Introduction

Hypertension is a pathological condition of high blood pressure in the arteries and is very common, affecting approximately 26% of people in the world and 30% of Korean adults.1,2 As hypertension is a major risk factor for cardiovascular and cerebrovascular diseases, as well as chronic kidney disease, various anti-hypertensive drugs, such as beta blockers, calcium channel antagonists, diuretics, angiotensin-converting enzyme inhibitors, and renin angiotensin receptor blockers have been developed and used for treating these disorders.2,7

Angiotensin II, a strong vasoconstrictor, is a pivotal mediator of hypertension. Recently, angiotensin II type 1 (AT1) receptor blockers (ARBs) have been attracting attention, and have become widely used to manage hypertension. Since ARBs specifically block the interaction between angiotensin II and the AT1 receptor, they are considered to be one of the most effective anti-hypertensive drugs. Fimasartan, 2-n-butyl-5-dimethylaminothiocarbonylmethyl-6-methyl-3-[(2-[1H-tetrazole-5-yl]biphenyl-4-yl)methyl]-pyrimidine-4(3H)-one, also known as BR-A-657, is a novel ARB with strong and selective AT1 receptor blocking activity.8–11 Its chemical structure is shown in Fig. 1(A). Fimasartan was developed by Boryung Pharm. Co., Ltd. in Korea and was approved by the Korean Food and Drug Administration for the treatment of hypertension in 2010. Through a number of pre- and clinical trials, its rapid and potent anti-hypertensive effect and safety have been reported.10,12–15

It is well established that ultra-performance liquid chromatography (UPLC: Waters Corp.) has higher sensitivity, intensity, and selectivity than other chromatographic methods, including high-performance liquid chromatography (HPLC).16–19 In addition, the UPLC method provides excellent chromatographic resolution, reduced run time, and good durability under high pressure, and it allows the measurement of small amounts of samples.16–19 Therefore, the UPLC-MS/MS method has been applied in various fields, such as pharmacokinetic analysis and metabolomics studies.20

Recent studies using HPLC have shown chromatographic analyses of fimasartan in rat and human plasma.12,13 However, a more rapid and sensitive ultra-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) method to determine fimasartan in human plasma has not been reported. In this study, a new UPLC-MS/MS method for the quantification of fimasartan in human plasma was developed, fully validated, and further applied to a pharmacokinetic study of fimasartan in healthy volunteers following oral administration.

Keywords Fimasartan, angiotensin II receptor blocker, UPLC-MS/MS, pharmacokinetic study

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applied to its pharmacokinetic study in healthy volunteers after oral administration.

Experimental

Reagents and chemicals

Fimasartan and internal standard (IS), BR-A-563, were supplied by Boryung Pharm. Co., Ltd. (Seoul, Korea). The structure of IS, which has been used in previous studies,12,13 is shown in Fig. 1(B). Normal human plasma was purchased from Bio Chemed services (Winchester, VA). HPLC-grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Formic acid was obtained from Sigma-Aldrich (St. Louis, MO). Distilled water was deionized and purified using a Milli-Q water purification system from Millipore (Bedford, MA).

Preparation of standard and quality controls

Stock solutions of fimasartan and IS were prepared in methanol at a concentration of 1 ng/mL. The fimasartan stock solution was diluted with methanol to generate working solutions with concentrations ranging from 30 to 10000 ng/mL. The concentration of the IS solution was 2 μg/mL. Calibration standard samples were prepared by the addition of 20 μL of a fimasartan working solution to 180 μL of blank plasma, yielding final concentrations of 3, 5, 10, 50, 100, 500 and 1000 ng/mL. Quality-control (QC) samples were prepared in the same way as the calibration standard, with three different final concentrations of 9 ng/mL (low, LQC), 250 ng/mL (middle, MQC), and 800 ng/mL (high, HQC). Analytical standards and QC samples were stored at 4°C.

Sample preparation

Blank human plasma samples were thawed at room temperature and centrifuged at 3000 rpm for 5 min. To a polypropylene tube containing 180 μL of blank plasma, 20 μL of a fimasartan working solution and 20 μL of IS were added and vortexed briefly. Then, 600 μL of acetonitrile was added to this mixture for protein precipitation. The mixture was vortexed for 5 min and centrifuged at 13200 rpm for 10 min. Clear supernatants were collected in a vial, and 5 μL of a sample was injected into the UPLC-MS/MS system for analysis.

