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Enantioselective determination of azelnidipine in human plasma using liquid chromatography–tandem mass spectrometry

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Abstract

A sensitive and simple method was developed for determination of the enantiomers of azelnidipine, (*R*)-(−)-azelnidipine and (*S*)-(+)-azelnidipine, in human plasma using chiral liquid chromatography with positive ion atmospheric pressure chemical ionization tandem mass spectrometry. Plasma samples spiked with stable isotope-labeled azelnidipine, [$^2H_6$]-azelnidipine, as an internal standard, were processed for analysis using a solid-phase extraction in a 96-well plate format. The azelnidipine enantiomers were separated on a chiral column containing α_1-acid glycoprotein as a chiral selector under isocratic mobile phase conditions. Acquisition of mass spectrometric data was performed in multiple reaction monitoring mode, monitoring the transitions from *m/z* 583 → 167 for (*R*)-(−)-azelnidipine and (*S*)-(+)-azelnidipine, and from *m/z* 589 → 167 for [$^2H_6$]-azelnidipine. The standard curve was linear over the studied range (0.05–20 ng/mL), with $r^2 > 0.997$ using weighted ($1/x^2$) quadratic regression, and the chromatographic run time was 5.0 min/injection. The intra- and inter-assay precision (coefficient of variation), calculated from the assay data of the quality control samples, was 1.2–8.2% and 2.4–5.8% for (*R*)-(−)-azelnidipine and (*S*)-(+)-azelnidipine, respectively. The accuracy was 101.2–117.0% for (*R*)-(−)-azelnidipine and 100.0–107.0% for (*S*)-(+)-azelnidipine. The overall recoveries for (*R*)-(−)-azelnidipine and (*S*)-(+)-azelnidipine were 71.4–79.7% and 71.7–84.2%, respectively. The lower limit of quantification for both enantiomers was 0.05 ng/mL using 1.0 mL of plasma. All the analytes showed acceptable short-term, long-term, auto-sampler and stock solution stability. Furthermore, the method described above was used to separately measure the concentrations of the azelnidipine enantiomers in plasma samples collected from healthy subjects who had received a single oral dose of 16 mg of azelnidipine.

Keywords: Azelnidipine; Enantiomer separation; Quantitative analysis; LC/APCI–MS/MS

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 channel antagonistic activity. Azelnidipine has two enantiomers due to an asymmetric carbon at the 4-position of the 1, 4-dihydropyridine ring (Fig. 1). In 2003, this drug was launched into the market as CALBLOCK ® in Japan. It is generally accepted that the (*R*)-(−) enantiomers of dihydropyridine calcium blockers possess intrinsic pharmacological activity [1], and it is therefore desirable to perform selective measurements of the enantiomers in their plasma concentrations. Previously, we have reported the plasma concentrations of azelnidipine and the metabolites following an oral administration of azelnidipine to healthy volunteers [2], while stereoselective measurement of the plasma levels of *R*-(−)- and *S*-(+)-enantiomers of azelnidipine has not yet been performed. Historically, numerous methods have been established for the quantitative determination of dihydropyridine calcium blockers as racemate in plasma, including high-performance liquid chromatography (HPLC) or gas chromatography coupled with mass spectrometry (HPLC or GC–MS) [3–9] or HPLC coupled with tandem mass spectrometry (LC–MS/MS) [10], and these were followed subsequently by the development of an assay of their enantiomers as in the case of amlodipine, felodipine and benidipine in human plasma, determined by LC–MS/MS using a chiral separation column [11–13].

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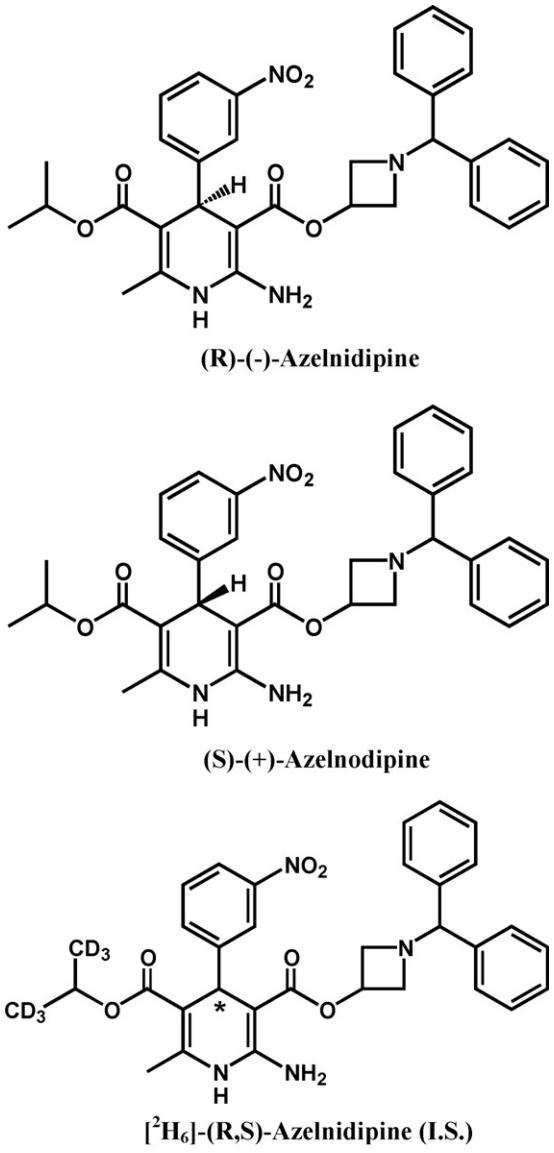

Fig. 1. Chemical structures of (*R*)-(−)-azelnidipine and (*S*)-(+)-azelnidipine, and the internal standard (I.S.).

