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Simultaneous determination of azelnidipine and two metabolites in human plasma using liquid chromatography-tandem mass spectrometry

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Abstract

A quantitative assay method by liquid chromatography/electrospray ionization-tandem mass spectrometry (LC/ESI-MS/MS) for the simultaneous determination of azelnidipine and its two metabolites, M-1 (aromatized form) and M-2 (hydroxylated form), in human plasma was developed and validated. Plasma samples, each of 1.0 mL, were extracted by a single step liquid–liquid extraction using a mixture of ethyl acetate and hexane (1:1, v/v), and analyzed by the LC/ESI-MS/MS method. Three analytes were separated by isocratic elution on a C_{18} column, and ionized using a positive ion electrospray ionization source. The ion transitions were monitored in selected reaction monitoring (SRM) mode. The chromatographic run time was 11 min per injection, with retention time of 3.6, 10.2 and 6.8 min for azelnidipine, M-1 and M-2, respectively. The calibration curves for azelnidipine, M-1 and M-2 well fitted to equations by a weighted ($1/X^2$) quadratic regression over the range of 0.5–40.0 ng/mL ($r^2 > 0.9979$). The intra- and inter-assay precisions (coefficient of variation: C.V.), calculated from quality control (QC) samples, were less than 8.7 and 8.4%, 3.8 and 4.7%, and 11.9 and 13.9%, respectively, for azelnidipine, M-1 and M-2. The accuracy was within ±9% for azelnidipine, within ±7% for M-1 and within ±16% for M-2. The overall recoveries for azelnidipine, M-1 and M-2 were 68.8–78.6%, 54.3–62.9% and 80.4–89.7%, respectively. All analytes evaluated demonstrated acceptable short-term, long-term, auto-sampler and stock solution stabilities. Furthermore, the method developed was successfully applied to pharmacokinetic studies on azelnidipine, M-1 and M-2 after an oral dose of 16 mg CALBLOCK® tablets (2 mg × 8 mg tablets) to healthy volunteers.
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Keywords: Azelnidipine; Two metabolites

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 dihydropyridine derivative with calcium antagonistic activity. Whereas all the existing dihydropyridine calcium blockers have two methyl groups located at the 2- and 6-positions of the dihydropyridine ring, one methyl group at the 2-position is substituted by an amino group in the azelnidipine molecule. After a conduct of a series of preclinical and clinical studies [1–5], this drug was launched into the market as CALBLOCK® in Japan in 2003. Chemical structures of azelnidipine, M-1 and M-2, the major metabolites of azelnidipine in human plasma and urine, and their stable isotope-labeled, internal standard (I.S.) substances, are shown in Fig. 1. Azelnidipine occurs as two enantiomers due to an asymmetric carbon at the 4-position of the 1,4-dihydropyridine ring. Numerous methods have been published for the quantitative determination of dihydropyridine calcium blockers in plasma, including gas chromatography combined with different detectors [6–8] or high performance liquid chromatography (HPLC) coupled with UV and MS/MS detection [9–12]. Regarding the simultaneous determination of the calcium antagonist and the metabolite, nicardipine and the aromatized nicardipine (pyridine metabolite) in human plasma have been measured by HPLC method [13]. A quantitative liquid chromatography/electrospray ionization-tandem mass spectrometry (LC/ESI-MS/MS) method suitable for the routine analysis of azelnidipine, M-1 and M-2 has not been reported yet. Therefore, this paper describes for the first time a sensitive, specific and rapid LC/ESI-MS/MS method for the simultaneous determination of azelnidipine and its two metabolites in human plasma. This assay method was successfully applied to pharmacokinetic studies of azelnidipine, M-1 and M-2 after a

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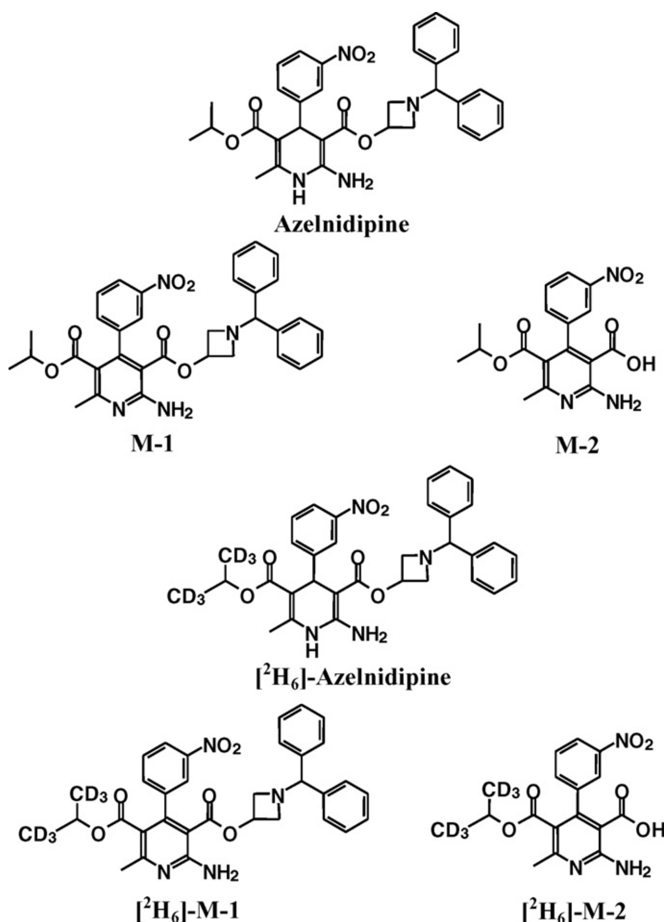

Fig. 1. Chemical structures of azelnidipine, M-1, M-2 and the internal standard (I.S.) substances.

single oral administration of CALBLOCK® tablets (16 mg) to healthy male volunteers.