Instruments and conditions

Liquid chromatography conditions. Liquid chromatography was performed on an ACQUITY™ UPLC system (Waters Corp., Milford, MA) consisting of a binary pump, a micro vacuum degasser, an autosampler, and a column oven compartment. The analytes were separated on a Phenomenex Kinetex C18 column (150 × 2.1 mm, 2.6 μm) (Phenomenex, Torrence, CA) with SecurityGuard ULTRA Cartridges for UHPLC C18 for 2.1 mm i.d. columns (Phenomenex, Torrence, CA), and the column oven was set at 40°C. Mobile phase A consisted of 5 mM ammonium acetate containing 0.1% formic acid, and mobile phase B consisted of acetonitrile containing 0.1% formic acid. The initial mobile condition was 60% mobile phase A and 40% mobile phase B for 0.5 min. Then, mobile phase B was increased from 40 to 90% for 0.5 min, and returned to the initial condition for 1 min, which was held for 2 min. The autosampler temperature was set at 10°C.

Mass spectrometry conditions. Mass spectrometry was performed on an API3200 Q trap triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA). Analyst software (Ver. 1.6.0) was used for system control and data processing. Electrospray ionization (ESI) was operated in the positive ionization mode to detect fimasartan. The ion spray voltage was set at 5500 V and the source temperature at 650°C. A multiple reaction monitoring (MRM) scan mode with a dwell time of 300 ms in each transition was used in the mass spectrometer.

Method validation

Calibration curves and linearity. The calibration curves were plotted using the peak-area ratio of fimasartan to IS as a function of the nominal concentration. Calibration curves were constructed at concentrations of 3, 5, 10, 50, 100, 500 and 1000 ng/mL, including the lower limit of quantitation (LLOQ). The linear regression equation and correlation coefficient were calculated using a weighted (1/x²) least-squares linear regression.

Accuracy and precision. The accuracy and precision were assessed by replicate analyses of four QC samples at concentrations of LLOQ, LQC, MQC, and HQC. The precision and accuracy of intra-day measurements were determined by five-replicate analysis on a single day. The inter-day accuracy and precision were determined by replicate analyses of four QC samples daily for five days. The accuracy was calculated as (mean measured concentration/nominal concentration)× 100, expressed as the percent deviation (% DEV). The precision was calculated as (standard deviation/mean measured concentration)× 100, expressed as the coefficient of variation (% CV).

Specificity. The specificity was determined by comparing chromatograms of plasma samples from six healthy individuals. The plasma samples for the specificity test were as follows: double blank (blank plasma), LLOQ (plasma spiked with fimasartan), blank (plasma containing IS) and LLOQ + IS. Recovery. The recovery assay was performed using three concentrations (9, 250, 800 ng/mL) of QC samples (n = 3). The extraction recovery was determined by calculating the ratio of peak area before (A) and after (Ar) sample extraction [R(%) = A/Ar × 100].

Carry-over, matrix effect, batch size, and system suitability. The carry-over test was performed using double blank plasma (without fimasartan and IS) and the highest calibration sample (ULOQ). Briefly, the double blank plasma sample was injected immediately following the ULOQ plasma sample injection. The matrix effect assay was assessed using six different origins of human plasma spiked with fimasartan (MQC). For the batch size test, a repetitive analysis of three QC samples (LQC, MQC, and HQC) was conducted with a set of calibration standards in a single run. The number of samples that are processed as a unit was set to 150. The system suitability was evaluated by replicate analysis (n = 5) of the MQC sample.

Stability. The stability of the fimasartan plasma samples was assessed by comparing the peak-area ratios of freshly prepared samples with samples maintained under various storage conditions. The stability of fimasartan was evaluated under four different conditions: short-term temperature stability at room temperature for 20 h, long-term temperature stability at −80°C for 200 days, post-preparative stability at 10°C for 24 h in an autosampler, and freeze-thaw stability for three cycles. The stock solution stability of fimasartan and IS was assessed under two different conditions: 24 h storage at room temperature and 24 days storage at a cold temperature (4°C).

Pharmacokinetic applications

The method described above was applied to a pharmacokinetic study to determine the plasma concentration of fimasartan after the administration of a single oral dose (120 mg) in six healthy
The study protocol was approved by the institutional review board of Kyungpook National University Hospital, Daegu, Korea, and the trial was registered in a public trial registry, ClinicalTrials.gov (NCT code: NCT01921946). The study was performed according to the recommendations of the Declaration of Helsinki and good clinical practice (GCP) guidelines. During the study period, the volunteers were not allowed to take any other drug. Blood samples were collected in sodium heparinized tubes, and the time points were 0, 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 24, 36 and 48 h after the administration of fimasartan. All plasma samples were stored at –80°C until analysis. The pharmacokinetic parameters for fimasartan in plasma were estimated using the WinNonlin Professional 5.3 software (Pharsight Corp., Mountain View, CA).

