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Determination of benidipine in human plasma using liquid chromatography–tandem mass spectrometry

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Abstract

We developed a method for determining benidipine, a dihydropyridine analogue calcium-channel blocker, in plasma using liquid chromatography–tandem mass spectrometry (LC–MS–MS). Benidipine and benidipine-d_5, an internal standard, were extracted from plasma using diethyl ether in the presence of 5 M NaOH. After drying the organic layer, the residue was reconstituted in acetonitrile and injected onto a reversed-phase C_{18} column. The isocratic mobile phase (acetonitrile–5 mM ammonium acetate, 90:10, v/v) was eluted at 0.2 ml/min. The ion transitions monitored in multiple reaction-monitoring mode were *m*/*z* 506–174 for benidipine and *m*/*z* 511–179 for the internal standard. The coefficient of variation of the assay precision was less than 13%, and the accuracy exceeded 92%, except at the limit of quantification, 0.05 ng/ml with 1 ml of plasma, when it was 85%. This method was used to measure the benidipine concentration in plasma from healthy subjects after a single 4-mg oral dose of benidipine. This method is a very simple, sensitive, and accurate way to determine the plasma benidipine concentration.

Keyword: Benidipine

1. Introduction

(±)-(*R**)-2,6-Dimethyl-4-(*m*-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylic acid (*R**)-1-benzyl-3-piperidinyl ester, methyl ester hydrochloride (benidipine hydrochloride) is a dihydropyridine analogue calcium-channel blocker, which has been used clinically as an antihypertensive and anti-anginal agent [1,2].

After a single oral dose of 8 mg benidipine, the peak concentration of benidipine in patients with essential hypertension was about 2 ng/ml at 1 h, and it declined to 0.1 ng/ml after around 8 h [3]. Therefore, a highly sensitive method is required to measure such low benidipine concentrations in plasma. Since benidipine was introduced recently, only a few analytical methods for physiological samples have been reported using gas chromatography–electron capture detection (GC–ECD) or GC–mass spectrometric detection (GC–MS) [4–6]. These methods are sensitive enough to detect benidipine in plasma; the detection limits were 0.02 and 0.2 ng/ml for GC–MS and GC–ECD, respectively. However, they require three or four re-extraction pretreatments to purify benidipine from plasma.

High-performance liquid chromatography with MS–MS detection has played a prominent role in analytic studies and has high sensitivity and accuracy. Therefore, we developed a new method for determining benidipine in plasma using LC–MS–MS. Our method is very simple, sensitive, and suitable for the pharmacokinetic study of benidipine.

2. Experimental

2.1. Reagents and materials

Benidipine hydrochloride and benidipine-D_5 hydrochloride (internal standard, IS) were kindly donated by Kyowa Hakko Kogyo Co. Ltd. (Shizuoka, Japan). HPLC-grade acetonitrile was purchased from Merck Co. (Darmstadt, Germany). All the other chemicals and solvents were of the highest analytical grade available.

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2.2. Preparation of standards and quality controls

Benidipine and IS were dissolved in methanol at 1 and 0.1 mg/ml, respectively. The benidipine standard solution was serially diluted with methanol and added at drug-free plasma to obtain concentrations of 0.05, 0.1, 0.5, 1, 5, and 10 ng/ml. A calibration graph was derived from the peak area ratio of benidipine to IS with a linear regression.

Quality controls were prepared daily in 1 ml of blank human plasma by adding 100 μl of standard solution. They were prepared for minimum (0.05 ng/ml), intermediate (0.5 and 1 ng/ml), and high (5 ng/ml) concentrations to evaluate the inter- and intra-day precision and accuracy of this assay method.

2.3. Characterization of the product ions using tandem mass spectrometry

One micromolar benidipine and IS solutions were infused into the mass spectrometer separately at a flow rate of 10 μl/min to characterize the product ions of each compound. The precursor ions, $[M + H]^+$, and the pattern of fragmentation were monitored using positive ion mode. The major peaks observed in the MS–MS scan were used to quantify benidipine and IS.

2.4. Analytical system

The plasma benidipine concentrations were quantified using liquid chromatography–mass spectrometry with a PE SCIEX API 3000 LC–MS–MS System (Sciex Division of MDS Inc., Toronto, Canada) equipped with an electrospray ionization interface used to generate positive ions $[M+H]^+$. The compounds were separated on a reversed-phase column (Luna C_{18}, 2 mm × 100 mm i.d., 3 μm particle size; Phenomenex, Torrance, CA, USA) with an isocratic mobile phase consisting of acetonitrile and 5 mM ammonium acetate buffer (90%:10% (v/v)). The mobile phase was eluted using an HP 1100 series pump (Agilent, Wilmington, DE., USA) at 0.2 ml/min.

