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Original Paper

Hydrophilic interaction chromatography-tandem mass spectrometric analysis of irbesartan in human plasma: Application to pharmacokinetic study of irbesartan

A hydrophilic interaction chromatography-tandem mass spectrometric method (HILIC/MS/MS) for the determination of irbesartan in human plasma was developed. Irbesartan and losartan (internal standard) were extracted from human plasma with ethyl acetate at acidic pH. The analytes were analyzed on a Luna HILIC column with the mobile phase of ACN–ammonium formate (50 mM, pH 6.5) (96:4, v/v) and detected by ESI MS/MS in the selected reaction monitoring mode. The standard curve was linear (r^2 = 0.9981) over the concentration range of 10–2500 ng/mL and the lower LOQ was 10 ng/mL using 100 μL of plasma sample. The CV and relative error for intra- and interassay at four QC levels were 2.9 to 8.1% and −2.7 to 2.3%, respectively. There were less absolute and relative matrix effects for irbesartan and losartan. The present method was successfully applied to the pharmacokinetic study of irbesartan after oral dose of irbesartan (150 mg tablet) to male healthy volunteers.

Keywords: HILIC/MS/MS / Human plasma / Irbesartan

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1 Introduction

Irbesartan, 2-butyl-3-[*p*-(*o*-1*H*-tetrazol-5-ylphenyl)benzyl]-1,3-diazaspiro[4.4]non-1-en-4-one, is an angiotensin II receptor antagonist used for the treatment of hypertension. Irbesartan may also delay the progression of diabetic nephropathy and is also indicated for the reduction of renal disease progression in patients with type 2 diabetes, hypertension and microalbuminuria or proteinuria [1, 2]. Irbesartan is also available as a combination formulation with a low dose of hydrochlorothiazide to achieve an additive antihypertensive effect.

For the determination of irbesartan in human plasma or urine samples, RP-HPLC methods with UV [3–7] and fluorescence [2, 8, 9] detection have been described using protein precipitation [2, 3, 5], SPE [4, 6–8], and liquid–liquid extraction [9] as clean-up procedures. RPLC/MS/MS methods [10–12] using gradient elution have been reported for the simultaneous determination of several angiotensin II receptor antagonists including irbesartan in biological fluids. Those methods required a large amount of plasma or urine samples (250–1000 μL) in order to obtain the high sensitivity and extensive chromatographic run times [2–12].

Hydrophilic interaction chromatography (HILIC) technique using polar stationary phases such as bare silica, amino and sulfobetaine-type zwitterionic columns, and low aqueous/high organic mobile phase has been shown to be a valuable tool for MS analysis of the polar or hydrophilic compounds that elute near the void in RPLC [13–20]. The increased retention of the analytes in HILIC allows elution outside the suppression region and less matrix effect in MS techniques. The high organic mobile phases in HILIC offers more favorable desolvation and ionization conditions, resulting in an increase of MS sensitivity. To the best of our knowledge, HILIC/MS/MS method for the determination of other angiotensin II receptor antagonists as well as irbesartan in human plasma has not been reported.

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Abbreviations: HILIC, hydrophilic interaction chromatography; **QC,** quality control; **RE,** relative error; **SRM,** selected reaction monitoring

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To develop the rapid, selective, and sensitive method for the quantification of irbesartan in human plasma, HILIC/MS/MS method was described and validated. The present method has been successfully applied to the pharmacokinetic study of irbesartan after oral administration of irbesartan (150 mg tablet) to 20 male volunteers.

2 Experimental

2.1 Materials and reagents

Irbesartan (purity, 100.6%) and losartan potassium (purity, 99.5%; internal standard) were supplied by Dong-A Pharm. (Yongin, Korea). ACN and ethyl acetate (HPLC grade) were obtained from Burdick & Jackson (Muskegon, MI, USA) and the other chemicals were of the highest quality available. Drug-free human plasma containing sodium heparin as the anticoagulant was obtained from healthy volunteers.