2. Materials and methods

2.1. Standard substances and reagents

Azelnidipine (molecular weight: 582.6, purity: 99.7%), M-1 (molecular weight: 580.6, purity: 99.2%), M-2 (molecular weight: 359.3, purity: 99.9%) and the internal standard substances, [$^2H_6$]-azelnidipine (molecular weight: 588.6, purity: 99.7%), [$^2H_6$]-M-1 (molecular weight: 586.6, purity: 99.6%) and [$^2H_6$]-M-2 (molecular weight: 365.3, purity: 99.8%) were synthesized by the Pharmaceutical Research Department of Ube Laboratory, Ube Industries Ltd. (Yamaguchi, Japan). Methanol of HPLC grade and ethylene glycol, acetic acid, sodium acetate and hydrochloric acid of reagent grade were obtained from Wako Pure Chemical Industries Co. Ltd. (Osaka, Japan). Ethyl acetate and hexane (Kanto Kagaku Co. Ltd., Tokyo, Japan) were of analytical grade. Purified water was passed through a Milli-Q® purification system (Millipore Corp. Bedford, Massachusetts, USA) before use. Blank plasma was prepared by centrifugation (3000 rpm for 10 min at 5 °C) of the blood obtained from five healthy volunteers, and stored frozen at −20 °C until used.

2.2. Liquid chromatography operating conditions

Separation by HPLC was conducted using a Waters 2690 Separations Module (Waters Corp. Maple Street, Milford, USA) with an Inertsil® ODS-3 C_{18} (5 μm) column (2.1 mm i.d. × 150 mm, GL Sciences Inc. Tokyo, Japan). A mixture of methanol, water and acetic acid (800:200:0.2, v/v/v) was used as the mobile phase at a flow rate of 0.2 mL/min. The temperature of the auto-sampler chamber was kept at 6 °C.

2.3. Mass spectrometry operating conditions

A Finnigan TSQ-7000, API-2 triple quadrupole mass spectrometer (Finnigan Corp., San Jose, CA, USA) and the HPLC system were interfaced by using an electro-spray ionization (ESI) source. The mass spectrometer was operated in the positive ion detection mode with the spray voltage set at 4.5 kV. Nitrogen was used as the sheath gas (80 p.s.i.) and auxiliary gas (3 L/min). The heated capillary temperature was set at 240 °C. For collision-induced dissociation (CID), argon was used as a collision gas at a pressure of 1.4 mTorr. Quantification was performed using selected reaction monitoring (SRM) of the transitions of m/z 583 → m/z 167 for azelnidipine, m/z 581 → m/z 167 for M-1, m/z 360 → m/z 318 for M-2, m/z 589 → m/z 167 for [$^2H_6$]-azelnidipine, m/z 587 → m/z 167 for [$^2H_6$]-M-1 and m/z 366 → m/z 318 for [$^2H_6$]-M-2, with a scan time of 0.3 s per transition. The collision offset energy was optimized at +30 eV for azelnidipine, +35 eV for M-1, +20 eV for M-2, +30 eV for [$^2H_6$]-azelnidipine, +35 eV for [$^2H_6$]-M-1 and +20 eV for [$^2H_6$]-M-2, respectively. The peak widths of precursor and product ions were maintained at a mass unit of 0.6 at half-peak height in the SRM mode.

2.4. Data acquisition

Data acquisition and analysis were performed using Xcaliber 1.2 software (Finnigan, San Jose, CA, USA). Post-acquisition quantitative analyses were performed using LCquan ™ software (Finnigan, San Jose, CA, USA). The calibration curves were constructed from the peak area ratios of each analyte to the I.S. versus plasma concentration using a $1/X^2$ weighted linear least-squares regression model. Concentrations of each analyte in the quality control (QC) samples or unknown samples were subsequently extrapolated from these calibration curves.

2.5. Preparation of stock solutions and standard solutions

The primary stock solution containing azelnidipine, M-1 and M-2 in methanol at the same time at a concentration of 1.0 mg/mL was prepared for the standard solutions to generate calibration curves and for QC samples, and stored at 5 °C. The primary stock solution was diluted hundredfold with methanol to produce the stock solution containing azelnidipine, M-1 and M-2 at a final concentration of 10 μg/mL. The stock solution of the internal standard substances, containing [$^2H_6$]-azelnidipine, [$^2H_6$]-M-1 and [$^2H_6$]-M-2 each at a concentration of 1.0 μg/mL

