phase. Under the present chromatographic conditions, the retention time was 7.9 min for azelnidipine and 3.4 min for IS. Representative selected-ion chromatograms are shown in Fig. 3.

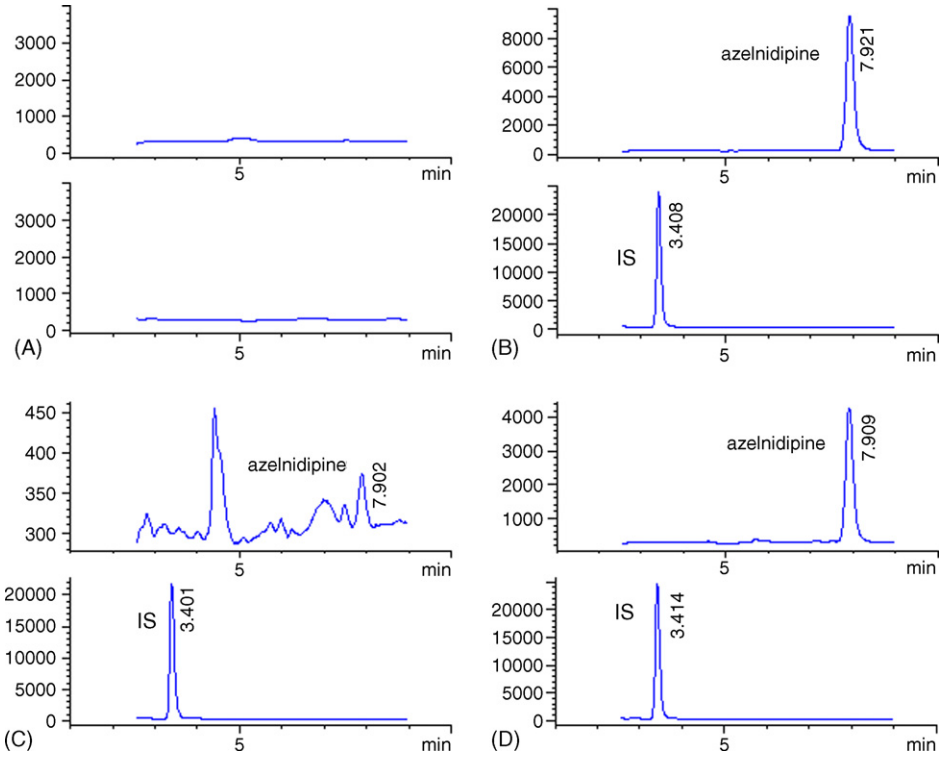

Fig. 3. Typical SIM chromatograms of blank plasma (A), plasma spiked with azelnidipine (10.26 ng/ml) and IS (B), LLOQ for azelnidipine in plasma (0.05 ng/ml) and IS (C), plasma obtained from a volunteer at 1.5 h after oral administration of 8 mg azelnidipine, the plasma concentration of azelnidipine was estimated to be 3.99 ng/ml (D).

3.3. Method validation [4,5]

3.3.1. Selectivity

Selectivity was assessed by comparing the chromatograms of six different batches of blank plasma with the corresponding spiked plasma. Fig. 3 showed the typical chromatograms of blank plasma, spiked plasma sample with azelnidipine and IS, and plasma sample from a healthy volunteer 1.5 h after an oral administration. Interferences from the matrices at the expected retention times of the target ions were not observed.

3.3.2. Linearity and lower limited of quantification

The azelnidipine calibration curve was constructed by plotting the peak area ratios (f) of azelnidipine to the IS versus the concentrations (C) of azelnidipine, using weighed least squares linear regression (weighing factor was $1/C$). Typical calibration curve for azelnidipine was $f = -0.0004670 + 0.09243C$, $r = 0.9994$. The calibration curve was linear over the concentration range 0.05–40 ng/ml for azelnidipine.

The presented LC–ESI-MS method offered an LLOQ of 0.05 ng/ml for azelnidipine. The LLOQ was defined as the lowest concentration on the calibration curve at which precision was within 20% and accuracy was within ±20%, and it was established using five samples independent of standards.

3.3.3. Precision and accuracy

Validation samples were prepared and analyzed on three separate runs to evaluate the accuracy, intra-run and inter-run precisions of the analytical method. The accuracy, intra-run and inter-run precisions of the method were determined by analyzing five replicates at 0.05, 0.2, 3 and 30 ng/ml of azelnidipine along with one standard curve on each of three runs. The results (see Table 1) demonstrated that the method was accurate and precise.

The accuracy of the assay was checked by preparation of QC samples at the start of the clinical study. These QC samples were assayed along with clinical samples in each run to monitor the performance of the assay and to assess the integrity and validity of the result of the unknown clinical samples analyzed.

3.3.4. Extraction recovery

When using the diethyl ether or acetic ether as the extraction solvent in the plasma sample preparation procedure, the higher extraction efficiency of the azelnidipine could be achieved, but the endogenous interference substances of the analytes were also extracted from the plasma. Using the mixture of cyclohexane–diethyl ether (1:1, v/v) as the extraction solvent can eliminate the interference of endogenous substances and

Table 1
Precision and accuracy of the assay for the determination of azelnidipine in human plasma ($n = 3$ runs, five replicates per run)

Added to plasma (ng/ml)	Mean measured concentration (ng/ml)	RE (%)	Intra-assay R.S.D.%	Inter-assay R.S.D.%
0.0513	0.0491	−4.3	9.5	11.0
0.2052	0.2129	3.8	5.0	9.1
3.078	3.033	−1.5	3.6	7.7
30.78	30.34	−1.4	2.5	2.9

meet the requirement of sensitivity for the assay. The extraction recovery of azelnidipine was evaluated by analyzing five replicates at 0.05, 0.2, 3 and 30 ng/ml of azelnidipine. Recovery was calculated by comparison of the peak areas of azelnidipine extracted from plasma samples with those of injected standards. The extraction recovery of the assay was 53.8 ± 4.1%.

