UPLC–MS/MS Method for Determination of Bepotastine in Human Plasma

Yun Kyong Choi1,2,†, Yoon Hee Chung2,†, Yun Sung Nam2, Da Young Kang1, Hohyun Kim1, Seo Eun Lee3, Hak Rim Kim4, Yong Seong Lee5 and Ji Hoon Jeong2*  

1Korea Medicine Research Institute, Inc., Gyeonggi-do 462-120, South Korea, 2College of Medicine, Chung-Ang University, 84 Heukseok-ro, Dongjak-gu, Seoul 156-756, South Korea, 3College of Medicine, Hanyang University, Seoul 133-791, South Korea, 4College of Medicine, Dankook University, and 5Hallym University College of Medicine, Seoul 150-950, South Korea  

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A sensitive and rapid method for quantitation of bepotastine in human plasma has been established using ultra performance liquid chromatography–electrospray ionization tandem mass spectrometry (UPLC–ESI–MS/MS). Valsartan was used as an internal standard. Bepotastine and internal standard in plasma sample were extracted using ethylacetate (liquid–liquid extraction). A centrifuged upper layer was then evaporated and reconstituted with the mobile phase of acetonitrile–5 mM ammonium formate (pH 3.5) (85:15, v/v). The reconstituted samples were injected into a phenyl column. Using MS/MS in the multiple reaction monitoring mode, bepotastine and valsartan were detected without severe interference from human plasma matrix. Bepotastine produced a protonated precursor ion ([M+H]+) at m/z 389 and a corresponding product ion at m/z 167. And the internal standard produced a protonated precursor ion ([M+H]+) at m/z 436 and a corresponding product ion at m/z 291. Detection of bepotastine in human plasma by the UPLC–ESI–MS/MS method was accurate and precise with a quantitation limit of 0.2 ng/mL. The validation, reproducibility, stability and recovery of the method were evaluated. The method has been successfully applied to pharmacokinetic studies of bepotastine in human plasma.

Introduction

Bepotastine besilate is a second-generation H₁-receptor antagonist developed in Japan (1), and is now widely used for allergic disorder in Asian countries including South Korea (2). Bepotastine has long-lasting, dose-dependent antihistamine effects (3), suppresses allergic inflammatory processes (4) and is indicated in the treatment allergic rhinitis, pruritus and chronic urticaria associated with skin diseases. Previous reports have demonstrated its excellent anti-allergic effects compared with other antihistamine such as ketotifen, cetirizine, epinastine and terfenadine (3, 5, 6).

In in vitro receptor-binding assays, bepotastine showed a potent, highly selective H₁-receptor antagonist activity with negligible affinity for other receptors, including adrenergic, serotoninergic, muscarinic, dopaminergic receptors. Multiple in vivo toxicology studies have demonstrated bepotastine to be safe with no significant effects on respiratory, circulatory, central nervous, digestive or urinary system (7). Furthermore, bepotastine was not associated with clinically significant changes in blood pressure, pulse rate, respiratory rate, body temperature and lab data in healthy volunteer (1).

Bepotastine is rapidly absorbed after oral administration, while the distribution to the brain is limited due to its poor penetrating ability to blood–brain barrier, predicting only minimal suppressive effects on the CNS (8, 9). Bepotastine undergoes very minimal metabolism and only trace amounts of metabolites were found in the urine in which most of bepotastine is excreted. In addition, bepotastine does not appear to accumulate in the body. The variation of drug concentration in plasma after p.o. administration to healthy volunteer was small, and plasma concentration was not significantly affected by food (1). Bepotastine showed relatively rapid onset of action than fexofenadine which is demonstrated by measuring t_max and in vivo effectiveness (10).

Although a number of publications have been made to evaluate clinical efficacy and safety of bepotastine, limited literatures are available for its estimation and pharmacokinetic profile in human (11, 12). Up to now, plasma concentration of bepotastine was measured using liquid chromatography–tandem mass spectrometry (LC–MS/MS) system (9, 12). On the other hand, ultra-high performance liquid chromatography (UPLC) coupled to MS was introduced recently. UPLC–MS/MS possesses high pressure pumps to accommodate the use of sub-2 μm particle size columns, with the sample injector system designed to handle fast injection cycles, low injection volumes, negligible carryover and temperature control which together contribute to rapid sample analysis (13). Hence, UPLC–MS/MS was chosen to provide for required fast, high-resolution separations having the necessary sensitivity and associated advantages over conventional LC–MS/MS systems.