at the same time, was prepared by dissolving 0.10 mg/mL of each I.S. in 100 mL methanol, and stored at 5 °C. The stock solution of I.S. was diluted tenfold with methanol to prepare the I.S. working solution (100 ng/mL). The standard solutions for the calibration curve of azelnidipine, M-1 and M-2 were prepared daily in methanol by appropriate dilution of the stock solution of azelnidipine, M-1 and M-2 at concentrations of 5.0, 10.0, 20.0, 50.0, 100.0, 200.0 and 400.0 ng/mL. Separately, the stock solution of azelnidipine, M-1 and M-2 was diluted with methanol to prepare the standard solutions for QC samples containing azelnidipine, M-1 and M-2 at concentrations of 5.0 ng/mL (Minimum: LLOQ), 15.0 ng/mL (Low), 150.0 ng/mL (Medium) and 300.0 ng/mL (High: ULOQ) to evaluate the intra- and inter-assay precision and accuracy of this assay method. All these working solutions, *i.e.*, the stock solution of azelnidipine, M-1 and M-2, the stock solution of I.S., the I.S. working solution, the standard solutions for the calibration curve of azelnidipine, M-1 and M-2 and the standard solutions for the QC samples were stored at 5 °C.

2.6. Standard samples for calibration curve and for QC samples

The standard samples for calibration curve containing azelnidipine, M-1 and M-2 simultaneously each at a final concentration of 0.5, 1.0, 2.0, 5.0, 10.0, 20.0 or 40.0 ng/mL together with three I.S. substances each at a final concentration of 10 ng/mL were prepared as follows. To a glass tube with screw cap, 10 μL of ethylene glycol, 100 μL of the I.S. working solution ([$^2H_6$]-azelnidipine, [$^2H_6$]-M-1 and [$^2H_6$]-M-2: 100 ng/mL in methanol) and 100 μL of either of the standard solutions for the calibration curve were added. The mixture was evaporated to dryness under a nitrogen gas stream at 40 °C in a Turbo Vap LV® evaporator (Zymark Corporation). To the residue, 1.0 mL of human plasma, 250 μL of 1.0 M acetate buffer and 50 μL of 0.2N hydrochloric acid at pH 4.0, were added. The mixture was stirred well using a vortex mixer, and extracted with 7 mL of a mixture of ethyl acetate and hexane (1:1, v/v) using a mechanical shaking for 10 min. The tubes were centrifuged at 3000 rpm for 10 min at 5 °C, and the organic layer was transferred to a round-bottomed polypropylene tube (FALCON?). The solvent was evaporated to dryness under a nitrogen gas stream at 40 °C in a Turbo Vap LV® evaporator. The residues were dissolved in 140 μL of methanol by ultrasonication for 3 min, and the solution was added with 60 μL of 0.2% acetic acid. After mixing the mixture well by a vortex mixer for 2 min, the tube was centrifuged at 3000 rpm for 5 min at 5 °C and the supernatant solution was transferred into a vial for auto-sampler. A 10 μL-aliquot of each sample was injected into the LC/MS/MS system. The QC samples containing azelnidipine, M-1 and M-2 at four different levels, minimum (0.5 ng/mL), low level (1.5 ng/mL), middle level (15 ng/mL) and high level (30 ng/mL) together with three I.S. substances (10 ng/mL) were prepared in triplicate in the same manner as described above for the standard samples for the calibration curve. The standard samples for calibration curve and the QC samples were prepared before use.

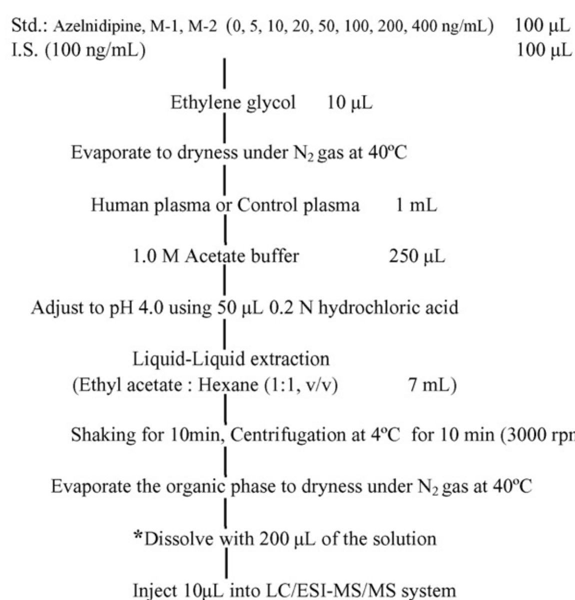

Fig. 2. Sample processing procedure for assay of azelnidipine, M-1 and M-2 in human plasma.

2.7. Procedure for plasma sample preparation

To a glass tube with screw cap, 10 μL of ethylene glycol, 100 μL of the I.S. working solution ([$^2H_6$]-azelnidipine, [$^2H_6$]-M-1 and [$^2H_6$]-M-2: 100 ng/mL in methanol) and 100 μL of methanol were added. The mixture was evaporated to dryness as described above. To the residue, 1.0 mL of human plasma, 250 μL of 1.0 M acetate buffer and 50 μL of 0.2N hydrochloric acid at pH 4.0 were added. The human plasma has been stored frozen at −80 °C and thawed at 25 °C for 10 min. Thereafter, the mixture was processed in the same manner as described above to prepare the final sample for analysis. A 10 μL-aliquot of each sample was injected into the LC/MS/MS system. The sample preparation procedure for the determination of azelnidipine, M-1 and M-2 is shown schematically in Fig. 2.

