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Quantification of isradipine in human plasma using LC–MS/MS for pharmacokinetic and bioequivalence study

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ABSTRACT

A highly sensitive and rapid method for the analysis of isradipine in human plasma using liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) was developed. The procedure involves a simple liquid–liquid extraction of isradipine and amlodipine (IS, internal standard) with methyl-t-butyl ether after alkaline treatment and separation by RP-HPLC. Detection was performed by positive ion electrospray ionization (ESI) in multiple reaction monitoring (MRM) mode, monitoring the transitions m/z 372.1 → m/z 312.2 and m/z 408.8 → m/z 237.9, for quantification of isradipine and IS, respectively. The standard calibration curves showed good linearity within the range of 10 to 5000 pg/mL ($r^2 \geq 0.9998$). The lower limit of quantitation (LLOQ) was 10 pg/mL. The retention times of isradipine (0.81 min) and IS (0.65 min) suggested the potential for high throughput of the proposed method. In addition, no significant metabolic compounds were found to interfere with the analysis. This method offered good precision and accuracy and was successfully applied for the pharmacokinetic and bioequivalence studies of 5 mg of sustained-release isradipine in 24 healthy Korean volunteers.

1. Introduction

Isradipine (methyl propan-2-yl 2,6-dimethyl-4- (8-oxa-7,9 diazabicyclo[4.3.0] nona- 2,4,6,9- tetraen- 5-yl)- 1,4-dihydropyridine-3,5-dicarboxylate) belongs to the dihydropyridine class of calcium-channel blockers and is commonly used to treat hypertension associated with various clinical conditions [1–6]. Isradipine acts by inhibiting the influx of extracellular calcium across both the cardiac and vascular cell membranes and has a high affinity for L-type channels located in vascular smooth muscle compared to those in the myocardium, thereby leading to a fairly specific and potent antihypertensive effect [7,8]. Unlike most other calcium channel blockers, isradipine is very effective at blocking calcium channels expressed by dopaminergic neurons. Isradipine antagonized the pressor effects of cocaine or methamphetamine [9–11], possibly via modulation of central dopaminergic neurotransmission [12,13]. Recent research has suggested that isradipine may have potential uses for treating Parkinson's disease (PD) [14]. In mouse model, isradipine rejuvenated ageing dopaminergic neurons, whose death leads to PD-like symptoms. The therapeutic effects of isradipine targeted substantia nigral neurons by blocking calcium channels and inducing a switch to sodium channel currents, which are a more youthful way of generating electrical signals [15].

The pharmacokinetics of isradipine have been reported following oral administration of immediate-release (IR) isradipine or sustained-release (SR) isradipine in healthy volunteers [16–19], cardiac patients [20], and cocaine-dependent individuals [21]. At an oral dose ranging between 2.4–20 mg, the pharmacokinetics of isradipine appeared to be linear [16,17]. However, at doses needed to reduce blood pressure, the side effects associated with the vasodilatory activity were usually mild, transient, and well-tolerated [7,19,21,22]. SR isradipine which has a significantly lower C_{max} and extended T_{max} compared to IR isradipine, exhibits a more favorable cardiovascular profile [18,21].

Numerous analytical methods have been developed to support clinical and pharmacokinetic studies of isradipine in biological fluids. Due to an extensive first-pass metabolism, the bioavailability of

