

## A Sensitive and Rapid LC-MS/MS Method for Determination of Berberine in Human Plasma

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**Abstract :** *Coptidis Rhizoma* (CR) has been used widely in traditional medicine to treat common diseases. This study aimed to develop a high-sensitivity liquid chromatography-tandem mass (LC-MS) spectrometry method for the evaluation of the pharmacokinetics of a new natural product that contain CR extract with the main bioactive compound, berberine, at trace concentrations. Human plasma samples were pretreated with methanol by a protein precipitation method. Berberine was analyzed on a Kinetex C18 column (2.1 mm × 50 mm, 100 Å, 1.7 μm) using a mobile phase of 10 mM ammonium formate/0.1% formic acid in water (A) and acetonitrile (B) (50:50, v/v) with a flow rate of 0.25 mL/min. The analyte was detected by using electrospray ionization in positive mode with multiple reaction monitoring (MRM). The method was sensitive, with a lower limit of quantification of 1 pg/mL, which has not been previously obtained. The method was validated (over the range of 1–50 pg/mL) and applied successfully for the pharmacokinetic study of human plasma samples.

**Keywords :** *Coptidis Rhizoma*, berberine, human plasma, LC-MS/MS, pharmacokinetic study

### Introduction

Since the early days of humankind, plants, herbs, and ethnobotanicals have been used for health promotion and the treatment of diseases.<sup>1</sup> It is estimated that 70,000 plant species are currently used for medicinal purposes.<sup>2</sup> Among the many herbal medicines, *Coptidis Rhizoma* (CR) is the dried rhizome of *Coptis chinensis* Franch, which belongs to the *Ranunculaceae* family.<sup>3</sup> It has been used widely in Asian countries, such as Korea, China, and Vietnam, for the treatment of vomiting, diarrhea, and abdominal pain.<sup>4</sup> Studies on CR have revealed its anti-inflammatory, antibacterial, antiviral, anti-Alzheimer, and anticancer activities.<sup>5-7</sup> Many types of secondary metabolites have been isolated from CR; the main components are protoberberine-

type alkaloids, such as berberine, palmatine, coptisine, epiberberine, jatrorrhizine, and columamine. Quantitative determination of these alkaloids is crucial for the quality evaluation of CR.<sup>8</sup> Many pharmaceutical products formulated from CR have been marketed; however, highly sensitive analysis is required owing to the trace quantities of alkaloids in the formulations.

Our study aimed to evaluate the pharmacokinetics of a new natural product that contain many ingredients of the CR extract and a berberine concentration of approximately 31.59 μg/27 mL (1.17 μg/mL). Although the determination of berberine in plasma has been studied by LC-MS methods, these studies did not reach the concentration range required for the analysis of the pharmacokinetics of this new product in human plasma.<sup>9-12</sup> Therefore, in this study, we aimed to (a) increase the sensitivity of the LC-MS/MS method to reduce the lower limit of quantification (LLOQ) to 1 pg/mL or below through the optimization of instrumental parameters and the pretreatment procedure and (b) reduce the volume of volunteers' plasma needed for the study.

### Experimental

#### Material

Berberine hydrochloride (100.0%) was purchased from USP (Rockville, MD, USA). The internal standard (IS), berberine hydrochloride-d<sub>6</sub>, was purchased from TRC Inc.

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(Toronto Research Chemicals, North York, Canada). HPLC-grade acetonitrile and methanol were obtained from Burdick & Jackson (SK Chemical, Seongnam, Korea). Ammonium formate and formic acid were purchased from Sigma-Aldrich (MO, USA). Ultrapure water was prepared by using a Milli-Q filtration system ( $18.2 \text{ M}\Omega \text{ cm}^{-1}$ ).

Human plasma was obtained from BioChemed Services (Winchester, VA, USA) with sodium heparin as an anticoagulant. The samples were stored at approximately  $-70^\circ\text{C}$  prior to analysis.