2.8. Method validation

The method was validated for precision (coefficient of variation, C.V.), accuracy, selectivity, specificity, linearity of the calibration curve, recovery, stability and reproducibility according to the recent paper by Shah *et al*. on bioanalytical method validation [14], using the standard samples for calibration curve over a concentration range of 0.5–40 ng/mL (seven concentrations) and five replicates of the QC samples at four concentration levels in three separate runs. Analyte stability was tested using the QC samples at a room temperature (25 °C) for short-term stability (6 h), at −80 °C in the freezer for a long-term stability (3 week) or at 6 °C in an auto-sampler for an auto-sampler stability (48 h). The stability of analytes after sample preparation and in the stock solutions (10 μg/mL) were also determined. For evaluation of the stability, the mean peak

area ratios of azelnidipine, M-1 and M-2 to I.S. were measured after storage and the residual ratio relative to the zero-time value were calculated in percentage to one decimal place. To calculate the extraction recoveries, the peak areas of azelnidipine, M-1 and M-2 relative to I.S. in the plasma sample after sample processing were determined, and compared to the peak areas of the analytes at corresponding concentrations without sample processing. The method specificity was evaluated by screening five lots of blank plasma containing sodium heparin.

2.9. Application to pharmacokinetic study

The LC/MS/MS method developed in the present study was used to investigate the plasma concentration–time profiles of azelnidipine, M-1 and M-2 after an oral administration of 16 mg of CALBLOCK® tablets (2 mg × 8 mg tablets). A clinical study on six healthy male volunteers was approved by the Institutional Review Board of Obara Hospital (Tokyo, Japan). Six healthy male volunteers with mean age of 21.7 ± 0.5 years, mean body weight of 63.7 ± 3.4 kg, and mean height of 172.3 ± 5.3 cm, participated in this study. The blood samples, each of 10 mL, were collected by venipuncture at 0 (pre-dose), 0.5, 1, 2, 3, 4, 6, 8, 12 and 24 h after administration. Following centrifugation (3000 rpm for 10 min at 5 °C), the plasma samples were collected and stored frozen at −80 °C until analysis. The pharmacokinetic parameters of azelnidipine, M-1 and M-2 were calculated by non-compartmental analysis using WinNonlin® (Pharsight Corp., Mountain View, CA, USA). The maximum plasma concentration (C_{max}) and the time of reaching C_{max} (t_{max}) were both obtained directly from the observed data. The area under the plasma concentration–time curve from time 0 to 24 h after administration ($AUC_{0–24}$) was calculated by the linear trapezoidal method. The terminal elimination rate constant (k_{el}) was estimated by linear least-squares regression of the last four points of the plasma concentration–time curve and the corresponding elimination half-life ($t_{1/2}$) was then calculated as $0.693/k_{el}$. The mean residence time ($MRT_{0–24}$) was calculated using $AUC_{0–24}$.

3. Results and discussion

3.1. Separation, specificity, sensitivity, and calibration curve range

Azelnidipine, M-1 and M-2 gave protonated precursor molecular ions $[M+H]^+$ in the positive ion-ESI mode in MS. The major ion observed was m/z 583 for azelnidipine, m/z 581 for M-1 and m/z 360 for M-2. The most intense product ions observed in the MS/MS spectra were m/z 167 for azelnidipine and M-1 and m/z 318 for M-2. The corresponding SRM ion spectra of azelnidipine, M-1 and M-2 are shown in Fig. 3. The retention times of azelnidipine, M-1 and M-2 in the chromatograms were 3.6, 10.2 and 6.8 min, respectively. Human blank plasma samples from five different subjects containing neither azelnidipine, M-1, M-2 or the I.S. substances were extracted as a true blank (double blank) and subjected to the analysis to detect any overlapping peaks with azelnidipine, M-1, M-2 and the I.S. substances. The blank plasma samples were added with the I.S. substances, and further spiked with azelnidipine M-1 or M-2 as a single blank. In the true blank plasma, there were no endogenous peaks overlapping the peaks of azelnidipine, M-1 and M-2 or the I.S. In the single blank plasma, there was no peak overlapping the peaks of azelnidipine, M-1 and M-2. The results demonstrated no interference in the quantification of azelnidipine, M-1 and M-2. The ratio of signal to noise obtained from the lower limit of quantification (LLOQ) sample (0.5 ng/mL) was at least 11 for azelnidipine, M-1 and M-2. Calibration curves for azelnidipine, M-1 and M-2 well fitted to the equations by quadratic regression with a weighting factor of the reciprocal of the concentration squared ($1/X^2$) in the concentration range of 0.5–40 ng/mL. Representative chromatograms of blank human plasma spiked only with the I.S. substances and blank human plasma spiked with azelnidipine, M-1, M-2 at the LLOQ and the I.S. substances are shown in Figs. 4 and 5.

3.2. Precision and accuracy

Table 1 shows the validation data on the precision (coefficient of variation, C.V.) and accuracy in the quantification of azelnidipine, M-1 and M-2 in the standard samples for calibration curve at seven concentrations. The C.V. values ($N = 5$) of the back-calculated concentrations in the standard samples for azelnidipine, M-1 and M-2 were 5.5, 2.3 and 2.2%, respectively, at the lowest concentration in the calibration curve and 1.1, 2.1 and 1.9%, respectively, at the highest concentration of the calibration curve.