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isradipine was demonstrated to be only 17–28%, although 90–95% of orally administrated isradipine (5–20 mg dose range) has been shown to be absorbed from the digestive tract [17,23]. Thus, the pharmacokinetics of isradipine absorption result in very low plasma concentrations. Specific radioimmuno-assay (RIA) techniques were reported early in the development of isradipine and had a relatively high sensitivity (LLOQ of 30–100 pg/ml), but were subject to interference from metabolites [16,17,20,24]. As isradipine is a heat-stable compound, plasma concentrations of isradipine have been determined by gas chromatography (GC) with or without derivatization. Several GC methods coupled with electron-capture detection (GC/ECD) with a LLOQ of 0.2 ng/ml [6], mass detection (GC/MS) with LLOQs of 0.04 ng/ml [25] and 0.1 ng/ml [19], and nitrogen-phosphorus detection (GC/NPD) with a LLOQ of 0.2 ng/ml [26] have been developed. However these methods, possess disadvantages such as low sensitivity [6,19,26], long analytical run times [6,26], or pre-column derivatization processes [25]. The quantified data from the HPLC techniques coupled with UV detection (HPLC/UV) [21,27,28], and electrochemical detection (HPLC/ECD) [29] exhibit a LLOQ of almost 0.5 ng/ml. In addition, a spectrofluorometric method was recently published, but this method offered low sensitivity (16 ng/ml LLOQ) and needed derivatization to enhance the fluorescent intensity because isradipine itself yields little native fluorescence [30].

Among the currently available bio-analytical techniques, LC–MS/MS is now firmly established as the primary tool for pharmaceutical analysis. The unique selectivity and sensitivity offered by MS/MS have made it the method of choice for performing bioanalysis of small molecular weight drugs [31,32]. Two decades ago, Barlett et al. [33] reported LC–MS method to determine the identity of a number of degradation products from the bulk drug form of isradipine (DynaCirc). However, this method had a long analytical run time of 22 min and there was no data on sensitivity or specificity.

Here, we present a rapid method for the determination of isradipine from human plasma with lower detection limit. The proposed LC–MS/MS method allowed a very sensitive and reliable determination of the pharmacokinetic and bio-equivalence of two SR isradipine formulations in healthy Korean volunteers.

2. Experimental

2.1. Chemicals and reagents

Isradipine ($C_{19}H_{21}N_3O_5$, MW 371.387) and amlodipine (IS, $C_{20}H_{25}ClN_2\ O_5$, MW 408.879) were purchased from Quimica Sintetia S.A (Madrid, Spain) and Sigma Co. (St. Louis, MO, USA), respectively (Fig. 1). The reference drug (Dynacirc SR capsule, 5 mg isradipine/capsule) was obtained from Daewoong Pharmaceutical Co., Ltd. (Seoul, South Korea) and the test drug (Dynadipine SR capsule, 5 mg isradipine/capsule) was provided by CTBIO Pharm Co., Ltd. (Seoul, South Korea). HPLC grade methanol and methyl-t-butyl ether were purchased from Sigma Co. while all other reagents and solvents used were of analytical grade. Ultra pure water was obtained from a Milli-Q system (Millipore Co., Walthan, MA, USA).

2.2. Calibration standards preparation

Stock solutions of isradipine and IS were prepared in methanol. A set of six non-zero calibration standards, ranging from 10 pg/mL to 5000 pg/mL, were prepared in blank human plasma. The quality control (QC) samples were prepared in blank human plasma at concentrations of 10 (LLOQ), 30 (low), 500 (medium) and 4000 pg/mL (high). Blank human plasma was tested before spiking to ensure that no endogenous interference was found proximal to the retention times of isradipine and IS.

2.3. Sample preparation

20 μl of IS solution and 0.5 mL of 1N sodium hydroxide were added to the 0.5 mL aliquot of human plasma samples and vortex mixed for 1 min. Five milliliters of methyl-t-butyl ether was added to all the tubes, which were extracted by agitating for 10 min. Subsequently, the tubes were frozen at −70 °C after centrifugation at 4000 rpm for 5 min. The upper organic layer was transferred to another set of clean glass tubes and then evaporated to dryness under N_2 gas at 40 °C. The dry residue was dissolved with 0.1 mL of a methanol:20 mM ammonium acetate mixture (90:10, v/v). Five microliters of the reconstituted upper layer was directly injected into the LC–MS/MS system.