### Instrumental conditions

The LC-MS/MS system consisted of an Agilent 1290 Infinity II UHPLC System G7120A (Agilent Technologies, Santa Clara, CA, USA) coupled with an AB SCIEX TRIPLE QUADTM 5500 (ABSCIEX, Foster City, CA, USA) equipped with a Turbo V Ion Spray source. The separation was performed on a Kinetex  $1.7 \mu\text{m}$  C18 column ( $2.1 \text{ mm} \times 50 \text{ mm}$ ,  $1.7 \mu\text{m}$ ,  $100 \text{ \AA}$ ) from Phenomenex (Torrance, CA, USA) using an isocratic elution of 10 mM ammonium formate/0.1% formic acid in water (A) and acetonitrile (B) (50:50, v/v) at a flow rate of 0.25 mL/min. The temperature of the column was  $35^\circ\text{C}$  and the injection volume was 20  $\mu\text{L}$ ; the autosampler was operated at  $15^\circ\text{C}$ . The positive ESI mode settings for the detection of berberine and IS were: ion source gas 1 (nebulizer gas), 40 psi; ion source gas 2 (turbo heater gas), 50 psi; curtain gas, 30 psi; collision gas, 7 psi; turbo-gas temperature,  $700^\circ\text{C}$ ; ion spray voltage, 5000 V. The electrical conditions, such as DP, CE, CXP, and EP, were optimized by auto infusion quantitative optimization to obtain the highest sensitivity for the precursor ion and the product ion (Supplemental Table S1). Analyst 1.6.3 software was utilized for LC-MS/MS system control and data processing.

### Preparation of standard solutions

Accurately weighed amounts of berberine hydrochloride and berberine- $d_6$  (internal standard, IS) were dissolved in methanol to prepare stock solutions of the compounds at 1000  $\mu\text{g/mL}$  and then stored at  $4^\circ\text{C}$ . Calibration standards (0.01, 0.02, 0.05, 0.1, 0.2, 0.5  $\text{ng/mL}$ ), quality control solutions (0.01, 0.03, 0.15, and 0.4  $\text{ng/mL}$ ), and the IS standard (0.5  $\text{ng/mL}$ ) were prepared from the dilution of a suitable volume of stock solution with methanol.

10  $\mu\text{L}$  of standard or QC solutions was spiked into 270  $\mu\text{L}$  of plasma in a 1.7 mL polypropylene tube. After vortex mixing for 3 mins, the mixture was centrifuged at 14000 rpm for 1 min; the resulting samples were stored at  $-70^\circ\text{C}$  prior to analysis.

### Preparation of plasma samples

Before analysis, the plasma samples were thawed at room temperature. Subsequently, a 300  $\mu\text{L}$  thawed plasma was placed in a 2 mL polypropylene tube, and 10  $\mu\text{L}$  of IS (0.5  $\text{ng/mL}$ ) was added, to which 1.25 mL of methanol was

added; the mixture was vortexed for 2 min, and then centrifuged at 14000 rpm for 5 min. 1 mL of the supernatant was transferred to a glass tube and completely dried at  $60^\circ\text{C}$  using a nitrogen stream. Subsequently, 100  $\mu\text{L}$  of 10 mM ammonium formate/0.1% formic acid in water: methanol (v/v, 50:50) was added to the supernatant in a completely dried glass tube and centrifuged at 14000 rpm for 5 min. For analysis, 20  $\mu\text{L}$  of the clear supernatant was injected into the LC-MS/MS.

### Application of the method

This method was applied for the detection and quantitation of berberine in plasma samples obtained from volunteers at 1, 2, 3, 4, 6, 8, 12, and 24 h after oral administration of syrup that contain many ingredients of the CR extract ( $n = 12$ ).