The intra-assay accuracy ranged from 97.8 to 102.3% for azelnidipine, from 95.0 to 103.3% for M-1, and from 95.4 to 103.9% for M-2. The precision and accuracy data for the QC samples are summarized in Table 2. For the QC samples at 0.5 and 30 ng/mL, the intra-assay C.V. values were 6.7 and 4.3%, respectively, for azelnidipine, 3.8 and 1.9%, respectively, for M-1, and 11.9 and 4.8%, respectively, for M-2. The intra-assay accuracy ranged from 92.2 to 109.5% for azelnidipine, from 94.2 to 105.4% for M-1, and from 84.0 to 97.7% for M-2. Inter-assay C.V., for the QC samples at 0.5 and 30 ng/mL, were 8.3 and 4.8%, respectively, for azelnidipine; 4.7 and 2.7%, respectively, for M-1 and 13.9 and 4.6%, respectively, for M-2. The inter-assay accuracy ranged from 91.4 to 102.5% for azelnidipine, from 93.2 to 106.8% for M-1, and from 92.2 to 97.8% for M-2.

3.3. Extraction recovery and matrix effect

The extraction recoveries of azelnidipine, M-1 and M-2 after processing the human plasma sample were determined using the QC samples at concentrations of 0.5, 1.5, 15 and 30 ng/mL. The data are summarized in Table 3. Extraction recovery ranged from 68.8 to 78.6% for azelnidipine, from 54.3 to 62.9% for M-1 and from 80.4 to 89.7% for M-2. The C.V. values in the recovery ratio at 0.5 and 30 ng/mL were 3.7 and 5.4%, respectively, for azelnidipine, 0.0 and 4.4%, respectively, for M-1, and 13.8 and 2.3%, respectively, for M-2. The effects of ion suppression on the peaks of azelnidipine, M-1 and M-2 were examined at the concentration of 40 ng/mL ($n = 2$), and were not more than 22% for azelnidipine, M-1 and M-2, respectively (data not shown).

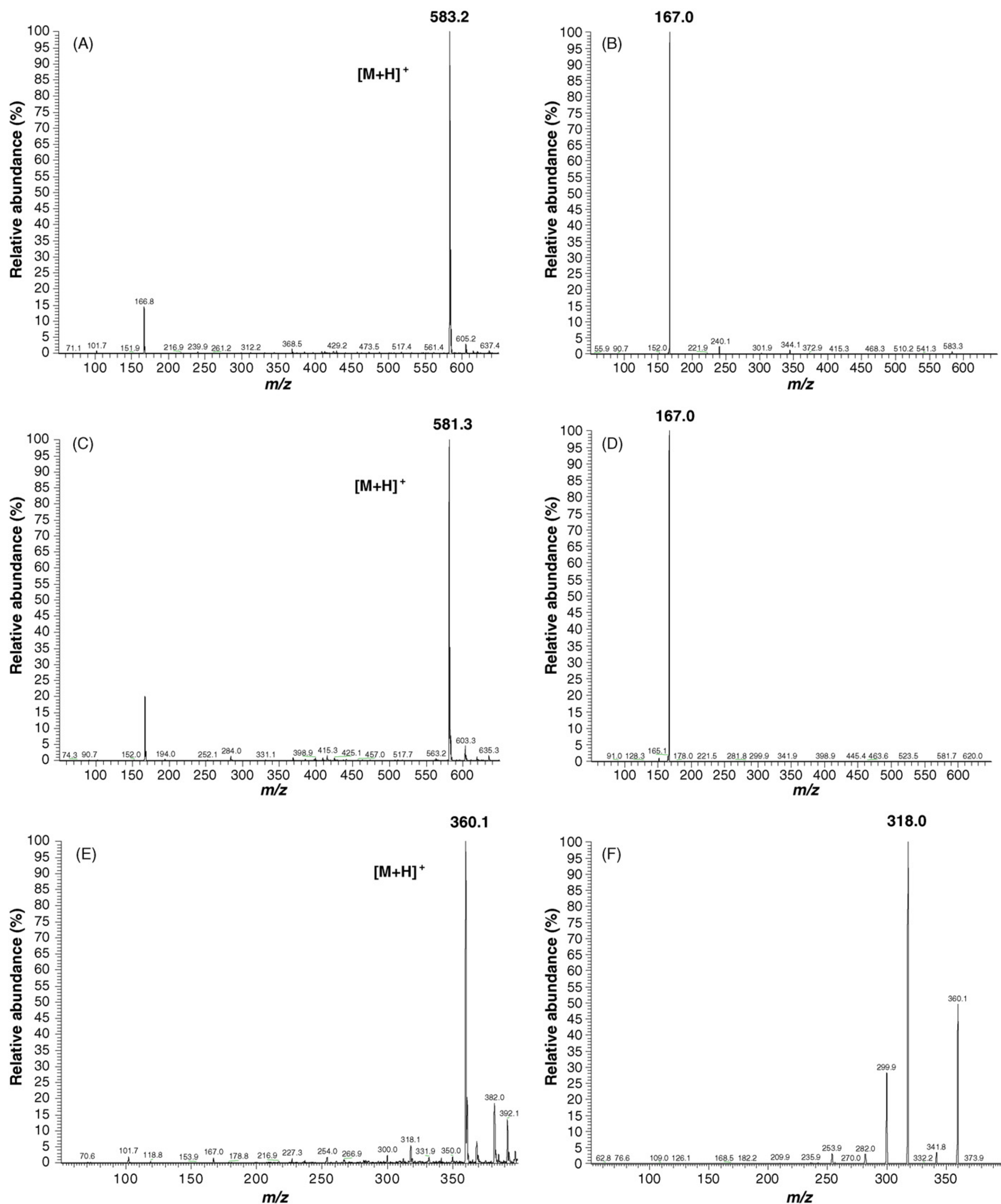

Fig. 3. Mass spectra of precursor ion of Azelnidipine (A), product ion of Azelnidipine (B), precursor ion of M-1 (C), product ion of M-1 (D), precursor ion of M-2 (E) and product ion of M-2 (F).