2.2 Preparation of calibration standards and quality control samples

Primary stock solutions of irbesartan and losartan (1 mg/mL) were prepared in DMSO. Working standard solutions of irbesartan were prepared by diluting primary stock solution with ACN. The internal standard working solution (2 μg/mL losartan) was prepared by diluting an aliquot of stock solution with ACN. All standard solutions were stored at *ca.* 4°C in 20 mL scintillation vial in the dark when not in use.

Human plasma calibration standards of irbesartan, *i.e.*, 10, 20, 100, 500, 1000, 2000, and 2500 ng/mL, were prepared by spiking the working standard solutions into a pool of drug-free human plasma. Quality control (QC) samples at 10, 30, 400, and 1800 ng/mL were prepared in bulk by adding 250 μL of the appropriate working standard solutions (0.2, 0.6, 8, and 36 μg/mL) to drug-free human plasma (4750 μL). The bulk samples were aliquoted (100 μL) into polypropylene tubes and stored at −80°C until analysis.

2.3 Sample preparation

Human blank plasma (100 μL), calibration standards, and QC samples were mixed with 10 μL of internal standard working solution and 200 μL of 50 mM HCl. The samples were extracted with 1000 μl of ethyl acetate in 1.5 mL polypropylene tubes by vortex mixing for 2 min at high speed and centrifuged at 10 000 × *g* for 5 min at 4°C. The organic layer (900 μl) was transferred and evaporated to dryness using vacuum concentrator at 30°C. The residues were dissolved in 400 μL of 96% ACN by vortex mixing for 2 min, sonicated for 3 min, transferred to injection vials, and 2 μL were injected into the LC/MS/MS system.

2.4 HILIC/MS/MS analysis

LC/MS/MS system consisted of a Nanospace SI-2 pump, SI-2 column oven, an SI-2 autosampler (Shiseido, Tokyo, Japan), and a tandem quadrupole mass spectrometer (TSQ Quantum Access, ThermoFisher Scientific, CA, USA). The separation was performed on a Luna HILIC column (3 μm, 100 × 2.0 mm i.d., Phenomenex, CA, USA) using a mixture of ACN–ammonium formate (50 mM, pH 6.5) (96:4 v/v) at a flow rate of 0.2 mL/min. The column and autosampler tray were maintained at 50 and 6°C, respectively. The analytical run time was 4.0 min. The ESI source settings for the analysis of irbesartan and losartan were as follows: spray voltage, 5.0 kV; vaporizer temperature, 250°C; capillary temperature, 330°C; sheath gas pressure, 35 psi; auxiliary gas pressure, 10 psi. The tube lens offsets for irbesartan and losartan were 71 and 58 V, respectively, in order to produce a strong protonated molecular ion (MH^+) without formation of adduct and fragment ions. Fragmentation of MH^+ for irbesartan and losartan was performed at collision energy of 22 and 20 V, respectively, by collision-activated dissociation with argon as the collision gas at a pressure setting of 1.5 on the instrument. Selected reaction monitoring (SRM) mode was employed for the quantification: m/z 429.02 → 206.91 for irbesartan and m/z 423.09 → 206.91 for losartan. The LC/MS/MS system control and data processing were performed by the Xcalibur® software (ThermoFisher Scientific).

2.5 Method validation

Batches, consisting of triplicate calibration standards at each concentration, were analyzed on three different days to complete the method validation. In each batch, QC samples at 10, 30, 400, and 1800 ng/mL were assayed in sets of six replicates to evaluate the intra- and interday precision and accuracy. The relative error (RE), percentage deviation of the mean from true values, and CV serves as the measure of accuracy and precision, respectively.

The matrix effect and recovery of irbesartan were assessed by analyzing three sets of standards at four concentrations, *i.e.*, 10, 30, 400, and 1800 ng/mL [21]. The absolute matrix effect for irbesartan was assessed by comparing the mean peak areas of the analyte at four concentrations spiked after extraction into plasma extracts originating from five different humans (set 2) to mean peak areas for neat solutions of the analyte in 96% ACN (set 1). The relative matrix effect was evaluated as a measure of the variability in the peak areas of the analyte spiked postextraction into five different plasma extracts (set 2)

expressed as CVs (%). Recoveries of irbesartan and losartan were determined by comparing mean peak areas of analytes spiked before extraction into the same five different plasma as set 2 (set 3) with those of the analytes spiked postextraction into different blank plasma lots at four concentrations (set 2).