In this study, we developed an ultrasenstive method by liquid chromatography atmospheric pressure chemical ionization tandem mass spectrometry (LC/APCI–MS/MS) for determination of (*R*)-(−)-azelnidipine and (*S*)-(+)-azelnidipine in human plasma, using deutellium-labeled azelnidipine ([$^2H_6$]-azelnidipine) as an internal standard and a chiral column containing α_1-acid glycoprotein as a stationary phase for chiral separation. Briefly, plasma samples were spiked with azelnidipine and [$^2H_6$]-azelnidipine, extracted by a solid-phase extraction using a 96-well plate format, and the plasma extracts were chromatographed on an HPLC system with the chiral column. Following separation, the (*R*)-(−)-azelnidipine, (*S*)-(+)-azelnidipine and the internal standard were selectively detected using APCI–MS/MS. The method has a lower limit of quantification (LLOQ) of 0.05 ng/mL for (*R*)-(−)-azelnidipine and (*S*)-(+)-azelnidipine, using a plasma sample volume of 1.0 mL. The LC/APCI–MS/MS method has a relatively short run time of 5 min/sample for high throughput. To the best of our knowledge, no quantitative LC/APCI–MS/MS methods suitable for the routine analysis of (*R*)-(−)- and (*S*)-(+)-isomers of azelnidipine have been reported yet.

This assay method was successfully applied to measurements of plasma concentrations of (*R*)-(−)-azelnidipine and (*S*)-(+)-azelnidipine after a single oral administration of 16 mg of azelnidipine (CALBLOCK® Tablets, 2 × 8 mg) to healthy male volunteers.

2. Materials and methods

2.1. Standard substances, reagents and blank plasma

(*R*)-(−)-azelnidipine and (*S*)-(+)-azelnidipine (molecular weight 582.6), and the internal standard substance, [$^2H_6$]-azelnidipine (molecular weight 588.6), were synthesized by the Pharmaceutical Research Department of Ube Laboratory, Ube Industries Ltd., (Yamaguchi, Japan). Methanol of HPLC grade and formic acid, ammonium acetate and hydrochloric acid of reagent grade were obtained from Wako Pure Chemical Industries Co. Ltd., (Osaka, Japan). Oasis® HLB 96-well plates were from Waters Corporation (Milford, MA U.S.A.). Purified water was further purified by a Milli-Q® purification system (Millipore Corporation, MA, U.S.A.) before use. Pooled blank plasma was prepared by centrifugation (3000 rpm, 10 min, 4 °C) of the blood obtained from five healthy male volunteers, and stored frozen at −80 °C until used. For the purpose of examining the specificity of the assay method, commercially available frozen plasma samples collected from six individuals with heparin sodium were used (Kohjin Bio Co. Ltd., Tokyo, Japan).

2.2. Operating conditions for HPLC with chiral column

Chiral separation by HPLC was conducted using a Waters 2690 Separations Module (Waters, Milford, MA, U.S.A.) with a chiral column containing α_1-acid glycoprotein (ULTRON ES-OVM, 4.6 mm in internal diameter and 150 mm in height with a particle size of 5 μm, Shinwa Chemical Industries Ltd., Tokyo, Japan). A mixture of methanol, water and 5 mM ammonium acetate (500/500/0.19, v/v/w) was used as a mobile phase at a flow rate of 1.0 mL/min for isocratic elution. The column temperature was maintained at 30 °C, and the temperature in the auto-sampler chamber was kept at 5 °C.

2.3. Operating conditions for mass spectrometer

A mass spectrometer (Micromass Quattro II: Micromass Manchester, UK) equipped with an atmospheric pressure chemical ionization (APCI) source in the positive ion mode was set up for data acquisition of transitions from *m/z* 583 → *m/z* 167 for (*R*)-(−)-azelnidipine and (*S*)-(+)-azelnidipine and from *m/z* 589 → *m/z* 167 for the internal standard by multiple reaction monitoring (MRM). Figs. 2–4 show the mass spectra (upper panel) and the product ion spectra (lower panel) obtained from (*R*)-(−)-azelnidipine, (*S*)-(+)-azelnidipine, and the internal standard, respectively. The proposed fragmentation (Fig. 5) is simply

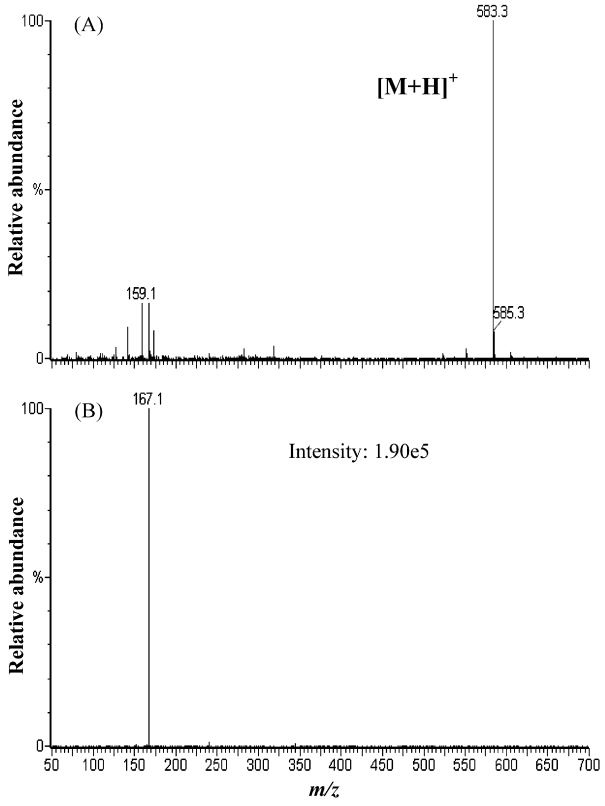

Fig. 2. APCI positive ion mass spectra of (*R*)-(−)-azelnidipine. (A) Mass spectrum. (B) Product ion spectrum.