The turboion spray interface was operated in the positive ion mode at 5500 V and 350 °C. The operating conditions were optimized by flow injection of a mixture of all analytes and were determined as follows: nebulizing gas flow, 1.04 l/min; auxiliary gas flow, 4.0 l/min; curtain gas flow, 1.44 l/min; orifice voltage, 80 V; ring voltage 400 V; collision gas (nitrogen) pressure, 3.58×10^{-5} Torr. Quantitation was performed by multiple reaction monitoring (MRM) of the protonated precursor ion and the related product ion for benidipine using the internal standard method with peak area ratios and a weighting factor of $1/x$. The mass transition used for benidipine and internal standard were m/z 506 → 174 and 511 → 179, respectively (collision energy 36 eV, dwell time 200 ms). Quadrupoles Q1 and Q3 were set on unit resolution. The analytical data were processed by Analyst software (version 1.2).

2.5. Sample preparation

Twenty microliters of internal standard (100 ng/ml) and 1 ml of 5 N NaOH were added to 1 ml of plasma, followed by 1-min liquid–liquid extraction with 5 ml of diethylether. The organic layer was separated and evaporated to dryness at ambient temperature in a Speed-Vac (Savant, Holbrook, NY, USA). The residue was reconstituted into 100 μl of acetonitrile by vortex mixing for 15 s; 10 μl of this solution was injected onto the column.

2.6. Validation procedure

The validation parameters were selectivity, extraction recovery, precision, and accuracy. Ten batches of blank heparinized human plasma were screened to determine the specificity. The extraction recovery of benidipine was calculated by comparing the peak area ratio measured for the standard solution considering condensation with that obtained for plasma extracts after the extraction procedure. The precision and accuracy of the intra- and inter-day assay validation were estimated using the inverse prediction of the concentration of the quality controls from the calibration curve.

2.7. Clinical application

Ten healthy subjects who gave written informed consent took part in this study. Health problems, drug or alcohol abuse, and abnormalities in laboratory screening values were exclusion criteria. This study was approved by the Institutional Review Board of Busan Paik Hospital (Busan, Korea). After an overnight fast, all the subjects were given a single 4-mg oral dose of benidipine. Blood samples (6 ml) were taken before and 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 5, 6, and 8 h after drug administration. The plasma was separated by centrifugation at 1000 × g for 10 min, and stored at −80 °C until analysis.

2.8. Pharmacokinetic analysis

The pharmacokinetic analysis was performed using noncompartmental methods. The area under the plasma concentration-versus-time curve (AUC) was calculated using the trapezoidal rule and extrapolated to infinity. The time course of the plasma benidipine concentration was used to determine the maximum plasma concentration (C_{max}) and the time (T_{max}) to reach C_{max}. The elimination rate constant (k_{el}) was obtained by the linear regression of the terminal phase and the calculated elimination half-life ($t_{1/2}$) was $0.693/k_{el}$.

3. Results and discussion

3.1. Mass spectra

Precursor ions for benidipine and benidipine-d_5, and their corresponding product ions, were determined from spectra