To evaluate the freeze/thaw stability and room temperature storage stability, the five replicates of QC samples at low and high concentrations (30 and 1800 ng/mL) were subjected to three freeze/thaw cycles or storage at room temperature for 4 h before processing. Postextraction batch integrity was determined by batch reinjection after 24 h storage in the autosampler.

2.6 Clinical application

The present method was applied to a pharmacokinetic study after an oral administration of irbesartan to male volunteers. The protocol was approved by an institutional review board at the Research Institute for Drug Development, Sungkyunkwan University (Suwon, Korea) and the informed consent was obtained from the subjects after explaining the nature and purpose details of the study. Twenty healthy volunteers, fasted for 12 h, received a single oral dose of irbesartan (Aprovel® tablet 150 mg, Sanofi-aventis Korea, Seoul, Korea) with 200 mL of water. Blood samples (3 mL) were withdrawn from the forearm vein at 0, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 24, and 48 h postdosing, transferred to Vacutainer® tubes (sodium heparin, BD, NJ, USA), and centrifuged at $3000 \times g$ for 10 min. The plasma samples were transferred to polypropylene tubes and stored at −80°C prior to analysis.

The maximum concentration (C_{max}) and the time to maximum concentration (T_{max}) were determined by visual inspection from each volunteer's plasma concentration–time curve for irbesartan. Area under the plasma concentration–time curve (AUC) from 0 to 48 h and terminal elimination half-life ($t_{1/2}$) were calculated using a noncompartment analysis (WinNonlin, Pharsight, Mountain View, CA, USA).

3 Results and discussion

3.1 HILIC/MS/MS

The ESI of irbesartan and losartan produced the abundant protonated molecular ions (MH^+) at *m*/*z* 429.02 and 423.09, respectively, without any evidence of fragmentation and adduct formation. MH^+ ions of irbesartan and losartan were selected as the precursor ions and were subsequently fragmented in MS/MS mode to obtain the product ion spectra, yielding the useful structural information (Fig. 1). Irbesartan produced the major product ion at *m*/*z* 206.91 *via* the loss of 2*H*-tetrazol-5-yl-biphenyl

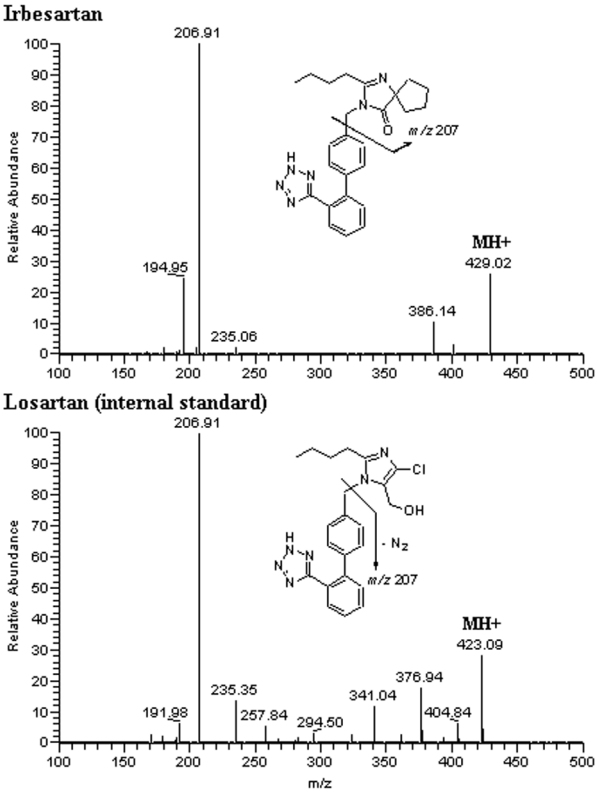

Figure 1. Product ion mass spectra of (a) irbesartan and (b) losartan.

moiety from MH^+ ion and the prominent product ion for losartan was *m*/*z* 206.91 due to the loss of 2-butyl-5-chloro-3*H*-imidazol-4-yl-methanol moiety and N_2 from MH^+ ion. Quantification of analytes was performed using the SRM mode due to the high selectivity and sensitivity of SRM data acquisitions, where the transition of the precursor ion to a product ion is monitored: *m*/*z* 429.02 → 206.91 for irbesartan and *m*/*z* 423.09 → 206.91 for losartan (internal standard).