In the present study, we describe a new, rapid and cost-saving method for estimation of bepotastine with UPLC–MS/MS. It could represent a useful analytic procedure to be used in pharmacokinetic studies of bepotastine.

Experimental

Chemicals and reagents

Bepotastine (Figure 1) was obtained from Hanlim Pharm. Co., Ltd. (Seoul, South Korea). HPLC grade acetonitrile and methanol, ethylacetate were purchased from SK chemicals (Ulsan, South Korea), and internal standard (IS) (Valsartan; Figure 1) from Ildong Pharm. Co., Ltd. (Seoul, South Korea) and formic acid from Junsei (Tokyo, Japan), and ammonium formate from Kanto (Tokyo, Japan). A stock solution of bepotastine (1 mg/mL) and valsartan (1 mg/mL) was prepared in the methanol. From these stock solutions, working standard solutions containing from 2 to 2000 ng/mL bepotastine were prepared by sequential dilution with the 50% methanol.
Sample preparation
Plasma specimens (50 µL) were pipetted into microcentrifuge tubes and spiked with 5 µL of 3 µg/mL IS solution. After adding 300 µL of 0.1% formic acid and 2 mL of ethylacetate, the plasma samples were vortexes at 5 min. And then the two phases were separated by 5 min of centrifugation at 4000g. The upper layer was transferred into another test tube and completely evaporated at 50°C under the nitrogen stream. The dry residue was reconstituted with 150 µL of 90% acetonitrile and then 5 µL of the reconstituted sample were injected into the UPLC–MS/MS system.

UPLC–MS/MS conditions
MS/MS was performed with a Xevo TQ triple quadruple mass spectrometer (Waters Co., Manchester, UK) equipped with an electrospray ion source. The sample (5 µL) was delivered into the ESI source by UPLC (Model Acquity UPLC, Waters Co., Milford, MA, USA) with a Hypersil GOLD Phenyl column (2.1 × 100 mm, 5 µm particle size). The mobile phase was composed of 5 mmol/L ammonium formate (pH 3.5 adjusted with formic acid) and acetonitrile (15:85, v/v) and was used after degassing. The flow rate was 300 µL/min and the total run time was 2.0 min.

The electrospray interface was maintained at 500°C. Nitrogen nebulization was performed with a nitrogen flow of 700 L/h. Argon was used as collision gas. Bepotastine and the IS were detected by the multiple reaction monitoring (MRM) scan mode with positive ion detection; the parameter settings were: capillary voltage at 3.0 kV, cone voltage at 30 V, extractor at 3 V, source temperature at 150°C, collision energy at 39 eV, multiplier at 529 V and dwell time of 0.20 s.

Mass calibration was performed by infusion of a mass calibration solution into the ionspray source. The peak widths of precursor and product ions were maintained at ~0.7 mass unit at half height in the MRM mode.

Validation procedures and calibration curves
To assess the intraday precision and accuracy of the method, five replicate analyses were performed on plasma standards containing four different concentrations (0.2, 0.6, 80 and 160 ng/mL) of bepotastine. Five replicate analyses of the same four samples were also performed to determine the initial interday precision and accuracy. The accuracy was expressed as [(mean observed concentration) – (spiked concentration)]/(spiked concentration) × 100%, with the precision expressed as relative standard deviation (RSD).

For the quality control (QC) samples, the appropriate QC working solution (2, 6, 800 and 1600 ng/mL; 50 µL) was added to 1.5 mL microcentrifuge tubes containing 0.45 mL human control plasma to yield QC concentrations of 0.2, 0.6, 80 and 160 ng/mL. The calibration curves (y = ax + b) were generated by a weighted linear least-squares regression of the peak area ratios (y) of the analytes to their IS versus the concentrations (x) of the calibration standards. Concentrations of analytes in QC samples were calculated using the resulting peak area ratios and the regression equations of the calibration curves. The bulk QC plasma samples were then vortexed, mixed, and 0.08 mL aliquots were transferred to 1.5 mL microcentrifuge tubes and capped, and stored at −70°C.

Pharmacokinetic assay
This study was carried out in accordance with the International Conference on Harmonization (ICH) Guideline for Good Clinical Practice and the ethical principles in the Declaration of Helsinki. The study protocol (permission number: Ca-1205, expiration: 2012.12.31) was approved by the Ethics Committee of College of Medicine, Chung-Ang University (Seoul, South Korea).

