Validated LC−MS/MS Assay for the Quantitative Determination of Fimasartan in Human Plasma: Application to Pharmacokinetic Studies

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A simple, rapid and sensitive liquid chromatography−tandem mass spectrometry (LC−MS/MS) method for the quantification of a newly developed antihypertensive agent, fimasartan (BR-A657, Kanarb®) in human plasma was developed and validated. Fimasartan and internal standard (IS, BR-A563) were extracted by simple protein precipitation using acetonitrile and separated on a Phenyl-Hexyl column (Luna™, 5 μm, 50 mm × 2.0 mm, Phenomenex) under the gradient conditions of mobile phase A (distilled water with 0.1% formic acid) and mobile phase B (100% acetonitrile with 0.1% formic acid) at a flow rate of 0.25 mL/min. Detection and quantification were performed by the mass spectrometer using multiple reaction monitoring mode at m/z 500.2→221.2 for fimasartan and m/z 524.3→204.9 for the IS. The assay was linear over a calibration range of 0.5–500 ng/mL with a lower limit of quantification of 0.5 ng/mL. The coefficient of variation of this assay precision was <14.9% and the accuracy exceeded 91.9%. This method provided the necessary sensitivity, linearity, precision, accuracy and specificity to allow the determination of fimasartan after oral administration to healthy Korean male volunteers in several drug−drug interaction studies conducted at the Clinical Trials Center of Seoul National University Hospital.

Introduction

Angiotensin II, a potent vasoconstrictor and the primary effector of the renin angiotensin system (RAS), plays an important role in the development of hypertension (1–3). Of the two major angiotensin II receptor subtypes identified (AT1 and AT2), the AT1 receptor mediates the pressor and growth-promoting effects of angiotensin II (4). Direct inhibition of RAS at the level of AT1 receptor induces vasodilation, reduces secretion of vasopressin and reduces production and secretion of aldosterone whereby in combination of these effects reducing the blood pressure (5). Some examples of clinically available angiotensin II receptor antagonists (ARBs) are losartan, valsartan, candesartan, telmisartan, irbesartan and olmesartan. These ARBs have advantages on angiotensin-converting enzyme (ACE) inhibitors, and various adverse effects such as dry cough and angioedema associated with the use of ACE inhibitors do not occur in the cases with ARBs (6, 7).

Fimasartan, 2-n-butyl-5-dimethylaminothiocarbonylmethyl-6-methyl-3-[(2’-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl]pyrimidin-4(3H)-one (Kanarb®, BR-A657, C27H31N7OS; Figure 1A), is a novel, non-peptide angiotensin II receptor antagonist with a selective AT1 receptor blocking effect, launched in September 2010, as an antihypertensive agent by Boryung Pharm. Co., Ltd. in Korea (8–9). In various preclinical studies, fimasartan has been shown to cause a dose-dependent decrease in the mean arterial pressure. It exerts a direct antihypertensive effect on vascular smooth muscle cells and an indirect antihypertensive effect on sodium retention, fluid retention and vascular tone as mediated by blockade of angiotensin II (10). Furthermore, this rapid antihypertensive effect is known to be more potent than that of losartan, the first marketed drug in ARBs.

To date only one liquid chromatography with tandem mass spectrometry method for the determination of fimasartan in rats has been reported (11). For sample preparation, the rat plasma sample (50 μL) was deproteinized with acetonitrile after the addition of IS (BR-A-563, 100 ng/mL), centrifuged, transferred to a polystyrene HPLC vial and mixed with 100 μL of distilled water. The assay ranged from 0.2 to 500 ng/mL. Retention times for fimasartan and IS in this article were 5.6 min and 5.9 min, respectively. The total run time was more than 10 min due to the presence of late-eluting interferences.

The objective of this study was to determine a more rapid, reliable and sensitive method to be used for the quantitation of fimasartan in human plasma using LC−MS/MS system to be applied in the determination of plasma fimasartan concentration in Korean volunteers.