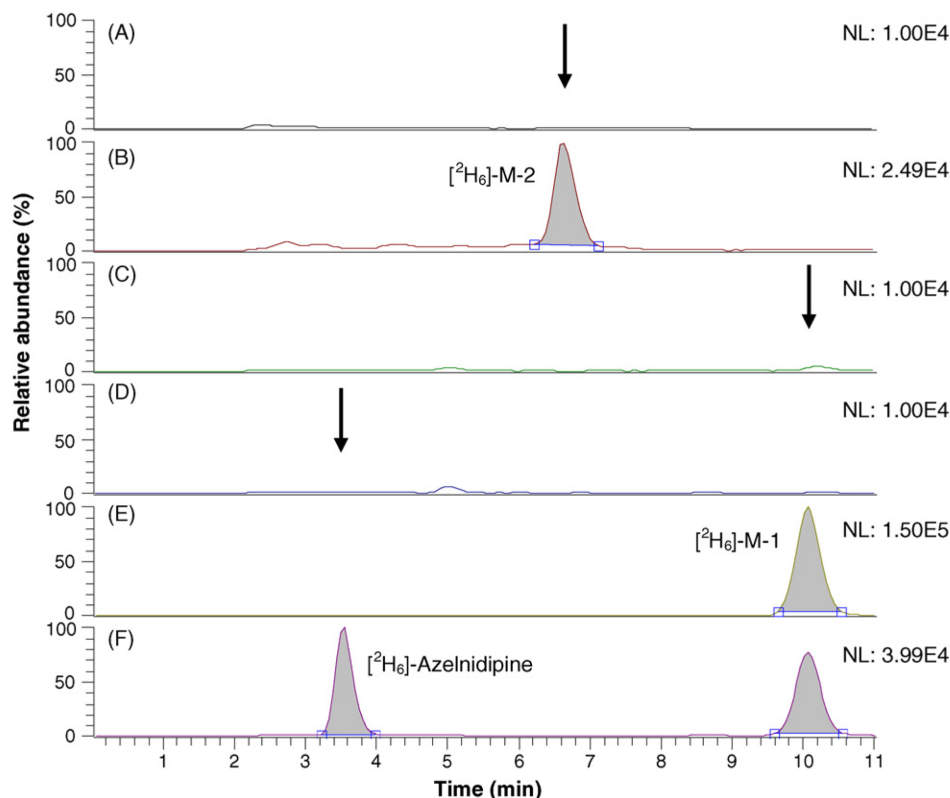

Fig. 4. SRM ion chromatograms of human plasma extract spiked with the I.S. substances only. (A) M-2: m/z 360 → m/z 318, (B) [$^2H_6$]-M-2: m/z 366 → m/z 318, (C) M-1: m/z 581 → m/z 167, (D) Azelnidipine: m/z 583 → m/z 167, (E) [$^2H_6$]-M-1: m/z 587 → m/z 167 and (F) [$^2H_6$]-azelnidipine: m/z 360 → m/z 318; ↓: Retention time of azelnidipine, M-1 and M-2.