Results and Discussion

Optimization of UPLC-MS/MS
A more rapid and sensitive assay method to quantify fimasartan in human plasma was established using the UPLC-MS/MS system. Solutions of fimasartan and IS were flown into the mass spectrometer under the gradient mobile-phase condition. Ion source/gas parameters were optimized to obtain enhanced sensitivity for the product ion. The mass spectra of fimasartan and IS exhibited strong protonated molecules [M+H]+ found at m/z 502.2 and 526.3, respectively. The transition of the precursors to the product ion was monitored at 502.2 → 207.1 and 526.3 → 207.1 for fimasartan and IS, respectively.

To verify that this UPLC-MS/MS equipment provides an adequate working system for chromatographic sample analysis, a system suitability assay was performed. We found that the system suitability met an acceptable criteria of 15%, and we then proceeded to analyze the samples.

Method validation
Specificity. Mass chromatograms of fimasartan and IS obtained from blank plasma and plasma spiked with fimasartan and IS are shown in Fig. 2. The retention times of fimasartan and IS were 2.91 and 2.95 min, respectively. The total run time was 4 min. In addition, each respective peak was distinct, and no significant peaks interfering with the analytes were detected for fimasartan and IS from six individual human plasma samples.

For the chromatographic analysis of fimasartan, recent studies using HPLC have been reported. Shin et al.12 analyzed fimasartan concentrations in rat plasma. In their study, the retention times for fimasartan and IS were 5.6 and 5.9 min, respectively. Furthermore, the total analysis time was more than 10 min because late-eluting interferences were present in the chromatogram. While our manuscript was being prepared for publication, Yoon et al.13 reported an HPLC-MS/MS method for the determination of fimasartan in human plasma. According to their study, the total run time was 6 min. Taken together, in comparison with these HPLC methods, the UPLC-MS/MS method developed in this study offered a faster analysis time and enhanced sensitivity.

Calibration curves and linearity. The calibration curves for fimasartan in human plasma were linear over the concentration range of 3 to 1000 ng/mL, and a representative calibration curve is shown in Fig. 3. The equation describing the calibration curve was $y = 0.0492x + 0.00894$; $y$ is the peak area ratio of fimasartan to the IS, and $x$ is the concentration of fimasartan. The correlation coefficients ($r$) of the calibration curves were over 0.9950.

Accuracy and precision. The intra- and inter-day accuracy (%, DEV) and precision (% CV) for the four QC samples are summarized in Table 1. The intra-day accuracy ranged from...
86.87 to 98.16%, and the inter-day accuracy ranged from 93.29 to 100.12%. The intra- and inter-day precision ranged from 2.00 to 3.09% and from 0.81 to 8.00%, respectively. The intra- and inter-day accuracy and precision of the four QC samples were within 15%, indicating that this UPLC-MS/MS technique provides a highly accurate and precise method for the quantification of plasma fimasartan.

**Recovery.** Fimasartan and IS were extracted from human plasma using a simple precipitation method with acetonitrile. The percentages of sample recovery at LQC, MQC, and HQC were 102.43, 97.87, and 95.10%, respectively (Table 2). The recovery was evaluated within 15% for accuracy and precision at each QC concentration, and found to be acceptable for chromatographic analysis.

**Carry-over and matrix effect.** The effect of carry over on blank human plasma samples was evaluated for fimasartan and IS. The carry-over values were below 0.2 and 0.05 for fimasartan and IS, respectively, showing that carry-over did not affect the chromatographic analysis. To evaluate the matrix effect on fimasartan analysis, a matrix effect test was performed using six blank plasma samples and IS samples.

**Conclusions**

We have developed and fully validated a new UPLC-MS/MS method suitable for analyzing up to 159 samples, including the calibration curve and QC samples.