Experimental

Chemicals and reagents

Fimasartan and BR-A563 (IS, Figure 1B) were kindly provided by the Research Laboratory of Boryung Pharm. Co., Ltd (Seoul, Korea). HPLC-grade acetonitrile and methanol were purchased from Fisher Scientific (PA, USA). Formic acid was purchased from Sigma (MO, USA). A Milli-Q® (Millipore Co., MA, USA) water purification system was used to obtain purified water used for the analysis. Blank human plasma was provided by the Blood Bank in Seoul National University Hospital and stored at approximately −70°C until use.

Instruments and chromatographic conditions

The quantification of fimasartan in human plasma was carried out with LC−MS/MS system using an Agilent 1200 series HPLC (Agilent Technologies, USA) coupled with an Applied Biosystems API3200 triple quadrupole mass spectrometer (Applied Biosystems, USA), equipped with an electrospray ionization source operated in negative ionization mode. The analytical column used was a Phenyl-Hexyl column (Luna™, 5 μm, 2.0 mm × 50 mm, Phenomenex) under the gradient conditions of mobile phase A (distilled water with 0.1% formic acid) and mobile phase B (100% acetonitrile with 0.1% formic acid) at a flow rate of 0.25 mL/min. Detection and quantification were performed by the mass spectrometer using multiple reaction monitoring mode at m/z 500.2→221.2 for fimasartan and m/z 524.3→204.9 for the IS. The assay was linear over a calibration range of 0.5–500 ng/mL with a lower limit of quantification of 0.5 ng/mL. The coefficient of variation of this assay precision was <14.9% and the accuracy exceeded 91.9%. This method provided the necessary sensitivity, linearity, precision, accuracy and specificity to allow the determination of fimasartan after oral administration to healthy Korean male volunteers in several drug−drug interaction studies conducted at the Clinical Trials Center of Seoul National University Hospital.
The samples were separated under the gradient condition using mobile phase A (double-distilled water with 0.1% formic acid) and mobile phase B (acetonitrile with 0.1% formic acid). The gradient condition used is described in Table I. The flow rate was maintained at 0.25 mL/min. The ion spray voltage was set at 4,000 V and at a temperature of 600°C. Voltages within the ion source were as follows: collision energy, entrance potential, collision cell exit potential and declustering potential of −46, −4, −4 and −50 V and −52, −4, −4 and −60 V for fimasartan and IS, respectively.

Preparation of the calibration standards and quality control samples

A stock solution of fimasartan was prepared in methanol at a concentration of 1 mg/mL. A subsequent dilution was made in 50% methanol, and thereafter working dilutions were prepared volumetrically at final concentrations across the range 25–25,000 ng/mL. All fimasartan solutions were stored in a refrigerator (4°C) using polypropylene tubes.

Plasma calibration standards were prepared on each day of analysis by the addition of 20 μL of working solutions to 1,000 μL of blank plasma to provide effective plasma concentrations of 0.5, 1, 5, 10, 50, 100 and 500 ng/mL. A second stock solution of fimasartan (1 mg/mL in methanol) was prepared from a separate weighing procedure, and dilutions were made in 50% methanol to form spiking solutions at 25, 100, 2,500 and 20,000 ng/mL for the preparation of quality control (QC) samples. These QC samples were prepared in drug-free human plasma at 0.5, 2, 50 and 400 ng/mL, respectively, and were used in the assay validation and subsequent sample analyses. The QC samples were stored as small aliquots in 1.5-mL polypropylene tubes at approximately −70°C until use.

Preparation of internal standard

A stock solution of internal standard (IS; BR-A563) was prepared at 100 μg/mL by appropriate dilution using methanol. This was stored at 4°C in a glass bottle. A 50 ng/mL of freshly prepared IS in acetonitrile was used for analysis.