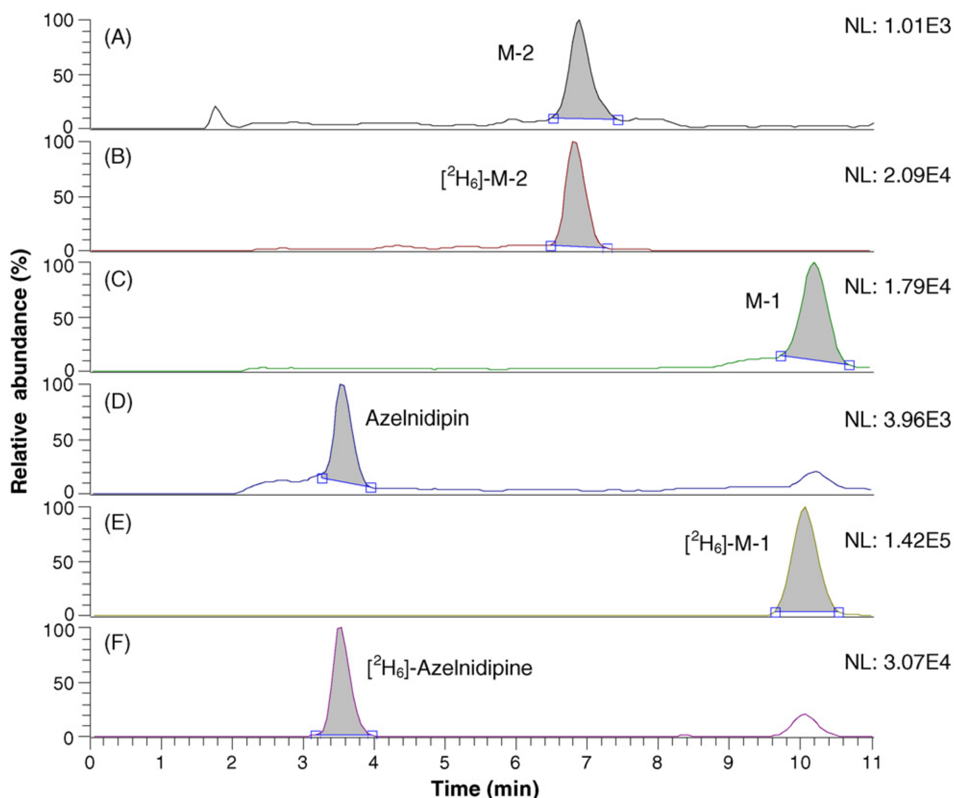

Fig. 5. SRM ion chromatograms of human plasma extract spiked with azelnidipine, M-1 and M-2 (0.5 ng/mL) and the I.S. substances (10 ng/mL). (A) M-2: m/z 360 → m/z 318, (B) [$^2H_6$]-M-2: m/z 366 → m/z 318, (C) M-1: m/z 581 → m/z 167, (D) azelnidipine: m/z 583 → m/z 167, (E) [$^2H_6$]-M-1: m/z 587 → m/z 167 and (F) [$^2H_6$]-azelnidipine: m/z 360 → m/z 318.

Table 1
Intra-assay precision and accuracy in assay of standard samples of azelnidipine, M-1 and M-2 for calibration curve ($N = 5$)

Nominal conc. (ng/mL)	Azelnidipine			M-1			M-2		
	Mean	C.V. (%)	Accuracy (%)	Mean	C.V. (%)	Accuracy (%)	Mean	C.V. (%)	Accuracy (%)
0.5	0.49	5.52	97.80	0.48	2.32	95.00	0.49	2.24	98.20
1	1.00	4.39	100.20	1.03	2.52	103.30	1.04	3.95	103.90
2	2.04	3.13	102.10	2.06	2.23	103.10	1.97	2.69	98.65
5	4.85	2.68	97.02	5.01	2.28	100.22	5.01	1.18	100.10
10	10.24	1.11	102.37	10.28	0.51	102.77	10.29	1.55	102.86
20	20.13	1.88	100.67	19.86	1.55	99.28	20.17	1.20	100.83
40	39.93	1.12	99.83	38.52	2.13	96.30	38.16	1.98	95.40

Table 2
Intra-assay and inter-assay precision and accuracy in assay of QC samples

Nominal conc. (ng/mL)	Azelnidipine			M-1			M-2		
	Mean	C.V. (%)	Accuracy (%)	Mean	C.V. (%)	Accuracy (%)	Mean	C.V. (%)	Accuracy (%)
Intra-assay ($N = 15$)									
0.5	0.46	6.73	92.20	0.47	3.80	94.80	0.42	11.91	84.00
1.5	1.64	8.76	109.53	1.58	1.77	105.4	1.41	4.96	94.13
15	14.93	2.57	99.56	14.71	2.26	98.05	14.66	3.39	97.75
30	29.72	4.35	99.05	28.28	1.94	94.28	27.61	4.85	92.03
Inter-assay ($N = 15$)									
0.5	0.46	8.32	91.40	0.47	4.72	93.20	0.47	13.92	93.40
1.5	1.54	8.45	102.53	1.60	3.31	106.87	1.46	5.76	97.20
15	14.94	3.55	99.63	15.04	2.36	100.26	14.67	3.56	97.82
30	28.56	4.84	95.19	28.65	2.77	95.51	27.68	4.64	92.27

Table 3
Summary of extraction recoveries of azelinidipine, M-1 and M-2 ($N = 3$)

Nominal conc. (ng/mL)	Azelnidipine			M-1			M-2		
	Mean	S.D.	C.V. (%)	Mean	S.D.	C.V. (%)	Mean	S.D.	C.V. (%)
0.5	68.89	2.55	3.70	58.18	0.00	0.00	87.45	12.07	13.80
1.5	78.42	7.95	10.13	60.20	1.22	2.03	80.70	1.68	2.09
15	78.65	3.78	4.81	62.98	0.95	1.50	80.46	9.79	12.16
30	78.20	4.23	5.41	54.33	2.39	4.40	89.78	2.09	2.33

Recovery calculated as [(peak area ratio of spiked sample after sample processing)/(peak area ratio of spiked sample without sample processing)] × 100.