The Luna HILIC column (3 μm, 2.0 mm i.d. × 100 mm, Phenomenex) with a mobile phase consisting of a mixture of ACN–ammonium formate (50 mM, pH 6.5) (96:4 v/v) resulted in chromatographic run time (4.0 min) with satisfactory separation of irbesartan and losartan. The primary HILIC chromatographic parameters are column chemistry, the type and composition of organic solvent, buffer pH, and ionic strength [13–20]. The use of ammonium formate pH 6.5 buffer resulted in the better retention and sensitivity of irbesartan and losartan compared to pH 3.0 and 4.5 buffers on a Luna HILIC column. Irbesartan showed better retention and peak shape on a Luna HILIC column compared to an Atlantis HILIC silica column. The high ACN content (96%) in mobile phase increased ESI efficiency for MS/MS detection and resulted in high sensitivity of irbesartan. The sensitivity for irbesartan in this HILIC/MS/MS method was 0.005 ng while

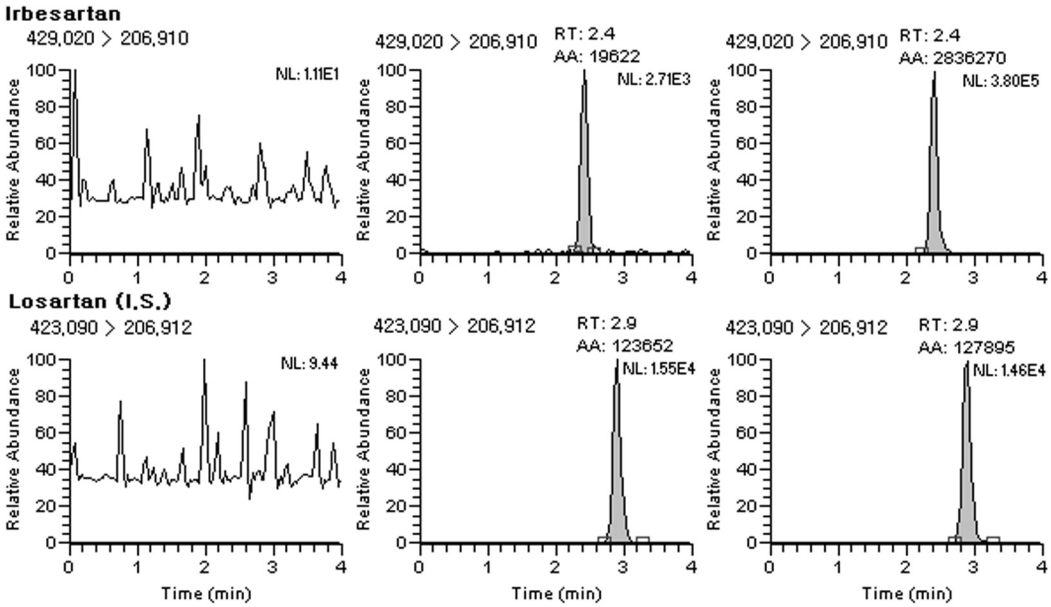

Figure 2. SRM chromatograms of (a) a human blank plasma, (b) a human plasma sample spiked with 10 ng/mL of irbesartan, and (c) a human plasma sample obtained 2 h after oral administration of irbesartan (150 mg tablet) to a male volunteer.

Table 1. Calculated concentrations of irbesartan in calibration standards prepared in human plasma ($n = 3$)

Statistical variable	Theoretical concentration (ng/mL)							Slope	Intercept	r^2
	10.0	20.0	100.0	500.0	1000.0	2000.0	2500.0			
Mean (ng/mL)	9.8	20.1	101.0	514.1	1005.5	1976.9	2498.9	0.0134	−0.0092	0.9981
CV (%)	10.5	6.6	7.6	3.7	4.5	5.7	4.2	8.1		
RE (%)	−2.0	0.5	1.0	2.8	0.6	−1.2	0.0			

those were 0.53 or 0.047 ng in RPLC/MS/MS methods [10, 11].