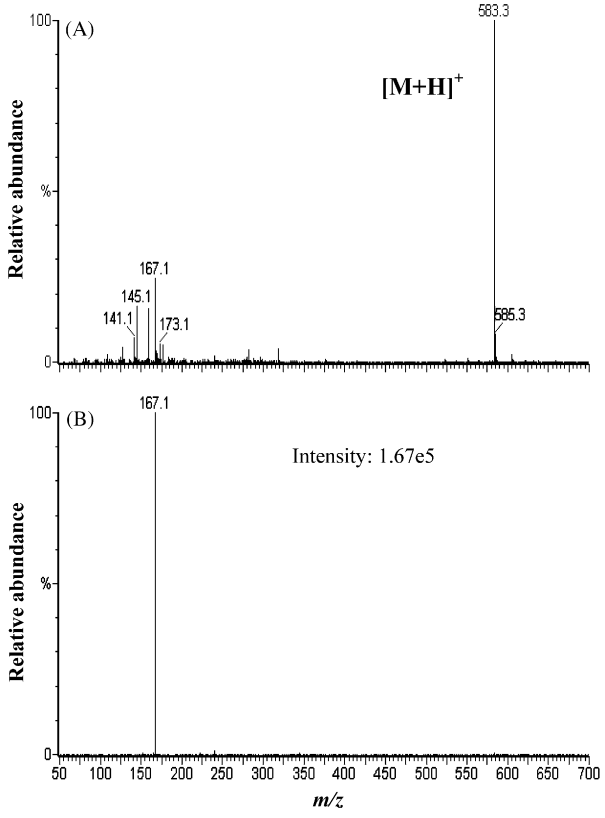

Fig. 3. APCI positive ion mass spectra of (*S*)-(+)-azelnidipine. (A) Mass spectrum. (B) Product ion spectrum.

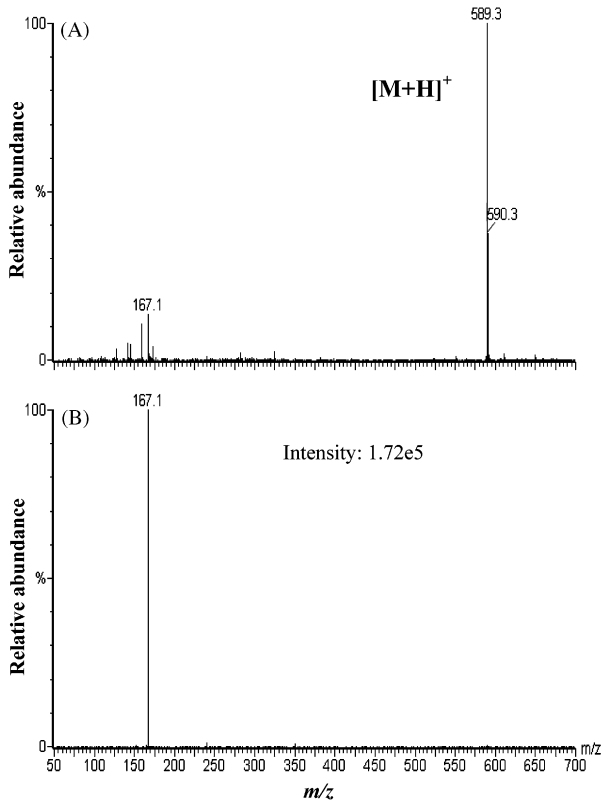

Fig. 4. APCI positive ion mass spectra of I.S. (A) Mass spectrum. (B) Product ion spectrum.

the production of the diphenylmethyl ion from the protonated molecular ion as the most abundant fragment ion in the transitions from *m/z* 583 → *m/z* 167 in (*R*)-(−)-azelnidipine and (*S*)-(+)-azelnidipine and from *m/z* 589 → *m/z* 167 in [$^2H_6$]-azelnidipine. The intensity of the peak detected in the APCI mode was greater than that detected in an electrospray ionization mode (data not shown). The operating conditions were optimized by using solutions of azelnidipine and the internal standard included a dwell time of 0.3 s, capillary voltage of 3.2 kV, cone voltage of 40 V, source temperature of 100 °C, desolvation temperature of 500 °C and collision energy of 26 eV.

2.4. Data acquisition

Data acquisition and analysis were performed using the software MassLynx (version 3.5) running under Windows NT (version 4.0). The calibration curves for (*R*)-(−)-azelnidipine and (*S*)-(+)-azelnidipine were constructed separately from the peak area ratio of each analyte to the internal standard versus the nominal plasma concentration using a linear least-squares regression with a weight of $1/x^2$. Concentrations of each analyte in the quality control (QC) samples or unknown samples were determined from these calibration curves.