Plasma sample preparation

An aliquot (50 μL) of the plasma sample containing fimasartan was pipetted into 1.5-mL polypropylene tubes and 100 μL of an IS (50 ng/mL in acetonitrile) was added and vortexed for 1 min. The tubes were then centrifuged at 13,000 rpm, at 4°C for 5 min to precipitate protein. A 100 μL of each supernatant was transferred into the injection vial and an aliquot of 5 μL was injected onto the LC–MS/MS system for analysis. All prepared samples were kept at 4°C until injection.

Assay validation

The validation parameters were specificity, linearity, sensitivity, accuracy, precision, recovery and stability in human plasma according to the US Food and Drug Administration (FDA) guidance for the validation of bioanalytical methods (12). Specificity in relation to endogenous compounds was established by confirming the absence of significant chromatographic peaks interfering with the analyte and IS in six different human plasma samples. Linearity was assessed on five occasions by processing plasma standard curves for fimasartan over the concentration range of 0.5–500 ng/mL. Peak area ratios (fimasartan:IS) were plotted against fimasartan concentrations for the standards, and

Table I

<table>
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<tr>
<th>Time (min)</th>
<th>Percentage of mobile phase in eluent</th>
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<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>0.00</td>
<td>60</td>
</tr>
<tr>
<td>1.02</td>
<td>20</td>
</tr>
<tr>
<td>2.02</td>
<td>20</td>
</tr>
<tr>
<td>2.05</td>
<td>60</td>
</tr>
<tr>
<td>6.02</td>
<td>60</td>
</tr>
</tbody>
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A, double-distilled water with 0.1% formic acid; B, acetonitrile with 0.1% formic acid.

Figure 1. Chemical structures of fimasartan (A) and IS (B; BR-A563).
calibration curves were generated by a weighted linear fit, using a weighting factor of $1/x^2$.

**Precision and accuracy**

Intra-assay precision and accuracy were assessed by the analysis of six QC samples at each of the low, medium and high concentrations on a single occasion, while inter-assay precision and accuracy were assessed over five occasions, by the analysis of six QC samples at each concentration on each occasion. Measured concentrations were determined by comparison to a full calibration curve analyzed concurrently on each occasion. Sample dilution precision and accuracy was assessed by demonstrating the ability to dilute samples with a concentration above the upper limit of the standard curve to within the analytical range of 0.5–500 ng/mL. Six replicates of a QC sample with fimasartan concentration of 4,000 ng/mL were diluted 10 times with drug-free human plasma and assayed and three freeze–thaw cycles prior to assay. The concentrations of fimasartan in each of the above tests were calculated by comparison to a freshly prepared standard curve and compared with baseline results.

**Application to pharmacokinetic studies**

Several drug–drug interaction studies of fimasartan and drugs with different mechanisms of action were conducted (14–18). These studies were approved by the Institutional Review Board (IRB) of Seoul National University Hospital (SNUH, Seoul, South Korea) and all procedures were performed in accordance with the recommendations of the Declaration of Helsinki (Sixth revision) on biomedical research involving human subjects, and the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use—Good Clinical Practice Guidelines. One example of these drug–drug interaction studies is an open-label, multiple-dosing, two-part, two-treatment, two-period, fixed-sequence crossover study with coadministration of fimasartan and amlodipine in 34 healthy male volunteers. The study protocol of this study was approved by the IRB of SNUH (permission number: 0904-008-277, 2009.06.09–2010.06.08) and before the start of subject enrollment, this trial had been registered in a public trial registry, ClinicalTrials.gov (www.clinicaltrials.gov, NCT code: NCT00938197). The aim of our study was to investigate the effect of amlodipine on the pharmacokinetics of fimasartan (Part A) and to investigate the effect of fimasartan on the pharmacokinetics of amlodipine (Part B). Twenty volunteers were enrolled in Part A of the study. The volunteers, who had given their written informed consent, were administered 120 mg of fimasartan alone for 7 days in Period 1, and they received 10 mg of amlodipine in Period 2 for 10 days, coadministered with 120 mg of fimasartan from day 4 of Period 2. Blood samples for pharmacokinetics of fimasartan were collected in a lithium heparin tube prior to drug administration and up to 24 h on day 7 (period 1) and day 21 (period 2) at 0 (before dosing), 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12 and 24 h after administration of the study drugs. Blood samples were centrifuged at ~3,000 rpm at 4 °C for 10 min, and the harvested plasma was aliquoted and stored below −70 °C until analysis.