2.4. LC–MS/MS conditions and quantifications

The LC system used was a nanospace series SI-2 3301 (Shiseido Co., Ltd., Japan) chromatograph equipped with an Nanospace SI-2 3133 autosampler and a Nanospace SI-2 3010 degasser and Peak Simple LC Data System (Lab Alliance Co., State College, PA, USA). The analytical column was a YMC Hydrosphere C_{18} (50 mm × 2 mm i.d., 3 μm; YMC Co., Ltd., Japan). The mobile phase consisted of methanol:20 mM ammonium acetate (90:10, v/v) and the flow rate was 0.25 mL/min. Detection was carried out on an API 5000 MS system (Applied Biosystems, Foster City, CA, USA) equipped with an electrospray interface (ESI) and operated in the positive ionization mode. The ion source parameters were set as the following: curtain gas = 20 p.s.i., GS1 = 20 p.s.i. and GS2 = 16 p.s.i., ion spray voltage = 5500 V, ion source temperature = 400 °C, collision activated dissociation (CAD) = 5. This system was set up in multiple reaction monitoring (MRM) mode, monitoring the transitions m/z 372.1 → m/z 312.2 and m/z 408.8 → m/z 237.9, for quantification of isradipine and IS, respectively. The strongest fragment of each compound was selected and used as the Q3 ion to be monitored. Data acquisition and analysis were performed using the analyst software version 1.4.2. (Applied Biosystems).

2.5. Assay validation

Assay validation was performed according to the FDA guidance for industrial bioanalytical method validation (2001) [34]. Linearity was determined using a linear least-squares regression with a weighting index of $1/x$, which was performed on the peak area ratios of isradipine and IS versus isradipine concentrations of the six human plasma standards. The sensitivity of the method was expressed as the lower limit of quantification (LLOQ) that could be quantitatively determined with acceptable accuracy and precision. The accuracy and precision were assessed by analyzing four concentrations of QC samples from five different validation batches and were calculated using one-way ANOVA.

2.6. Pharmacokinetic and bioequivalence studies in healthy volunteers

A randomized, single-dose, two-period, two-sequence, and crossover design was used for the assessment of the pharmacokinetics and bioequivalence. Isradipine was randomly given to 24 healthy volunteers of both sexes (10 female and 14 male). Participants had not taken other medications (including over-the-counter

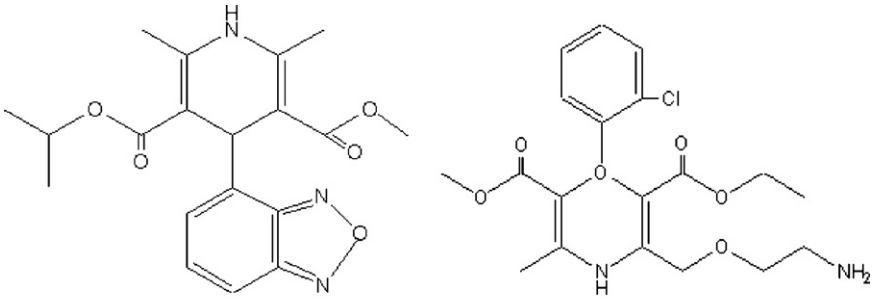

Fig. 1. Chemical structures of (A) isradipine (MW = 371.387 g/mol, $C_{19}H_{21}N_3O_5$, methyl propan-2-yl 2,6-dimethyl-4- (8-oxa-7,9-diazabicyclo [4.3.0] nona- 2,4,6,9- tetraen-5-yl)- 1,4-dihydropyridine- 3,5-dicarboxylate) and (B) amlodipine (IS, MW = 408.879 g/mol, $C_{20}H_{25}ClN_2O_5$, 3-ethyl-5-methyl-2-(2-aminoethoxymethyl)-4-(2-chlorophenyl)-1,4-dihydro-6-methyl-3,5- pyridinedicarboxylate).