2.5. Preparation of stock solutions and standard solutions

A primary stock solution containing 1.0 mg/mL of (*R*)-(−)-azelnidipine and 1.0 mg/mL of (*S*)-(+)-azelnidipine was

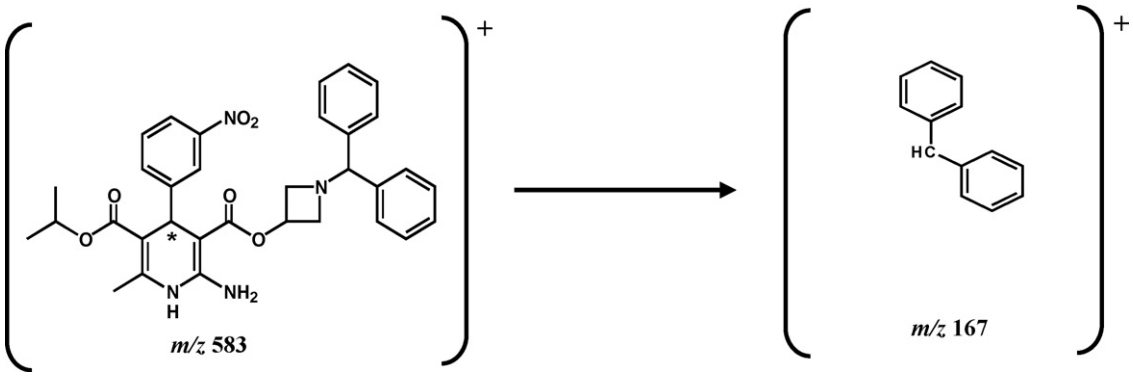

Fig. 5. Proposed fragmentation pathway for (*R*)-(−)-azelnidipine and (*S*)-(+)-azelnidipine.

prepared in methanol, and diluted with methanol to prepare a stock solution containing 10 μg/mL of (*R*)-(−)-azelnidipine and 10 μg/mL of (*S*)-(+)-azelnidipine, which was stored at 5 °C. The standard solutions of (*R*)-(−)-azelnidipine and (*S*)-(+)-azelnidipine for the calibration curves were prepared daily by appropriate dilution of the stock solution with methanol at concentrations of 0.5, 1.0, 5.0, 20, 50, 100 and 200 ng/mL. The standard solutions for the QC samples for evaluation of the intra-assay and inter-assay precision and accuracy were prepared by diluting the stock solution with methanol at minimum (LLOQ 0.5 ng/mL), low (1.5 ng/mL), medium (60 ng/mL), and high (ULOQ 140 ng/mL) concentrations. In a similar manner, a primary stock solution containing 1.0 mg/mL of the internal standard substance ($[^2H_6]$-azelnidipine) was prepared in methanol, and diluted with methanol to prepare a stock solution containing 1.0 μg/mL of the internal standard, which was stored at 5 °C. A working solution of the internal standard containing 100 ng/mL of $[^2H_6]$-azelnidipine was prepared immediately before use by diluting the stock solution of the internal standard with methanol.

2.6. Standard samples for calibration curves and QC samples

The standard samples for the calibration curves containing 0.5, 1, 5, 20, 50, 100 and 200 ng/mL of (*R*)-(−)-azelnidipine and (*S*)-(+)-azelnidipine were prepared by mixing 1.0 mL of the control plasma with 0.1 mL of one of the above-mentioned standard solutions. The QC samples were prepared at four different concentrations of (*R*)-(−)-azelnidipine and (*S*)-(+)-azelnidipine, i.e. minimum, low, medium, and high concentrations (0.5, 15, 60, and 140 ng/mL, respectively). The QC samples were prepared daily by adding 0.1 mL of one of the standard solutions for QC samples to 1.0 mL of the control plasma ($n = 5$ for each concentration).

2.7. Procedure for plasma sample preparation

A 1.0 mL-aliquot each of the plasma samples, collected from six healthy male volunteers after oral administration of 16 mg of azelnidipine (CALBLOCK®), was placed in a polypropylene 96-well (2 mL) collection plate (Waters Corporation, MA). To each well, 0.98 mL of 0.01 N hydrochloric acid, 0.1 mL of methanol, and 0.02 mL of the working solution of the internal standard was added, and the collection plate was mechanically shaken to mix the samples well. The plasma samples were transferred manually to an Oasis HLB 96-well plate with each well containing 30 mg of adsorbent (Waters Corporation, MA), which had been pre-conditioned by washing each well twice with 1.0 mL of methanol, followed by washing each well twice with 1.0 mL of distilled water. Solid phase extraction of the plasma samples was performed by a sample suction using a vacuum manifold and vacuum source. After solid phase extraction, each well was washed slowly (0.5 mL/min) twice with 1.0 mL of distilled water containing 5% methanol by suction. The Oasis HLB 96-well plates were thoroughly dried by vacuum (10–15 mm Hg) for 2 min, and the analytes were eluted with 1.0 mL of methanol containing 0.1% formic acid. Each elute was collected into a 96-well (2 mL) collection plate, and evaporated to dryness under a nitrogen gas stream at 40 in a Turbo Vap® 96 evaporator (Zymark, Hopkinton, MA). To the residue, 0.1 mL of methanol and 0.1 mL of 5 mM ammonium acetate were added successively. At each time after the additions, the plate was shaken mechanically for 2–3 min. The collection plate was set in an auto-sampler, and a 20 μL-aliquot of the sample was injected into the LC/APCI–MS/MS system. To generate the standard curves, the standard samples for the calibration curves (0.1 mL) were transferred into the wells of the 96-well collection plate and processed in the same manner as described above. The QC samples were also processed in the same manner as described above. To prepare a zero plasma sample, which was used in each run as the standard sample at a concentration of 0 ng/mL, 1 mL of the control plasma was processed in the same manner as described above. As a double blank or a true blank sample to examine the specificity of the assay method, 1 mL of the control plasma was processed in the same manner as described above with the exception of 0.02 mL of the working solution of the internal standard, which was replaced with 0.1 mL of methanol.