3.4. Stability

The stability tests of the analytes were designed to cover anticipated conditions for preservation of the clinical samples. Stability was assessed at four concentrations (0.5, 1.5, 15 and 30 ng/mL) as shown in Table 4. The short-term (6 h, 25 °C), long-term (3 week, −80 °C) and auto-sampler stability (48 h, 6 °C) indicated reliable stability behavior of all three analytes under

Table 4
Summary of stability test on azelnidipine, M-1 and M-2 ($N = 5$)

Nominal conc. (ng/mL)	Azelnidipine				M-1				M-2			
	0.5	1.5	15	30	0.5	1.5	15	30	0.5	1.5	15	30
6 h, short-term at 25 °C												
Residual (%)	83.08	105.88	96.99	95.27	92.31	93.75	97.32	96.83	86.84	95.46	99.52	102.45
C.V. (%)	1.85	3.33	0.93	2.48	2.08	0.67	2.21	2.29	3.03	2.86	3.09	0.29
3 weeks, long-term at −80 °C												
Residual (%)	88.89	86.54	93.70	90.29	82.54	88.89	104.44	101.04	94.81	104.76	99.04	100.72
C.V. (%)	12.50	0.74	2.22	5.73	3.85	3.75	3.76	2.44	2.74	2.73	2.17	3.10
48 h, auto-sampler at 6 °C												
Residual (%)	103.70	95.83	97.14	95.99	100.00	100.00	103.45	104.30	89.87	95.71	91.30	93.74
C.V. (%)	3.57	4.89	1.71	2.90	4.00	1.89	0.27	2.89	2.82	2.69	2.70	4.30

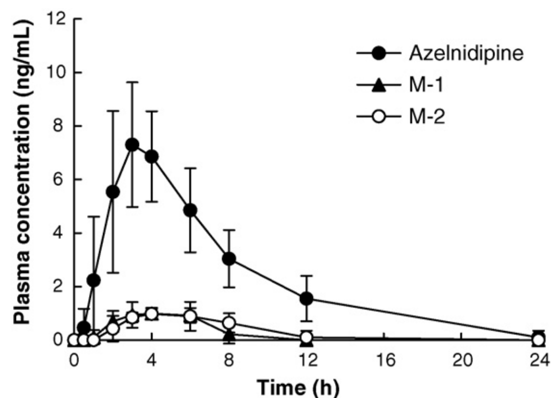

Fig. 6. Mean plasma concentrations of azelnidipine, M-1 and M-2 after an oral dose of 16 mg CALBLOCK® tablets to healthy male volunteers (mean ± S.D., $n = 6$).

the experimental conditions evaluated. The short-term stability, expressed as percentages of the corresponding nominal values, was 83.0–105.8% for azelnidipine, 92.3–97.3% for M-1 and 86.8–102.4% for M-2, allowing us to conclude that the analytes in the processed samples are stable for at least 6 h at room temperature (25 °C). The long-term stability was 86.5–93.7% for azelnidipine, 82.5–104.4% for M-1 and 94.8–104.7% for M-2. Therefore, it was concluded that azelnidipine, M-1 and M-2 in the plasma samples are stable for at least 3 weeks when stored frozen in a freezer (−80 °C). The auto-sampler stability ranged from 95.9 to 103.7% for azelnidipine, from 100.0 to 104.3% for M-1 and from 89.8 to 95.7% for M-2, and demonstrated that azelnidipine, M-1 and M-2 are stable for at least 48 h at 6 °C. The stability of azelnidipine, M-1 and M-2 in the stock solutions (10 μg/mL), stored at 5 °C for 0, 2 and 3 weeks, was also investigated. The results after 3 weeks were 97.8% for azelnidipine, 89.7% for M-1 and 113.9% for M-2, demonstrating that the analytes in the stock solutions are stable (data after storage for 0 and 2 weeks not shown).

3.5. Application of the assay method to pharmacokinetic study

The assay method established in the present study was successfully applied to determine the plasma concentrations of azelnidipine, M-1 and M-2 after single oral administration of 16 mg of CALBLOCK® tablets (2 mg × 8 mg tables) to six healthy non-smoking male volunteers. The assay method was robust as the analysis of more than 60 plasma samples was achieved without problem. The mean plasma concentration–time curves of azelnidipine, M-1 and M-2 are presented in Fig. 6. The pharmacokinetic parameters (C_{max}, t_{max}, $AUC_{0–24}$, $t_{1/2}$ and $MRT_{0–24}$) are presented in Table 5.

Table 5
Pharmacokinetic parameters of azelnidipine, M-1 and M-2 after an oral dose of 16 mg CALBLOCK® tablet to six male volunteers (mean ± S.D.)

Parameter	Azelnidipine	M-1	M-2
C_{max} (ng/mL)	7.70 ± 1.78	1.12 ± 0.23	1.11 ± 0.34
T_{max} (h)	3.33 ± 1.37	4.33 ± 1.86	4.50 ± 1.22
$t_{1/2}$ (h)	4.03 ± 1.18	5.41 ± 1.38	7.06 ± 2.16
$MRT_{0–24}$ (h)	5.52 ± 1.01	4.19 ± 0.57	4.88 ± 0.94
$AUC_{0–24}$ (ng h/mL)	49.15 ± 19.45	4.76 ± 2.12	5.63 ± 3.48

4. Conclusions

An LC/MS/MS method for the simultaneous determination of azelnidipine, M-1 and M-2 in human plasma has been successfully developed and validated in the present study. The method was simple, rapid and robust. The lower limit of quantification was 0.5 ng/mL for azelnidipine, M-1 and M-2 using a 1.0 mL-sample aliquot. The analytes were stable in the freezer, at room temperature, and in the auto-sampler without showing any significant degradation for the period evaluated. The method was suitable for simultaneous routine quantification of azelnidipine, M-1 and M-2 in human plasma over a concentration range of 0.5–40 ng/mL, and has been successfully used as the assay method in the pharmacokinetic studies in humans.

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