The typical HILIC/MS/MS SRM chromatograms of a blank human plasma and a human plasma sample spiked with irbesartan at 10 ng/mL were shown in Fig. 2. There was no interference peak at the retention times of irbesartan (2.4 min) and losartan (2.9 min) in the analysis of blank plasma samples from 20 volunteers, indicating the selectivity of the present method. The retention times of irbesartan and losartan were reproducible throughout the experiment and no column deterioration was observed after analysis of 500 human plasma samples. Sample carryover effect was not observed.

3.2 Method validation

Calibration curves were obtained over the concentration range of 10–2500 ng/mL for irbesartan in human plasma. Linear regression analysis with a weighting of 1/concentration gave the optimum accuracy (RE: −2.0 to 2.8%) and precision (CV: ≤10.5%) of the corresponding calculated concentrations at each level (Table 1). The low CV value (8.1%) for the slope indicated the repeatability of the method (Table 1).

Table 2 shows a summary of intra- and interday precision and accuracy data for QC samples containing irbesartan. Both intra- and interassay CV values ranged from 2.9 to 8.1% at four QC levels. The intra- and interassay RE values were −2.7 to 2.3% at four QC levels. These results indicated that the present method has the acceptable accuracy and precision. The LOQ was set at 10 ng/mL for irbesartan using 100 μL of human plasma. Representative chromatogram at the LOQ is shown in Fig. 2b and the S/N for irbesartan are higher than 20.

The liquid–liquid extraction of irbesartan and losartan using ethyl acetate at acidic pH resulted in better recovery compared to the use of methyl *tert*-butyl ether and dichloromethane as extraction solvent [9]. The extraction recoveries of irbesartan and losartan were 63.3 and 70.8%, respectively (Table 3).

The absolute matrix effects, the mean peak areas of set 2 to those of set 1 multiplied by 100, were 100.7% for irbesartan and losartan (Table 3). A value of 100% indicates that the response in the solvent and in the plasma

Table 2. Precision and accuracy of irbesartan in human plasma QC samples

Statistical variable	Intraday (n = 6)				Interday (n = 3)			
QC (ng/mL)	10.0	30.0	400.0	1800.0	10.0	30.0	400.0	1800.0
Mean (ng/mL)	10.2	29.5	403.7	1785.8	10.2	30.7	391.6	1750.8
CV (%)	8.1	6.0	5.4	4.8	2.9	4.1	3.7	3.1
RE (%)	2.0	−1.7	0.9	−0.8	2.0	2.3	−2.1	−2.7

Table 3. Absolute matrix effect and recovery of irbesartan and losartan (internal standard) in five different lots of human plasma

Nominal concentration (ng/mL)	Absolute matrix effect[a] (%)		Recovery[b] (%)	
	Irbesartan	Losartan	Irbesartan	Losartan
10.0	100.8	100.7	62.2	70.4
30.0	98.6	101.3	62.5	69.6
400.0	101.6	101.0	65.7	73.5
1800.0	101.8	99.9	62.9	69.8
Mean	100.7	100.7	63.3	70.8

[a] Absolute matrix effect expressed as the ratio of the mean peak area of an analyte spiked postliquid–liquid extraction (set 2) to the mean peak area of same analyte standards (set 1) multiplied by 100.

[b] Recovery calculated as the ratio of the mean peak area of an analyte spiked before liquid–liquid extraction (set 3) to the mean peak area of an analyte spiked after liquid–liquid extraction (set 2) multiplied by 100.

extracts were the same and no absolute matrix effect was observed. A value of <100% indicates the ionization suppression and a value of >100% indicates the ionization enhancement. Absolute matrix effects for irbesartan and losartan were little observed. The relative matrix effect was assessed based on direct comparison of the peak areas of irbesartan and losartan spiked postextraction into plasma extracts originating from five different human plasma samples (set 2). The CVs of determination of set 2 at different concentrations were 2.3–4.6% for irbesartan and 2.2–3.6% for losartan. This variability seemed to be comparable to CVs of peak areas of standards injected directly in 96% ACN (set 1) (2.3–4.6%), indicating that there was no relative matrix effect for irbesartan and losartan.