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 FDA guidance for bioanalytical method validation for human studies [14], over a concentration range of 0.05–20 ng/mL using standard samples for the calibration curves at seven different concentrations ($n = 5$) and for the QC samples at four different concentrations ($n = 5$). The stability of the analytes was tested using the QC samples at 25 °C (short-term stability, up to 6 h), at −80 °C in a freezer (long-term stability, up to 3 weeks) or at 5 °C in an auto-sampler (up to 48 h). Post-preparative stability and stock solution stability (10 μg/mL) were also determined. Extraction recoveries of (*R*)-(−)-azelnidipine and (*S*)-(+)-azelnidipine were calculated by comparing the peak areas of the extracts of the QC samples, reconstituted in the mobile phase for HPLC, to the peak areas of the extracts of blank plasma spiked at corresponding concentrations after extraction. To investigate the ion suppression, the peak area of the blank plasma extract, which was spiked with 20 ng/mL of (*R*)-(−)-azelnidipine, 20 ng/mL of (*S*)-(+)-azelnidipine and 5 ng/mL of the internal standard, and reconstituted in the mobile phase for HPLC, was compared with the peak area of the mobile phase containing 20 ng/mL of (*R*)-(−)-azelnidipine, 20 ng/mL of (*S*)-(+)-azelnidipine and 2 ng/mL of the internal standard. The specificity of the assay method was evaluated by screening commercially available plasma samples collected separately from six individuals.

2.9. Application to pharmacokinetic study

The assay method by LC–MS/MS developed in the present study was used to investigate the plasma concentration-time profiles of (*R*)-(−)-azelnidipine and (*S*)-(+)-azelnidipine after an oral administration of 16 mg of azelnidipine (CALBLOCK® Tablets 8 mg, 2 tablets) to healthy volunteers in a clinical study conducted in Obara Hospital (Tokyo, Japan). Six healthy male volunteers with mean ages of 21.7 ± 0.5 years, mean body weights of 63.7 ± 3.4 kg, and mean heights of 172.3 ± 5.3 cm, participated in the study. Blood (5 mL) was removed by venipuncture prior to administration and 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), the plasma was collected and stored at −80 °C until analysis. The pharmacokinetic parameters of (*R*)-(−)-azelnidipine and (*S*)-(+)-azelnidipine were calculated by non-compartmental analysis using WinNonlin® (Pharsight Corporation, Mountain View, CA, USA). The maximum plasma concentration (C_{max}) and the time to reach C_{max} (T_{max}) were obtained directly from the data collected. The area under the plasma concentration–time curve from time 0 to 24 h after administration ($AUC_{0\text{-}24}$) was calculated by a 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 ($MRT_{0\text{–}24}$) was calculated using $AUC_{0\text{–}24}$.

3. Results and discussion

3.1. Separation of (R)-(−)-azelnidipine and (S)-(+)-azelnidipine by chiral column

In separating the enantiomers by HPLC equipped with a chiral column containing α_1 acid glycoprotein, the proportion of the organic modifiers (generally methanol), the buffer concentration (generally ammonium acetate), and the buffer pH in the mobile phase strongly influence the retention time and enantioselectivity in HPLC, as well as the intensity in the subsequent mass spectrometric detection [15,16]. Regarding the proportion of organic solvent in the mobile phase, we used 50% methanol in ammonium acetate, as this concentration has been generally employed in chiral chromatography using a column containing α_1 acid glycoprotein [15,16]. The addition of higher concentrations of ammonium acetate in the mobile phase up to 10 mM resulted in a lower resolution between (*R*)-(−)-azelnidipine and (*S*)-(+)-azelnidipine with a shorter retention time and a slightly increased signal intensity in LC–MS/MS analysis (data not shown). We selected 2.5 mM ammonium acetate in the mobile phase in order to achieve the highest resolution of the azelnidipine enantiomers. Experiments to examine the effect of pH ranging from 4 to 7 on the resolution of the azelnidipine enantiomers demonstrated that the retention of the azelnidipine enantiomers increases with increasing pH without much difference in the resolution (data not shown). We decided to use 2.5 mM ammonium acetate containing 50% methanol without pH adjustment in an isocratic mode as the mobile phase, since the pH of the unadjusted mobile phase containing 2.5 mM ammonium acetate and 50% methanol was about 7.0. Under these conditions, the azelnidipine enantiomers were resolved almost completely within 5 min with (*S*)-(+)-azelnidipine being eluted earlier than (*R*)-(−)-azelnidipine as shown later (Fig. 7).

3.2. Specificity, sensitivity, and calibration curve range

Human blank plasma samples collected from six different subjects, used as a double blank (true blank) sample to examine the specificity of the assay method, were extracted and analyzed by the assay method for (*R*)-(−)-azelnidipine and (*S*)-(+)-azelnidipine as described in the materials and methods (Fig. 6). As shown in Fig. 6, there were no peaks at retention times of (*R*)-(−)-azelnidipine, (*S*)-(+)-azelnidipine and the internal standard substance in the extract of human blank plasma. Separately, in the measurement of the QC sample at a lower limit of quantification (LLOQ) (0.05 ng/mL), two peaks of (*R*)-(−)-azelnidipine and (*S*)-(+)-azelnidipine together with those of the internal standard substance (100 ng/mL) were clearly observed as shown in Fig. 7, with a signal to noise ratio of more than 100, demonstrating the high sensitivity of this assay method. The calibration curves were fitted to quadratic regression with a weighting factor of the reciprocal of the squared concentration ($1/x^2$) for (*R*)-(−)-azelnidipine and (*S*)-(+)-azelnidipine in the concentration range of 0.05–20 ng/mL. Data on the calibration curves are described in the following section.