drugs) 2 weeks prior to or during the study period. The study was carried out according to the Declaration of Helsinki [35] for biomedical research involving human subjects and the Rules of Good Clinical Practice [36]. Subjects were informed of the aims and risks of the study by the clinical investigator. Based on this description, they provided written informed consent before participating in the study. In addition, the Institutional Review Board of Hanyang University Medical Center approved the protocol prior to the start of the study. Twenty-four volunteers aged between 19–27 years (22.0 years ± 2.6 years), with heights between 157.5–182.0 cm (169.5 cm ± 7.1 cm) and with body weights between 41.9–83.0 kg (63.8 kg ± 11.8 kg) who were non-alcoholics and free from disease, were assessed as having a healthy status by clinical evaluations including a physical examination and the following laboratory tests: albumin, alkaline phosphatase, ALT, AST, blood glucose, creatinine, BUN, total cholesterol, protein, total bilirubin, Hb, Hct, total and differential white cell counts, and routine urinalysis. After screening and 2 weeks of a washout period, the volunteers were confined for two periods of approximately 96 h. Each confinement was separated by a period of 1 week. During each period, the participants were hospitalized at the Hanyang University Medical Center at 17:00 pm and had an evening meal before 20:00 pm. After an overnight fasting, they received a test or reference drug (single 5 mg isradipine/capsule) at 7:00 am along with 240 mL of water. Subjects were then fasted for additional 4 h. A standard lunch and evening meal were provided at 4 and 10 h after dosing. Liquid consumption was allowed *ad libitum* after lunch except for liquid that contained xanthine and acidic beverages including tea, coffee, and cola. Before and at 0, 0.33, 0.66, 1, 1.5, 2, 3, 4, 6, 9, 12, 24, 36, and 48 h after dosing, blood pressure, heart rate, and body temperature were recorded. Blood samples (9 mL) were withdrawn by an indwelling catheter into heparin-containing tubes from a suitable antecubital vein. The blood samples were centrifuged at 3000 rpm for 10 min at room temperature and plasma was stored at −70 °C until analysis. The total plasma isradipine levels were determined and the maximal concentration (C_{max}) and T_{max} were determined by visual inspection from each subject's plasma concentration of isradipine versus time plots. Other pharmacokinetic parameters were calculated in the following manner. The area under the plasma concentration versus the time curve ($AUC_{infinity} = \int^{t} C_t\,dt + C_{last}/\lambda$, λ = terminal phase slope) was calculated for the total isradipine level (C_t) using the linear trapezoidal rule extrapolated to infinity according to a pharmacokinetic analysis multi-lines fittings where C_{last} was the last measurable concentration and the terminal phase slope (λ) was obtained from the least square fitted terminal log-linear portion of the plasma concentration versus time profile [37]. The terminal half-life ($T_{1/2}$) was calculated by $0.692/\lambda$ [38].

3. Results and discussion

3.1. Separation

The molecular structures of isradipine and IS are shown in Fig. 1. The simple preparation procedures including the liquid–liquid extraction of isradipine with methyl-t-butyl ether after alkaline treatment, evaporation of extracted sample, and reconstitution with the mobile phase were used before reverse phase HPLC separation. The retention times of isradipine and IS were 0.81 and 0.65 min, respectively (Fig. 2). Blank human plasma had no significant endogenous peaks at the retention time of isradipine or IS (Fig. 2A). Blank plasma spiked with 100 ng/mL of IS (Fig. 2B), blank plasma spiked with 10 pg/mL of calibration standard of isradipine and 100 ng/mL of IS (Fig. 2C), and the subject's plasma taken after 3 h of a single oral administration of 5 mg isradipine spiked with IS (Fig. 2D) showed an excellent peak shape for isradipine and IS.