The stabilities of processing (three freeze/thaw cycles and 4 h storage at room temperature) and chromatography (reinjection) were evaluated with QC samples at 30.0 and 1800 ng/mL and shown to be of insignificant effect (Table 4). QC samples that went through three freeze/thaw cycles showed the acceptable accuracy (RE: −8.3∼ −5.5%) and precision (CVs: ≤7.9%). QCs showed the acceptable accuracy (RE: −9.7∼ −5.0%) and precision (CVs: ≤7.4%) when exposed to room temperature for 4 h.

Table 4. Stability of samples (n = 5)

Statistical variable	Theoretical concentration (ng/mL)	
	30.0	1800.0
Three freeze and thaw cycles		
Mean (ng/mL)	27.5	1700.7
CV (%)	4.7	7.9
RE (%)	−8.3	−5.5
Short-term stability (4 h storage at room temperature)		
Mean (ng/mL)	26.8	1710.1
CV (%)	1.9	7.4
RE (%)	−9.7	−5.0
Postpreparative stability (24 h at 4°C)		
Mean (ng/mL)	31.6	1869.0
CV (%)	4.2	3.7
RE (%)	5.3	3.8

The reanalysis of the reconstituted extracts stored for 24 h at 4°C showed the acceptable accuracy (RE: 3.8∼5.3%) and precision (CVs: ≤4.2%) for QC samples.

3.3 Clinical application

This method has been successfully applied to the analysis of 260 plasma samples in a pharmacokinetic study of irbesartan. Representative chromatograms of the extract of a plasma sample obtained 2 h after oral administration of irbesartan at a dose of 150 mg to human are shown in Fig. 2c. Figure 3 shows mean plasma concentration profiles of irbesartan obtained after a single oral dosing of irbesartan (150 mg tablet) to 20 healthy male volunteers. C_{max}, T_{max}, $AUC_{0-48\,h}$, and $t_{1/2}$ of irbesartan were 2150.3 ± 515.7 ng/mL, 1.1 ± 0.7 h, 8193.9 ± 2461.3 ng/h/mL, and 8.6 ± 4.1 h, respectively.

4 Concluding remarks

A rapid, sensitive, and reliable HILIC/MS/MS method for the determination of irbesartan in human plasma has been successfully developed. Irbesartan and losartan were extracted from 100 μL of plasma samples using ethyl acetate at acidic pH as sample preparation. This method demonstrated acceptable sensitivity (LOQ:

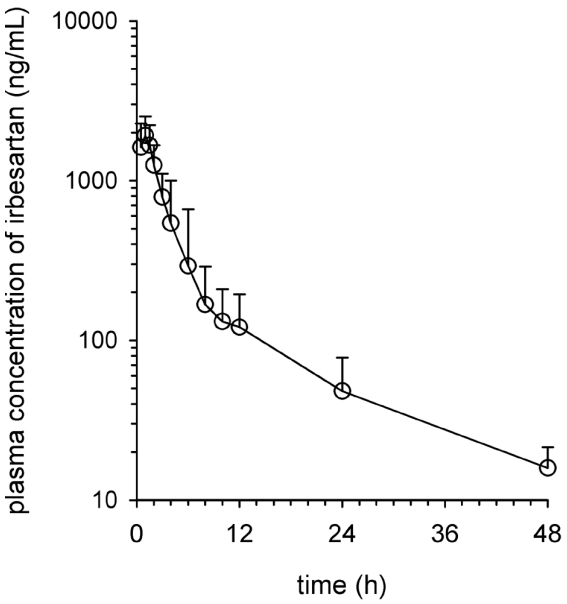

Figure 3. Mean plasma concentration–time plot of irbesartan after a single oral dose of irbesartan (150 mg tablet) to 20 male volunteers. Each point represents the mean ± SD.

10.0 ng/mL), precision, accuracy, selectivity, recovery and stability, and minor matrix effects. The present method was successfully applied to the determination of irbesartan in plasma samples obtained after an oral administration of irbesartan (150 mg tablet) in 20 male volunteers.

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The authors declared no conflict of interest.

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