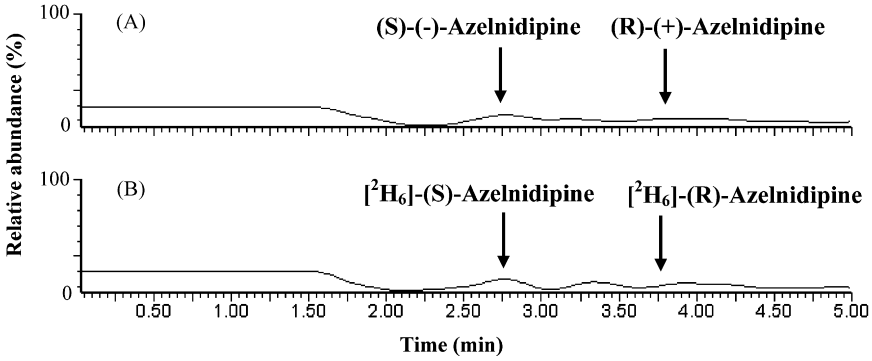

Fig. 6. MRM ion chromatograms of human blank plasma extract. (A) (*R*)-azelnidipine and (*S*)-azelnidipine: *m/z* 583 → *m/z* 167. (B) [$^2H_6$]-(*R* and *S*)-azelnidipine: *m/z* 589 → *m/z* 167 (▼): retention time.

3.3. Precision and accuracy

Table 1 shows the validation data on the precision (coefficient of variation, C.V.) and accuracy in the assay of each standard sample for the calibration curves. The C.V. values of the back-calculated standard sample at 0.05 ng/mL ($N = 5$) were low, at 5.3 and 6.7% for (*R*)-(−)-azelnidipine and (*S*)-(+)-azelnidipine, respectively, and those values of the back-calculated standard sample at 20 ng/mL were 3.2 and 2.3%, respectively. The accuracy ranged from 95.4 (at LLOQ) to 90.0% for (*R*)-(−)-azelnidipine and from 110.0 (at HLOQ) to 109.5% for (*S*)-(+)-azelnidipine. The precision and accuracy data for the QC samples are summarized in Table 1. For the QC samples at 0.05 (LLOQ) and 14 ng/mL, the intra-assay C.V. values were 1.2 and 3.4%, respectively, for (*R*)-(−)-azelnidipine and 2.8 and 2.4%, respectively, for (*S*)-(+)-azelnidipine ($N = 5$). The accuracy ranged from 117.0 (at LLOQ) to 101.6% for (*R*)-(−)-azelnidipine and from 104.8 (at LLOQ) to 105.0% for (*S*)-(+)-azelnidipine. The inter-assay C.V for the QC samples at 0.05 and 14 ng/mL were 8.2 and 6.2%, respectively, for (*R*)-(−)-azelnidipine and 4.6 and 5.8%, respectively, for (*S*)-(+)-azelnidipine ($N = 15$). The accuracy ranged from 108.0 to 104.1% for (*R*)-(−)-azelnidipine and from 107.0 to 105.2% for (*S*)-(+)-azelnidipine.

3.4. Extraction recovery and matrix effect

The extraction recovery was determined by comparing the peak areas of the extracts of the QC samples at 0.05, 0.15, 6 and 14 ng/mL to the peak areas of the extracts of the blank plasma spiked at the corresponding concentrations after extraction. The extraction recovery values from human plasma at four concentrations ranged from 79.7, 79.2, 72.5 to 71.4%, respectively, for (*R*)-(−)-azelnidipine and from 77.8, 84.2, 72.2 to 71.7%, respectively, for (*S*)-(+)-azelnidipine. For the QC samples at 0.05 (LLOQ) and 14 ng/mL, the C.V. values in the extraction recovery were 1.9 and 5.0%, respectively, for (*R*)-(−)-azelnidipine and 1.5 and 4.0%, respectively, for (*S*)-(+)-azelnidipine. The effects of ion suppression on the peaks of (*R*)-(−)-azelnidipine and (*S*)-(+)-azelnidipine were examined at the concentration of 20 ng/mL ($n = 3$), and were within 17% for (*R*)-(−)-azelnidipine and (*S*)-(+)-azelnidipine, respectively.

3.5. Stability

The stability tests of (*R*)-(−)-azelnidipine and (*S*)-(+)-azelnidipine were designed to cover the anticipated storage conditions of the clinical samples. The stability data are summarized in Table 2. The short-term, long-term and auto-sampler

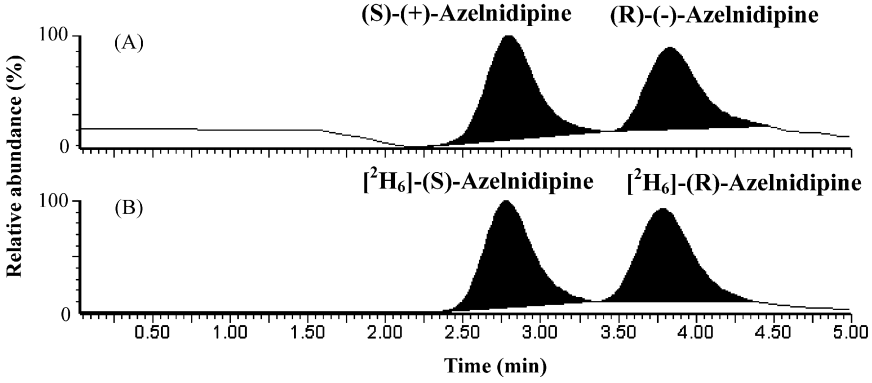

Fig. 7. MRM ion chromatograms of human plasma extract spiked with (*R*)-(−)-azelnidipine and (*S*)-(+)-azelnidipine at 0.05 ng/mL and the internal standard substance at 100 ng/mL. (A) (*R*)-(−)-azelnidipine and (*S*)-(+)-azelnidipine: *m/z* 583 → *m/z* 167. (B) [$^2H_6$]-(*R*,*S*)-azelnidipine: *m/z* 589 → *m/z* 167.