For the pharmacokinetic assay, 7.11 mg dose of bepotastine besilate and salicylate was administered orally to 24 subjects. Each volunteer was judged to be in good health through medical story, physical examination and routine laboratory tests. The subjects were orally administered a single dose of bepotastine besilate and salicylate formulations after recommended wash out period of 7 days with 240 mL of water. Blood samples of this assay were taken, using heparin vacutainer collection tubes, 0, 0.33, 0.67, 1, 1.5, 2, 3, 4, 6, 8, 10, 12 and 24 h after ingestion. Human plasma was obtained by centrifugation at 2000g for 10 min. The plasma specimens were stored at −70°C before analysis.

Results
Mass spectrometry
Figure 2a shows the full scan first quadrupole positive ion spectrum of bepotastine, whereas Figure 3a shows that of the IS. These formed protonated precursor ion [M+H]+ as major ion peaks. These spectrums were obtained from a working standard solution (1 µg/mL). Bepotastine produced a protonated precursor ion ([M+H]+) at m/z 389 with a major product ion at m/z 167. On the other hand, IS produced a protonated precursor ion ([M+H]+) at m/z 436, with a major product ion at 291. Figures 2b and 3b show the product ion spectrum of bepotastine and the IS, respectively. The most abundant product ions (m/z 167 for bepotastine and m/z 291 for valsartan) were selected for MRM analysis. MRM mode was used for quantitation and achieved very high sensitivity and selectivity.

UPLC
Figure 4a shows the UPLC–MS/MS chromatogram of bepotastine in human blank plasma. No interference was observed in drug-free human plasma samples at the retention times of bepotastine and valsartan. Figure 4b shows the chromatogram of quantitation limit of bepotastine in human plasma. In Figure 4c,
Figure 2. Precursor (a) and production (b) ion scan of bepotastine.
Figure 3. Precursor (a) and production (b) ion scan of valsartan.
Figure 4. The chromatogram of bepotastine in human plasma. (a) Blank plasma, (b) plasma spiked with bepotastine (0.2 ng/mL) and IS, and (c) a volunteer plasma sample, 10 h after single oral dose of 7.11 mg bepotastine.
there is representative chromatogram of bepotastine and IS from a volunteer at 10 h.

**Method validation**

**Matrix effects**
The effect of the matrix was also evaluated. A matrix blank is a representative biological sample that is free of the target analytes. A spiked matrix blank is a control sample that has been fortified with the target analytes at a defined, relevant level (matrix effect = [response of postextracted spike/response of unextracted sample]). The absence of a matrix effect is indicated by a response ratio of 1.0. If responses are different, a matrix effect is present. The present study was unable to detect a matrix effect.

**Precision and accuracy**
The intraday precision, expressed as RSD (%), was 1.3–3.9% for 0.2, 0.6, 80 and 160 ng/mL standard concentrations, based on five replicate analyses at each concentration level. The intraday accuracy, expressed as a percentage of nominal values was measured as −9.5 to 8.0% for four standard concentrations, based on five replicate analyses at each concentration level. Table I shows the measured intraday precision and accuracy of bepotastine in human plasma. The interday precision was measured as 5.4–7.0% for four standard concentrations, based on five replicate analyses at each concentration level. The interday accuracy was measured as −5.3 to 12.2% for four standard concentrations, based on five replicate analyses at each concentration level. Table II shows the measured interday precision and accuracy of bepotastine in human plasma.

**Recovery and stability**
Analyte recovery from a sample matrix (also called extraction efficiency) is a comparison of the analytical response from an amount of analyte added to and extracted from the sample matrix (preextraction spike) with that from a postextraction spike. (% recovery = (response of extracted spike)/(response of postextracted spike) × 100). The % recovery of liquid–liquid extraction (LLE) was measured as 31.6–38.4% for 0.6, 80 and 160 ng/mL standard concentrations, with three replicates at each concentration level. The stability of bepotastine and IS was evaluated in the dissolution solvent and in human plasma. It was found that bepotastine and IS were stable for the duration of the experiment (Table III).

**Calibration curve and sensitivity**
Standard calibration curves (reproducibility) were constructed on different working days (5 days) using the human plasma. The response was linear throughout the concentration range of the study, with the coefficient of determination ($r^2$) always >0.9917. The correlation equations was $y = 0.00783x + 0.00011$ (± 0.0008) in human plasma.