To avoid the interference from exogenous/endogenous compounds co-eluted with the target compound, MS/MS detection (termed tandem MS detection) was performed. From full-scan mass spectra via the Q1 mass filter, the protonated molecular ions, $[M+H]^+$, at *m/z* 372.1 for isradipine and *m/z* 408.8 for IS were chosen for the precursor ion. As reported by Bartlett et al. [33], isradipine possessed a specific affinity for sodium and ammonia and produced strong peaks at *m/z* 389.1 $[M+NH_4]^+$ and *m/z* 394.1 $[M+Na]^+$. The MS/MS fragmentation was achieved by introducing the $[M+H]^+$ ions into the second quadrupole (Q2) cell with the optimum collision energy set of 15.0 eV for isradipine and 17.0 eV for IS. After collision-induced dissociation, the MS/MS transition *m/z* 372.1 → *m/z* 312.2 for isradipine and *m/z* 409 → *m/z* 238 for IS were selected. The most abundant ions in the product ion mass spectrum at *m/z* 312.2 for isradipine and *m/z* 238.0 for IS were monitored for quantification.

3.2. Method validation and linearity of calibration

The standard calibration curves showed good linearity within the range of 10 (LLOQ) to 5000 pg/mL of isradipine in human plasma ($y = 0.00049x + 0.03325$, $r^2 \geq 0.9998$). The retention time of isradipine (0.81 min.) and IS (0.65 min.) suggested the opportunity for high throughput screening of the proposed method. Intra- and inter-day precisions and accuracies were determined by analyzing QC samples against a calibration curve, on the same day ($n = 5$) and on different days ($n = 5$). As shown in Table 1, this method allowed good precision and accuracy. The coefficient of variation values of both intra- and inter-day results