Table 1
Precision and accuracy of standard samples for calibration curve and QC samples

Nominal concentration (ng/ml)	(*R*)-(−)-azelnidipine			(*S*)-(+)-azelnidipine		
	Mean	C.V. (%)	Accuracy (%)	Mean	C.V. (%)	Accuracy (%)
Intra-assay ($N=5$)						
0.05	0.0477	5.3	95.4	0.045	6.7	90.0
0.1	0.0866	1.7	86.6	0.096	3.1	96.0
0.5	0.468	1.3	93.6	0.468	1.7	93.6
2	2.04	0.9	102.0	2.01	1.2	100.5
5	5.20	1.9	104.0	5.15	1.8	103.0
10	10.8	3.3	108.0	10.7	2.5	107.0
20	22.0	3.2	110.0	21.9	2.3	109.5
Intra-assay ($N=5$)						
0.05	0.0585	1.2	117.0	0.0524	2.8	104.8
0.15	0.152	3.1	101.3	0.150	2.4	100.0
6	6.07	4.8	101.2	6.19	4.4	103.2
14	14.2	3.4	101.6	14.7	2.4	105.0
Inter-assay ($N=15$)						
0.05	0.054	8.2	108.0	0.0535	4.6	107.0
0.15	0.155	3.3	103.3	0.156	4.0	104.0
6	6.24	4.3	104.0	6.27	3.6	104.5
14	14.6	6.2	104.1	14.7	5.8	105.2

stabilities indicated sufficient stability behavior of both enantiomers under the experimental conditions examined. The QC samples at four different concentrations (0.05, 0.15, 6 and 14 ng/mL) were used to determine the stability of the azelnidipine enantiomers in the plasma sample. The back calculated values from time 0 to 6 h, expressed as residual values of the corresponding nominal values were 93.8–97.1% for (*R*)-(−)-azelnidipine and 92.0–98.5% for (*S*)-(+)-azelnidipine which allowed us to conclude that the processed samples are stable for at least 6 h at room temperature (25 °C). The stability results for (*R*)-(−)-azelnidipine and (*S*)-(+)-azelnidipine showed that the residual values of the samples stored in a freezer (−80 °C) for 3 weeks were from 96.6 to 108.4% for (*R*)-(−)-azelnidipine and from 95.4 to 111.4% for (*S*)-(+)-azelnidipine. Therefore, it was concluded that the plasma samples containing the azelnidipine enantiomers are stable for at least 3 weeks when stored in a freezer (–80 °C). The results of the auto-sampler stability test ranged from 86.8 to 101.0% for (*R*)-(−)-azelnidipine and from 90.7 to 100.0% for (*S*)-(+)-azelnidipine, and demonstrated that both enantiomers are stable for at least 48 h at 6 °C. The stability of the stock solution containing 10 μg/mL of (*R*)-(−)-azelnidipine and 10 μg/mL of (*S*)-(+)-azelnidipine in methanol was also investigated at 5 °C. The results of the stock solution stability test were 93.9% for (*R*)-(−)-azelnidipine and 99.5% for (*S*)-(+)-azelnidipine at 5 °C for 3 weeks.

3.6. Application to pharmacokinetic study

The assay method for the azelnidipine enantiomers established in the present study was successfully applied to determine plasma concentrations of (*R*)-(−)-azelnidipine and (*S*)-(+)-azelnidipine, after single oral administration of 16 mg of azelnidipine tablets to six healthy male volunteers (CALBLOCK®, 2 × 8 mg tablets). Representative chromatograms obtained by MRM from an unknown plasma sample collected from a healthy male volunteer at 4 h after administration are shown in Fig. 8. The mean plasma concentration–time curves of (*R*)-(−)-azelnidipine and (*S*)-(+)-azelnidipine are presented

Table 2
Summary of stability tests for (*R*)-(−)- and (*S*)-(+)-azelnidipine ($N=3$)

Nominal concentration (ng/ml)	(*R*)-(−)-azelnidipine				(*S*)-(+)-azelnidipine			
	0.05	0.15	6	14	0.05	0.15	6	14
6 h, short-term at 25 °C								
*Residual (%)	93.8	94.1	94.1	97.1	96.3	92.0	93.3	98.5
C.V. (%)	4.1	3.0	5.1	3.6	4.1	1.7	1.5	4.2
3 weeks, long-term at −80 °C								
*Residual (%)	96.6	106.8	107.4	108.4	95.4	107.3	102.4	111.4
C.V. (%)	0.4	3.3	2.6	1.7	3.8	8.3	5.2	5.3
48 h, auto-sampler at 6 °C								
*Residual (%)	86.8	101.0	99.7	98.4	97.1	90.7	100.0	99.7
C.V. (%)	17.2	13.0	2.1	4.3	12.1	13.6	3.2	5.8

*Residual (%), expressed as mean percentage change from time zero (nominal concentration).