On the basis of a signal-to-noise ratio (S/N) of 10, the lower limit of quantitation (LLOQ) for bepotastine was found to be 0.2 ng/mL on injection of 5 μL of sample into the UPLC–MS/MS system. The LLOQ is defined as the lowest concentration of the analyte that can be measured with a coefficient of variation and accuracy both <20%. These LLOQ values were sufficient for pharmacokinetic studies (Table IV).

**Pharmacokinetic application**
Figure 5 shows the concentration of bepotastine in human plasma–time curve. Pharmacokinetic parameters were similar between the bepotastine besilate and salicylate products and the AUC agreed with those published pharmacokinetic studies (Table V).

**Discussion**
We have developed a rapid and sensitive method for detecting bepotastine in human plasma, using LLE and UPLC–ESI/MS/MS for pharmacokinetic studies. Under electrospray ionization
condition, bepotastine and valsartan exhibit a fairly high sensitivity in positive ion detection mode rather than in negative ion detection mode. The analysis for compounds with basic sites (e.g., amines), as for bepotastine and valsartan, should be performed at a low pH using positive ion detection. UPLC–MS/MS is still limited to conditions that are suitable for mass spectrometry operations. There are restrictions on pH, solvent choice, solvent additives and flow rates for UPLC to achieve optimal ESI–MS/MS sensitivity. For the chromatographic analysis and electrospray ionization of bepotastine and valsartan, we initially attempted to develop a reversed-phase chromatographic method with methanol or acetonitrile as mobile phase. Acetonitrile was used instead of methanol, because acetonitrile affords better sensitivity and resolution in the analysis of bepotastine and IS. The amount of acetonitrile in mobile phase was optimized at 85%. The pH of the 5 mM ammonium formate phase was optimized at pH 3.5 by use of formic acid. In this method, ammonium acetate buffer was not used because it was shown that good sensitivity and peak symmetry. Generally, when we used ammonium acetate buffer it was found that much higher ion intensities and peak symmetry were achieved in the presence of ammonium acetate. Under these conditions, the sensitivity of bepotastine and IS was very high.

Chromatography might result in co-elution of bepotastine and IS with endogenous interferences, which might not be detected by MS/MS but which might affect the ionization efficiency of the analytes. This effect can lead to decreased reproducibility and accuracy for an assay and failure to reach the desired limit of quantitation. It is reported that the extent of ionization suppression seen is much more severe with electrospray ionization than with atmospheric pressure chemical ionization. Therefore, the analytes need to use a postextraction spiked matrix blank and compare the results with an analytical standard in solution to determine the influence of the matrix on the analysis. In the present study, a matrix effect was not detected. Determining the concentration of bepotastine in human plasma has been applied to pharmacokinetic studies by use of LLE with UPLC–MS/MS. Figure 5 indicates that the proposed method is suitable for pharmacokinetic studies to determine the concentration of bepotastine in human plasma.

### Table V

Pharmacokinetic Parameters of Bepotastine after Oral Administration

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Bepotastine Besilate (mean ± SD)</th>
<th>Salicylate (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C\textsubscript{max} (ng/mL)</td>
<td>101.39 ± 25.56</td>
<td>99.55 ± 30.88</td>
</tr>
<tr>
<td>T\textsubscript{max} (h)</td>
<td>0.93 ± 0.27</td>
<td>1.13 ± 0.35</td>
</tr>
<tr>
<td>AUC\textsubscript{0-2} (ng h/mL)</td>
<td>393.16 ± 96.29</td>
<td>380.04 ± 114.28</td>
</tr>
<tr>
<td>AUC\textsubscript{0-1} (ng h/mL)</td>
<td>396.53 ± 96.92</td>
<td>383.53 ± 114.84</td>
</tr>
<tr>
<td>T\textsubscript{1/2} (h)</td>
<td>3.85 ± 0.51</td>
<td>3.82 ± 0.60</td>
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</tbody>
</table>

### Conclusion

A highly sensitive and specific UPLC–MS/MS method for the determination of bepotastine in human plasma has been developed and validated, with a lower quantitation limit of 0.2 ng/mL, which is better than that attained by HPLC–UV. Validation experiments have shown that the assay has good precision and accuracy over a wide concentration range (0.2–200 ng/mL), and no interference caused by endogenous compounds was observed. This simple, rapid and robust assay enables the complete processing of large samples for pharmacokinetic studies of bepotastine in biological fluid.

### References

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**Figure 5.** Pharmacokinetic profile of bepotastine besilate and salicylate in human plasma in 24 healthy male volunteers after oral administration of 7.11 mg tablet.
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