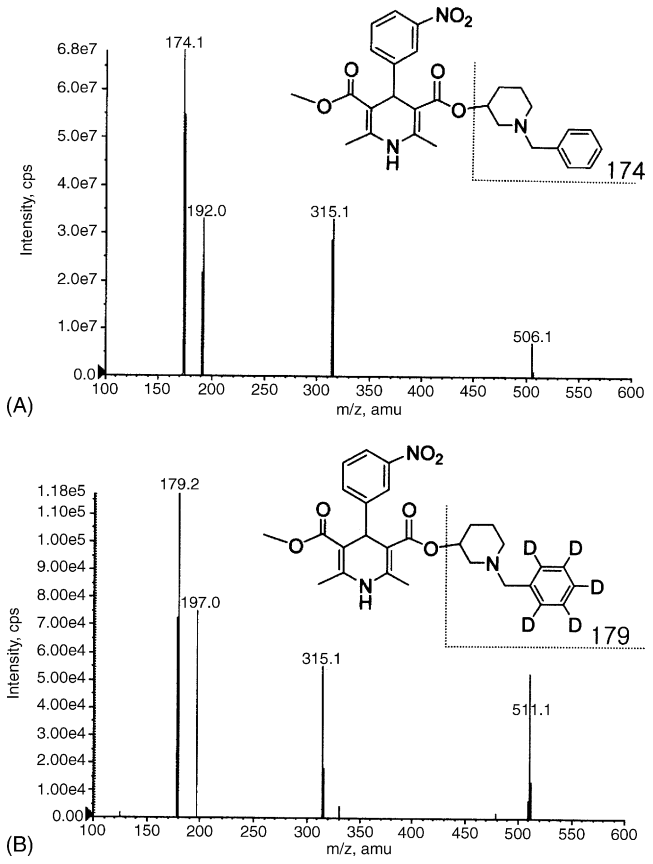

Fig. 1. Mass–mass spectra of benidipine (A) and benidipine-d_5 (B) using electrospray ionization mode.

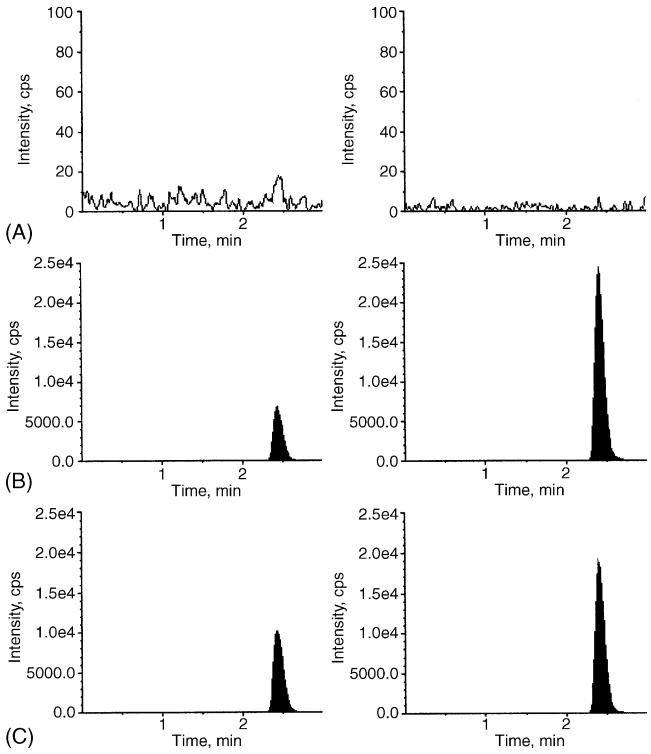

Fig. 2. Chromatograms of benidipine (left) and benidipine-d_5 (right). (A) blank plasma, (B) plasma spiked with 0.5 ng/ml benidipine and benidipine-d_5, (C) plasma sample equivalent to 0.83 ng/ml from a volunteer 0.75 h after the oral dose.

obtained during the infusion of standard solutions into a mass spectrometer using an electrospray ionization source, which operated in positive ionization mode with collision nitrogen gas in Q2 of a MS–MS system. Benidipine and benidipine-d_5 mainly produced protonated molecules at *m/z* 506 and 511, respectively. Both product ions were scanned in Q3 after collision with nitrogen in Q2 at *m/z* 174 and 179 for benidipine and benidipine-d_5, respectively. These are the most sensitive product ions for quantification (Fig. 1). 1-Benzyl piperidine and 1-benzyl piperidine-d_5 are thought to be the fragment ions with molecular weights of 174 and 179, respectively [7].

3.2. Determination of benidipine

There were no interfering peaks at the elution times for either benidipine (2.7 min) or IS (2.5 min). Fig. 2A and B show typical chromatograms for blank plasma and plasma spiked with both compounds (0.5 ng/ml benidipine, 100 ng/ml IS), respectively. The plasma sample from a volunteer is shown in Fig. 2C. The extraction efficiencies of saturated $NaHCO_3$ solution and 1 N NaOH were examined. However, neither was as good as 5 N NaOH for recovery.

Previous studies used three liquid–liquid extractions before injecting the extracts onto the GC–MS system and followed this by back extraction in acidic conditions. The benidipine in the aqueous layer was finally re-extracted with diethylether after neutralization with alkaline solution [4–6]. We found that liquid–liquid extraction in alkaline solution could remove the benidipine from plasma, and that this was much simpler than the pretreatment procedure used for GC–MS.