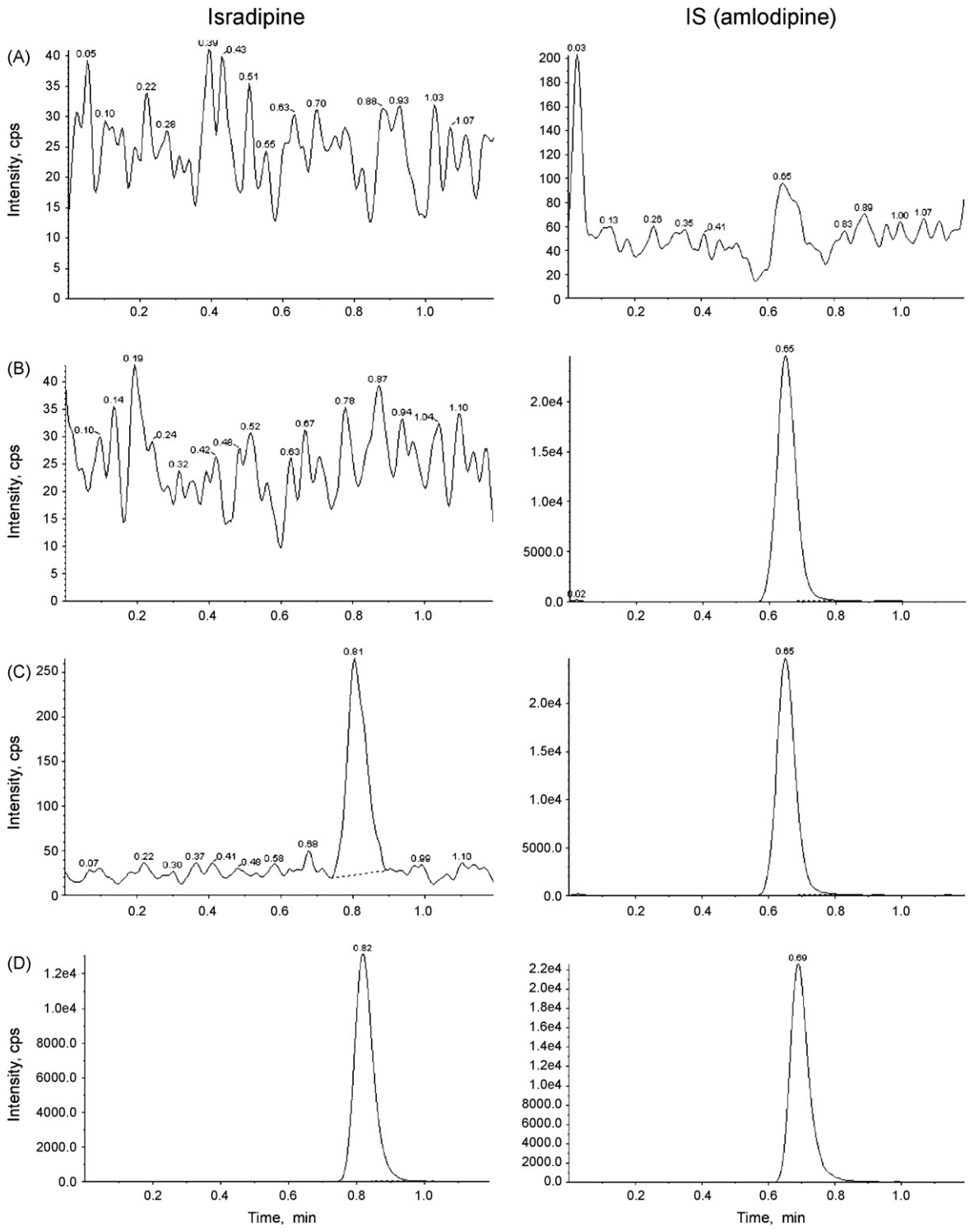

Fig. 2. Chromatograms of (A) blank plasma, (B) with IS (100 ng/mL), (C) with isradipine (LLOQ, 10 pg/ml) and IS, and (D) human plasma taken 3 h after a single oral administration of 5 mg isradipine spiked with IS.

were below 5.67% and 8.97%, respectively. Intra- and inter-day accuracies were 86.78%–101.82% and 81.07%–103.31%, respectively. Under the described analytical conditions, the lowest limit of quantification (LLOQ), defined as the lowest concentration of isradipine at which both the precision and accuracy were less than or equal to 20% (Guidance for industry, 2001) [34], was 10 pg/mL.

3.3. Clinical pharmacokinetic and bioequivalence study in healthy subjects

The proposed method was applied for the determination of isradipine in plasma samples for the purpose of establishing the pharmacokinetic and bioequivalence study of 5 mg SR isradipine capsule in 24 healthy Korean volunteers. The pharmacokinetic

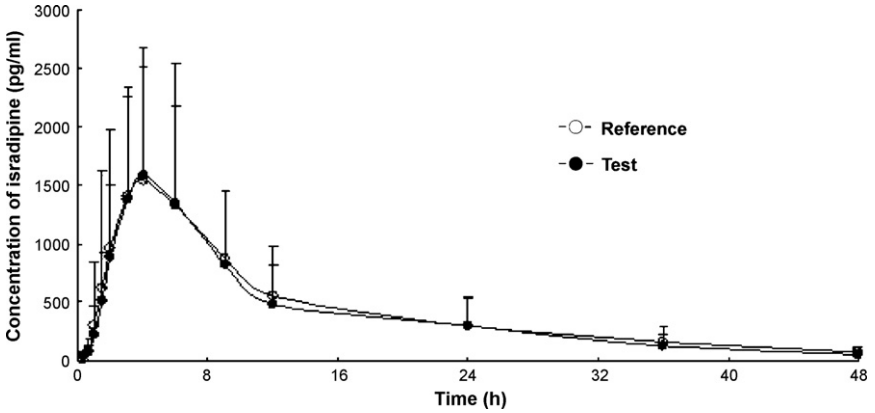

Fig. 3. Profiles of mean (±S.D., $n = 24$) plasma concentrations versus time of two isradipine (reference vs. test) formulations in 24 healthy volunteers after oral administration of single 5 mg SR isradipine capsule.

Table 1
Precision and accuracy for analysis of isradipine in human plasma, $n = 5$.

Nominal concentration (pg/mL)	Precision (CV %)		Accuracy (%)	
	Intra-day	Inter-day	Intra-day	Inter-day
10(LLOQ)	5.67	8.97	92.12	81.07
30	2.22	7.82	86.78	88.62
500	1.68	1.60	100.49	103.31
4000	0.87	2.62	101.82	98.24

LLOQ = lower limit of quantification; CV = coefficient of variation.