**Results**

**Mass spectrometry**

The product ion spectra and fragmentation patterns for fimasartan and IS are shown in Figure 2. Mass transitions of the ions of fimasartan and IS were $m/z$ 500.2 $> 221.2$ and $m/z$ 524.3 $> 204.9$, respectively. The mass parameters were optimized by observing the maximal response of the product ions.

**Chromatography**

The chromatographic conditions and sample preparation for the proposed method were optimized to suit the pharmacokinetic studies. Figure 3 shows typical mass chromatograms of human drug-free blank plasma (A); blank plasma with IS (B); blank plasma spiked with fimasartan corresponding to a concentration of

**Lower limit of quantitation**

The lower limit of quantification (LLOQ) is defined as the lowest concentration of analyte that can be determined with acceptable precision (within 20%) and accuracy (80–120%) under the experimental conditions. The LLOQ was determined using the signal-to-noise ratio of 5:1 by comparing test results from samples with known concentrations (0.5 ng/mL) of analytes with blank samples.

**Matrix effect and extraction recovery**

Three different QC samples, 2, 50 and 400 ng/mL, were used to evaluate matrix effects and the extraction recovery of fimasartan and IS. All assays were performed in quadruplicate. Considering analyte peak areas of diluted (or neat) standard solutions spiked into plasma extracts after extraction as $A$, the corresponding analyte peak areas of diluted (or neat) standard solutions spiked into plasma before extraction as $C$, the matrix effects and extraction recovery were as follows (13):

\[
\text{Matrix effect} (\%) = \frac{A}{B} \times 100 \\
\text{Extraction recovery} (\%) = \frac{C}{A} \times 100.
\]

The matrix effects and extraction recovery of IS were evaluated using the same method.

**Stability**

To ensure the reliability of the results in relation to handling and storing of plasma samples and stock standard solution, stability studies were assessed at two different concentrations, 2 and 400 ng/mL. On-tray (autosampler carousel) stability of fimasartan was assessed by injecting the second set of QC samples ~24 h after the injection of the first set. To assess the stability of fimasartan in plasma during sample handling, quadruplicate samples of low and high concentration QC samples were prepared and allowed to stand at room temperature for 24 h prior to assay. Stability of fimasartan in plasma under the conditions of freezing and thawing was tested by the analysis of quadruplicate samples of QCs following passage through one and three freeze–thaw cycles prior to assay. In each case, samples were frozen at −70 °C for at least 24 h and then thawed at room temperature prior to assay. The concentrations of fimasartan in each of the above tests were calculated by comparison to a freshly prepared standard curve and compared with baseline results.
0.5 ng/mL (C) and plasma sample obtained from a volunteer 30 min after the oral administration of 120 mg of fimasartan (93.02 ng/mL) at day 7 (D). A simple protein-precipitation extraction method was applied in this study, which resulted in clean and sharp peaks with high resolution. The IS, BR-A563, structurally and chemically similar to fimasartan, provided most appropriate chromatographic and analytical results.

**Selectivity and specificity**

Using the chromatographic conditions described above, the retention time ranges observed during assay validation were 2.95–3.02 min for fimasartan and 2.91–2.95 min for IS, with a total run time of <6 min and high precision observed for within-batch retention times. Specificity in relation to endogenous compounds was confirmed by the absence of any significant chromatographic peaks interfering with the analyte and IS following their addition to six individual blank human plasma samples.