3.3.5. Stability

The stability of azelnidipine in plasma was studied under a variety of storage and handling conditions using a set of plasma samples at 0.05 and 30 ng/ml of azelnidipine. The short-term temperature stability was assessed by analyzing three aliquots of each of the low and high concentration samples that were thawed at room temperature and kept at this temperature for 10 h. Freeze–thaw stability (at −20 °C in plasma) was checked through three freeze-and-thaw cycles. Three aliquots at each of the low and high concentrations were stored at −20 °C for 24 h and thawed unassisted at room temperature. When completely thawed, the samples were refrozen for 24 h under the same conditions. The freeze–thaw cycles were repeated three times, and then analyzed on the third cycle. The long-term stability was determined by analyzing three aliquots of each of the low and high concentrations stored at −20 °C for 20 days. All the plasma samples were stored and handled with the protection from light. The results of stability experiment showed that no significant degradation occurred at ambient temperature for 10 h, −20 °C in plasma for 20 days and during the three freeze–thaw cycles for azelnidipine plasma samples.

3.4. Application of the method to a pharmacokinetic study in healthy volunteers

The method was successfully applied to determine the plasma concentration of azelnidipine up to 96 h after an oral administration of 8 mg azelnidipine to 12 healthy Chinese volunteers. The mean plasma concentration–time curve of azelnidipine was shown in Fig. 4. The main pharmacokinetic parameters of azelnidipine in 12 volunteers were calculated and summarized in Table 2. The maximum plasma concentration (C_{max}) of azelnidipine in healthy Chinese volunteers after oral administration of 8 mg azelnidipine was 5.1 ± 2.2 ng/ml, and the time to it (t_{max}) was 2.9 ± 1.4 h. The elimination half-life ($t_{1/2}$) of azelnidipine in healthy Chinese volunteers was 25.0 ± 4.3 h. Koike et al. [1] described the pharmacokinetic profiles of azelnidipine in healthy Japanese volunteers after a single oral administration under fasting conditions. Their experiment results showed that the C_{max} and AUC of azelnidipine in healthy Japanese volunteers increased dose-dependently, the t_{max} of azelnidipine for Japanese volunteers after oral administration of 8 mg azelnidipine was 3.2 ± 0.3 h, the C_{max} was 11.8 ± 1.4 ng/ml, and the $t_{1/2}$ was determined to be 23.1 ± 8.1 h. These results show that there are no significant differences in the two pharmacokinetic parameter $t_{1/2}$ and t_{max} between Chinese and Japanese volunteers. But the difference in C_{max} is significant.

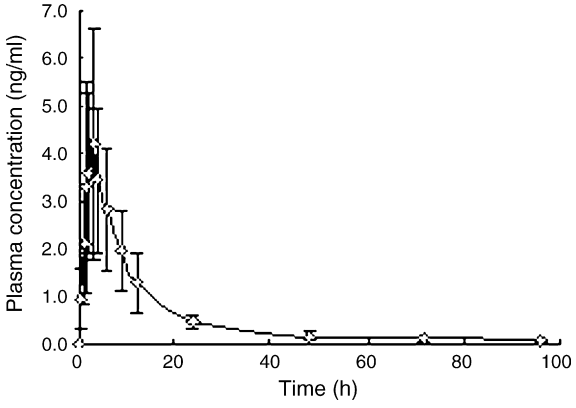

Fig. 4. Mean plasma concentration–time profile of azelnidipine after an oral administration of 8 mg azelnidipine to 12 healthy volunteers.

Table 2
Mean pharmacokinetic parameters of azelnidipine for 12 volunteers after oral administration of 8 mg azelnidipine tablet

Parameters	Test tablet
k_{el} (h^{-1})	0.028 ± 0.005
$t_{1/2}$ (h)	25.0 ± 4.3
C_{max} (ng/ml)	5.1 ± 2.2
t_{max} (h)	2.9 ± 1.4
$AUC_{0–96}$ (h ng/ml)	53.6 ± 17.1

4. Conclusions

A sensitive LC–ESI-MS method for the quantification of azelnidipine in human plasma was developed and validated. No significant interferences caused by endogenous compounds were observed. The method is sensitive and selective with a LLOQ of 0.05 ng/ml. This simple and sensitive assay is suitable for the pharmacokinetic study and bioavailability evaluation of azelnidipine formulations, and can also be used as a therapeutic drug monitoring method in clinic to check the plasma concentration of azelnidipine in hypertensive patients.

References

[1] H. Koike, T. Kimura, T. Kawasaki, T. Sada, T. Ikeda, A. Sanbuissho, H. Saito, Annu. Rep. Sankyo Res. Lab. 54 (2002) 1–64.
[2] K. Kuramoto, S. Ichikawa, A. Hirai, S. Kanada, T. Nakacachi, T. Ogihara, Hypertens. Res. 26 (2003) 201–208.
[3] J. Ma, S. Kishida, G. Wang, K. Meguro, H. Imuta, H. Oonuma, H. Iida, T. Jo, H. Takano, T. Morita, R. Nagai, T. Nakajima, J. Cardiovasc. Pharmacol. 47 (2006) 314–321.
[4] Guidance for Industry, Bioanalytical Method Validation, US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), May 2001.
[5] L. Ding, X. Huang, J. Yang, X.J. Bian, Z.X. Zhang, G.Y. Liu, J. Pharm. Biomed. Anal. 40 (2006) 758–762.