Table 2
Pharmacokinetic parameters (mean ± S.D. of $n = 24$) of 2 types of 5 mg isradipine capsules based on its plasma concentrations.

Parameters	Reference	Test
$AUC_{48\,h}$ (ng h/mL)	19.882 ± 12.367	18.707 ± 12.254
AUC_{∞} (ng h/mL)	21.421 ± 12.901	19.654 ± 12.488
Extrapolation (%)	7.4 ± 7.5	5.6 ± 5.6
C_{max} (ng/mL)	1.908 ± 1.190	1.889 ± 1.215
T_{max} (h)	4.3 ± 1.5	4.3 ± 2.1
$T_{1/2}$ (h)	13.7 ± 13.2	9.8 ± 3.9

AUC = area under plasma concentration-time curve, C_{max} = maximal plasma concentration that is independent to sampling time, T_{max} = time for the maximal plasma concentration, $T_{1/2}$ = elimination half-life.

parameters for the reference and test drug obtained are described as follows. The profiles of the plasma isradipine concentration versus time are shown in Fig. 3. Plasma concentrations of isradipine were in the standard curve range and remained above the LLOQ (10 pg/mL) for the entire sampling period except for one subject at 0.66 h, one subject at 1 h, one subject at 24 h, and one subject at 36 h after dosing. Even though the bioavailability of orally administered isradipine was characterized by considerable interindividual variation [16,19,21], the plasma profiles of the mean isradipine concentration versus time after oral administration of a single dose of both formulations in 24 subjects exhibited closely similar patterns. The mean estimated pharmacokinetic parameters derived from the plasma concentration profiles of isradipine are shown in Table 2. The bioequivalence parameters almost overlapped between the test and reference samples, with the only exception of mean elimination half-life ($T_{1/2}$) that showed a certain difference 13.7 ± 13.2 (range 2.6–72.2) h for the reference versus 9.8 ± 3.9 (range 4.8–20.5) h for the test. The difference was from considerable interindividual variation. Especially, one subject (subject no: B3) who participated in the reference group but had an extraordinary long elimination half time ($T_{1/2}$) of 72.2 h which contributed to the greater difference. A large variation in clearance is usually seen in a high clearance drug with low bioavailability such as isradipine [20]. The mean ratio of the AUC_{48} divided by AUC_{∞} was 92.8% for the reference and 95.64% for the test. The 90% confidence interval (CI) of the test/reference percent ratios were 98.1% (91.31–105.46%) for C_{max} and 93.5% (83.56–104.84%) for $AUC_{48\,h}$. No differences between the reference and test drugs were detected (p-value greater than 0.05 for the formulation effect in all tested parameters). Since a 90% CI for C_{max} and AUCs ratios were ranging within the limits of the FDA (80–125%), it was determined that the two types of 5 mg SR isradipine formulations were considered to be bioequivalent.

In previous reports, absorption of an orally administrated IR isradipine capsule in the fasting condition was rapid with 1.2–2.0 h of T_{max} and 6.1–13.8 h of elimination half-life ($T_{1/2}$) [16–18,21]. SR isradipine capsules took about 3–4 times longer (6 h) for the T_{max} compared to IR isradipine. Our experimental results in a Korean population showed faster T_{max} and half-life than previous oral studies using SR isradipine.

4. Conclusion

The proposed method of combining a simplified plasma extraction procedure and a highly sensitive HPLC/ESI-MS/MS method provided a rapid and sensitive detection technique for isradipine in human plasma. We achieved a lower LLOQ (10 pg/ml) and shorter retention times (0.81 min. for isradipine, 0.65 min for IS) than previous reports. The precision and accuracy for calibration and QC samples were well within the acceptable limits. This method was sensitive enough to monitor isradipine plasma concentrations up to 48 h after dosing and provided us with a successful application in pharmacokinetics and bioequivalence study of the two different isradipine 5 mg SR capsule formulations in 24 healthy Korean volunteers.

Acknowledgements

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