**Quantification and calibration curve**

Analysis of the results from the intra- and inter-day occasions of accuracy and precision (n = 5) across the 0.5–500 ng/mL range was performed using a weighted (1/x^2) linear regression method. The correlation coefficients (r) ranged between 0.9972 and 0.9991 in five batches. The precision and accuracy of calibration curves over these five occasions ranged 2.0–6.2% and 96.4–105.6%, respectively.

**Accuracy and precision**

Precision was assessed in terms of the relative standard deviation of the measured concentrations, while accuracy was assessed in terms of the mean error in the replicate set. The intra- and inter-batch precisions of the QC samples were below 14.9 and 10.5%, respectively, and the intra- and inter-batch accuracies were 91.6–102.7% and 91.9–96.8%, respectively (Table II).

**Lower limit of quantitation**

The LLOQ of this method was found to be 0.5 ng/mL for fimasartan in human plasma with CV of 14.9% and an accuracy of 99.6% (Table II). The LLOQ was determined based on a signal-to-noise ratio of 5:1.

**Matrix effect and extraction recovery**

Three QC samples, 2, 50 and 400 ng/mL, were used to evaluate the effects of the sample matrix on fimasartan ionization. The mean percentage of the matrix effect of fimasartan was 95.8%, indicating no significant matrix effect for fimasartan. The extraction recovery of fimasartan in human plasma was determined to be 78.8%. For the IS, the mean percentage of the matrix effect and the extraction recovery at 50 ng/mL were 100.5 and 104.5%, respectively. The low matrix effects and highly reproducible recoveries demonstrated the reliability of this method for bioanalysis.

**Stability**

Table III shows the results of investigations on the stability of fimasartan under various conditions tested throughout the
validation process. The tests were designed to cover anticipated conditions which might be encountered during sample handling and processing. The experiments showed fimasartan to be stable at room temperature for 24 h, through three freeze–thaw cycles, in processed extracts at 4°C for 24 h, and in stored plasma samples for up to 28 months at −70°C (data not shown).

Pharmacokinetic study
The method described above was applied for the analysis of plasma samples from healthy male human volunteers who were administered fimasartan as part of their participation in a

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Intra- and Inter-day Precision and Accuracy Data for Assay of Fimasartan in Human Plasma (n = 6)

<table>
<thead>
<tr>
<th>Fimasartan Added</th>
<th>Precision Measured (ng/mL)</th>
<th>CV (%)</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-day</td>
<td>0.5</td>
<td>0.498 ± 0.075</td>
<td>14.9</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.83 ± 0.088</td>
<td>4.79</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>51.4 ± 1.53</td>
<td>2.97</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>377.4 ± 21.7</td>
<td>5.75</td>
</tr>
<tr>
<td>Inter-day</td>
<td>0.5</td>
<td>0.477 ± 0.044</td>
<td>9.26</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.87 ± 0.196</td>
<td>10.5</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>48.4 ± 2.02</td>
<td>4.18</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>367.7 ± 10.2</td>
<td>2.79</td>
</tr>
</tbody>
</table>
The developed and validated LC–MS/MS method was successfully applied in drug–drug interaction studies of fimasartan in Korean volunteers.

**References**


**Conclusion**

This study reports a selective, simple, and rapid LC–MS/MS assay for the determination of concentration of fimasartan in human plasma with an analytical range of 0.5–500 ng/mL. This validated assay was sufficiently sensitive for the successful measurement of fimasartan in human plasma samples collected during several clinical studies conducted at the Seoul National University Hospital Clinical Trials Center and should be easily transferred with minimal change and implemented in other laboratories performing plasma sample analysis using similar equipment.
13. Matuszewski, B.K., Constanzer, M.L., Chavez-Eng, C.M.; Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS; *Analytical Chemistry*; (2003); 75: 3019–3030.