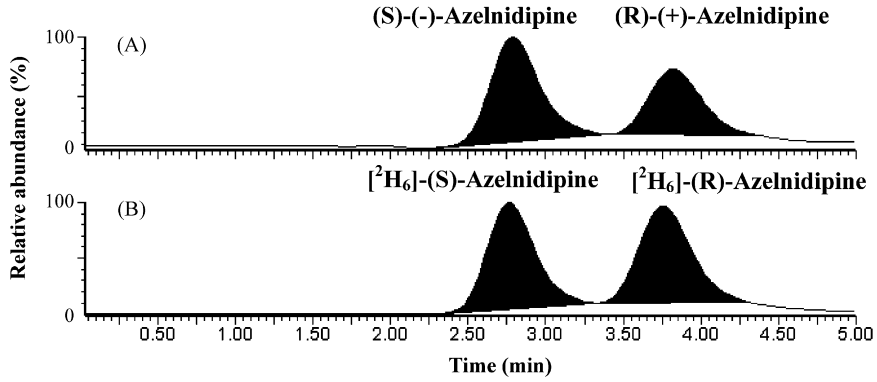

Fig. 8. MRM ion chromatograms of unknown human plasma sample from a volunteer, at 4 h after an oral dose of 16 mg CALBLOCK ® (2 × 8 mg tablets). (A) (*R*)-(−)-azelnidipine and (*S*)-(+)-azelnidipine: *m/z* 583 → *m/z* 167. (B) [$^2H_6$]-(*R*,*S*)-azelnidipine: *m/z* 589 → *m/z* 167.

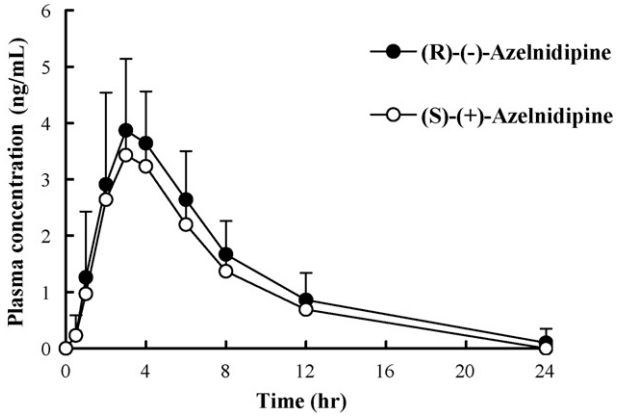

Fig. 9. Mean plasma concentrations of (*R*)-(−)-azlnidipine and (*S*)-(+)-azlnidipine after an oral dose of 16 mg CALBLOCK® (2 × 8 mg tablets) to healthy male volunteers (mean ± SD, *n* = 6).

in Fig. 9. The corresponding pharmacokinetic parameters (C_{max}, T_{max}, $t_{1/2}$, $MRT_{0–24}$) and $AUC_{0–24}$ values are presented in Table 3. The mean plasma concentrations of (*R*)-(−)-azelnidipine were equivalent to those of (*S*)-(+)-azelnidipine. The C_{max}, T_{max}, $MRT_{0–24}$ and $AUC_{0–24}$ values were not significantly different between the azelnidipine enantiomers as the (*R*)-(−)-azelnidipine and (*S*)-(+)-azelnidipine reached C_{max} values of 3.33 ± 1.37 ng/mL and 3.50 ± 1.38 ng/mL, respectively, with $t_{1/2}$ values at the terminal phase of 4.39 ± 1.80 h and 3.72 ± 0.78 h, respectively, $AUC_{0–24}$ values of 26.97 ± 11.76 h and 21.34 ± 7.59 h, respectively, and $MRT_{0–24}$ values of 5.65 ± 1.27 h and 5.10 ± 0.59 h, respectively. The t_{max}, value for (*R*)-(−)-azelnidipine was exactly the same as that for (*S*)-(+)-azelnidipine in five subjects with the exception of one subject. The results showed that the mean *R*/*S* ratio of the azelnidipine enantiomers in the pharmacokinetic parameters (C_{max}, T_{max}, $t_{1/2}$, $MRT_{0–24}$, and $AUC_{0–24}$) ranged from 0.96- to 1.25-fold.

Table 3
Main pharmacokinetic parameters of (*R*)-(−)-azelnidipine and (*S*)-(+)-azelnidipine after oral dose of 16 mg CALBLOCK® (2 × 8 mg tablets) to healthy male volunteers

Parameter	(*R*)-(−)-azelnidipine	(*S*)-(+)-azelnidipine	Ratio (*R*/*S*)
C_{max} (ng/mL)	4.10 ± 0.94	3.60 ± 0.87	1.15 ± 0.09
T_{max} (h)	3.33 ± 1.37	3.50 ± 1.38	0.96 ± 0.10
$t_{1/2}$ (h)	4.39 ± 1.80	3.72 ± 0.78	1.16 ± 0.30
$MRT_{0–24}$ (h)	5.65 ± 1.27	5.10 ± 0.59	1.11 ± 0.26
$AUC_{0–24}$ (ng h/mL)	26.97 ± 11.76	21.34 ± 7.59	1.25 ± 0.31

Mean ± SD.

4. Conclusion

An LC–MS/MS method for the quantification of (*R*)-(−)-azelnidipine and (*S*)-(+)-azelnidipine in human plasma was developed and fully validated according to the FDA guidance. The combination of techniques employed, including solid-phase extraction, use of stable isotope-labeled internal standard, and chiral LC and MS/MS, resulted in a robust, ultrasensitive, accurate and precise method for the determination of the azelnidipine enantiomers in human plasma at concentrations from 0.05 to 20 ng/mL. The lower limit of quantification was 0.05 ng/mL for (*R*)-(−)-azelnidipine and (*S*)-(+)-azelnidipine using a 0.1 mL-aliquot of the plasma sample. Using a 96-well extraction plate, the sample preparations could be achieved easily in less than 1 h, and the analysis by LC–MS/MS could be completed within 8 h, with a 5.0 min injection-to-injection interval. The freezer, room temperature, and auto-sampler stability of the analytes demonstrated no significant degradation over the time evaluated. This method was successfully used to provide bioanalytical supporting data in a human study on the pharmacokinetics of (*R*)-(−)-azelnidipine and (*S*)-(+)-azelnidipine.

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