Analysis using liquid chromatography coupled to tandem mass spectrometry has been widely applied to detect very low concentrations of calcium antagonists at a picogram per milliliter scale [8–10] and to identify their metabolites [11]. In human plasma, the limit of quantitation of each amlodipine enantiomer was found to be 0.1 ng/ml [8]. Both enantiomers were removed from plasma using a solid-phase extraction method and separated using a chiral stationary phase. Barnidipine and nifedipine have been assayed in human samples at levels as low as 0.03 and 0.5 ng/ml, respectively [9,10]. Walles et al. recently characterized five new metabolites of verapamil in human heart tissue using electrospray LC–MS–MS [11].

3.3. Linearity and detection limit

The calibration curve provided a reliable response from 0.05 to 10 ng/ml. The mean equation of the regression line was $y = (0.693 \pm 0.013)x + (0.0064 \pm 0.0008)$ ($r^2 > 0.995$). The limit of detection was 0.02 ng/ml at a signal-to-noise

Table 1
The precision and accuracy of the intra-day benidipine assay ($n = 6$)

Added (ng/ml)	Measured (ng/ml) (mean ± S.D.)	CV (%)	Accuracy (mean ± S.D.) (%)
0.05	0.047 ± 0.006	12.5	85 ± 11
0.5	0.48 ± 0.04	8.2	94 ± 7
1	0.97 ± 0.10	10.2	98 ± 6
5	4.75 ± 0.38	7.8	97 ± 4

Table 2
The precision and accuracy of the inter-day benidipine assay ($n = 6$)

Added (ng/ml)	Measured (mean ± S.D.) (ng/ml)	CV (%)	Accuracy (mean ± S.D.) (%)
0.05	0.045 ± 0.004	10.3	88 ± 5
0.5	0.46 ± 0.04	8.8	92 ± 6
1	0.98 ± 0.05	5.4	98 ± 3
5	4.85 ± 0.22	4.6	97 ± 5

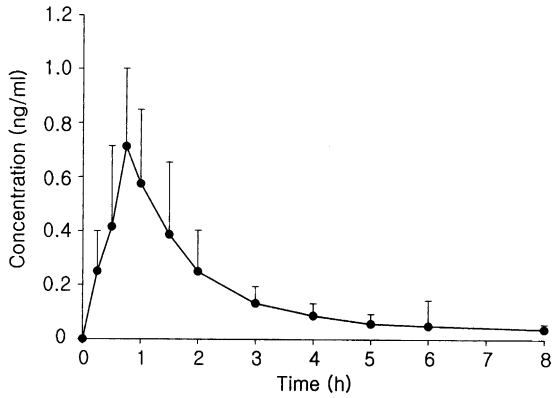

Fig. 3. Time course of the plasma benidipine concentration in healthy subjects after a single 4-mg oral dose. Each point represents the mean ± S.D. ($n = 10$).

(S/N) ratio of 3, which is as sensitive as that of the previous GC–MS method [4].

3.4. Precision and accuracy

The intra- and inter-day precision and accuracy of our method are listed in Tables 1 and 2, respectively. The coefficients of variation of the precision of the intra- and inter-day validation were less than 12.5 and 10.3%, respectively. The accuracy of the method exceeded 92%, except at the limit of quantitation, when it was 85%. The mean recovery in the range from 0.05 to 5 ng/ml exceeded 93%.

Table 3
Pharmacokinetic parameters of benidipine in 10 healthy subjects after a single 4-mg oral dose

Parameters	Mean ± S.D.
C_{max} (ng/ml)	1.07 ± 0.49
T_{max} (h)	0.63 ± 0.23
$AUC_{8\,h}$ (ng h/ml)	1.50 ± 0.63
$t_{1/2}$ (h)	1.53 ± 0.20

3.5. Pharmacokinetics of benidipine

Fig. 3 shows the time course of the benidipine plasma concentration after a single 4-mg oral dose. The pharmacokinetic parameters are listed in Table 3. The C_{max} was 1.07 ± 0.49 ng/ml at 0.63 ± 0.23 h. $AUC_{8\,h}$ was 1.50 ± 0.63 ng h/ml at more than 95% of AUC_{inf}. The half-life calculated from the terminal phase was 1.53 ± 0.20 h.

In conclusion, our LC–MS–MS method is a very simple, sensitive, and accurate way to determine the plasma benidipine concentration, and is suitable for clinical pharmacokinetic studies of this drug at low doses.

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