**Table 1** Intra- and inter-day accuracy and precision of fimasartan (n = 5)

<table>
<thead>
<tr>
<th>Nominal concentration/ ng mL⁻¹</th>
<th>Accuracy, %</th>
<th>Precision, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intra-day</td>
<td>Inter-day</td>
</tr>
<tr>
<td>3</td>
<td>86.87</td>
<td>95.80</td>
</tr>
<tr>
<td>9</td>
<td>95.38</td>
<td>99.71</td>
</tr>
<tr>
<td>250</td>
<td>98.16</td>
<td>100.12</td>
</tr>
<tr>
<td>800</td>
<td>89.18</td>
<td>93.29</td>
</tr>
</tbody>
</table>

**Table 2** Results of a recovery test of fimasartan

<table>
<thead>
<tr>
<th>Nominal concentration</th>
<th>LQC (9 ng mL⁻¹)</th>
<th>MQC (250 ng mL⁻¹)</th>
<th>HQC (800 ng mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Testa</td>
<td>Referenceb</td>
<td>Testa</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>0.643 ± 0.04</td>
<td>0.628 ± 0.01</td>
<td>15.864 ± 0.22</td>
</tr>
<tr>
<td>CV, %</td>
<td>6.34</td>
<td>1.98</td>
<td>1.40</td>
</tr>
<tr>
<td>Mean recovery, %</td>
<td>102.43</td>
<td>97.87</td>
<td>95.10</td>
</tr>
<tr>
<td>Mean recovery CV, %</td>
<td>3.76</td>
<td></td>
<td></td>
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</table>

a. Peak area ratio of extracted samples.  b. Peak area ratio of unextracted samples.

**Table 3** Stability of fimasartan under four different conditions (n = 3)

<table>
<thead>
<tr>
<th>Nominal concentration</th>
<th>Short-term, for 20 h</th>
<th>Long-term for 200 days</th>
<th>Post-preparative for 24 h</th>
<th>Freeze-thaw for 3 cycles</th>
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</thead>
<tbody>
<tr>
<td>LQC (9 ng mL⁻¹)</td>
<td>Mean ± SD</td>
<td>CV, %</td>
<td>Mean ± SD</td>
<td>CV, %</td>
</tr>
<tr>
<td></td>
<td>9.03 ± 0.13</td>
<td>1.45</td>
<td>9.43 ± 0.22</td>
<td>2.28</td>
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<td></td>
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<td></td>
<td>9.54 ± 0.47</td>
<td>4.97</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>9.73 ± 0.16</td>
<td>1.61</td>
</tr>
<tr>
<td>HQC (800 ng mL⁻¹)</td>
<td>Mean ± SD</td>
<td>CV, %</td>
<td>Mean ± SD</td>
<td>CV, %</td>
</tr>
<tr>
<td></td>
<td>731.40 ± 5.60</td>
<td>0.77</td>
<td>775.65 ± 17.05</td>
<td>2.20</td>
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<td></td>
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<td></td>
<td>750.53 ± 11.97</td>
<td>1.60</td>
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<td></td>
<td></td>
<td></td>
<td>763.47 ± 2.03</td>
<td>0.27</td>
</tr>
</tbody>
</table>

**Stability.** The results of the fimasartan stability experiments are summarized in Table 3. The fimasartan samples were stable at room temperature for 20 h, in an autosampler at 10°C for 24 h, and over three freeze-thaw cycles. QC samples kept in a freezer below −80°C were stable for 200 days. In addition, a fimasartan stock solution was stable for 24 h at room temperature (RT) and 24 days when refrigerated (4°C). Data on the fimasartan stock solution stability are given in Table 4.

**Pharmacokinetic application**

This validated UPLC-MS/MS method was applied to a pharmacokinetic study of fimasartan. Six healthy volunteers were each administered 120 mg of fimasartan, and plasma samples were collected for up to 48 h after oral administration. The mean plasma concentration-time curve for fimasartan is shown in Fig. 4. Pharmacokinetic parameters were estimated using the WinNonlin 5.3 software. Since the estimated values below LLOQ are considered to be zero for pharmacokinetic and statistical analysis, we set the values below LLOQ to zero (36 and 48 h).

The maximum plasma concentration (C max) and the area under the curve (AUC0–24) were 302.74 ± 159.43 ng/mL at 0.5 h (T max) and 801.66 ± 246.01 ng/mL, respectively.

**Conclusions**

We have developed and fully validated a new UPLC-MS/MS method suitable for analyzing up to 159 samples, including the calibration curve and QC samples.
method for the quantification of fimasartan, a newly developed anti-hypertensive drug, in human plasma. This analytical method provided improved sensitivity and specificity, and also reduced analysis time compared to conventional HPLC. Furthermore, this fully validated method was successfully applied to a pharmacokinetic analysis of fimasartan in human plasma samples obtained from clinical trials conducted at Kyungpook National University Hospital and was found to be sufficiently sensitive for bioanalytical applications.

Acknowledgements

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