The study protocol was approved by the review board at Chungnam National University Hospital (CNUH 2017-06-068) on 2018. 08. 04, each participant was provided informed consent to the sampling, in accordance with the review board indications and in accordance with the World Medical Association Declaration of Helsinki (as revised in 2013), the International Conference on Harmonization (ICH) Guidelines for Korean Good Clinical Practices and was approved by the Ministry of Food and Drug Safety.<sup>13-15</sup>

## Results

### LC-MS/MS

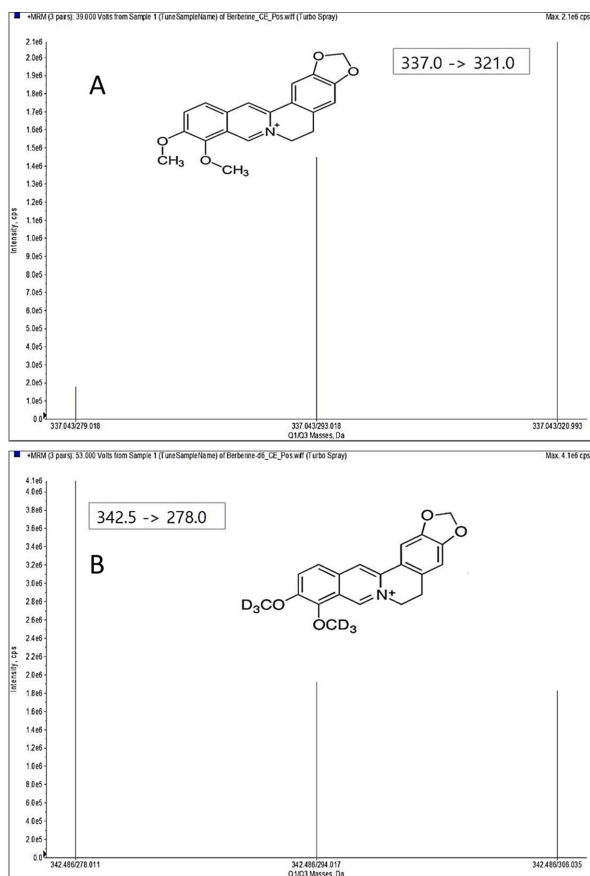
The precursor ion of berberine ( $[\text{M} + \text{H}]^+$ ) was at  $m/z = 337.0$  and a product ion was at  $m/z = 321.0$ . The precursor ion of the internal standard was at  $m/z = 342.5$  ( $[\text{M} + \text{H}]^+$ ) and the product ion was at  $m/z = 278.0$  (Figure 1).

The Kinetex  $1.7 \mu\text{m}$  C18 ( $2.1 \text{ mm} \times 50 \text{ mm}$ ,  $1.7 \mu\text{m}$ , Phenomenex) and Luna C18 ( $2.0 \times 50 \text{ mm}$ ,  $5 \mu\text{m}$ , Phenomenex) columns were tested in preliminary experiments. The Kinetex column, which resulted in better peak shape, selectivity, and reproducibility, was chosen for further experiments. A guard column C18 ( $4 \times 2.0 \text{ mm}$ , Phenomenex) was also used to prevent contamination of the column from the high quantities of plasma used.

Methanol and acetonitrile were analyzed at different ratios with ammonium formate or ammonium acetate buffer at varying strengths to select suitable mobile phase conditions. It was observed that using an isocratic elution of 10 mM ammonium formate/0.1% formic acid (A) and acetonitrile (B) (v/v, 50:50) provided the best sensitivity, peak shape, and analysis time (Figure 2).

### Validation

The present method was validated in accordance with "Guidance for Industry Bioanalytical Method Validation" published by the US FDA and the "Guidelines for Validation of Biological Sample Analysis".<sup>16</sup>



**Figure 1.** Product ion scan spectra of berberine (A) and berberine- $d_6$  (B).

*Selectivity and lower limit of quantification*

No interfering peaks from endogenous compounds were observed at 0.95 min (the retention times of berberine and IS). The total chromatographic run time was 3 min (Figure 2). The LLOQ concentration was 1.0 pg/mL; at this value, the precision and variance of accuracy were less than 20%, and the signal-to-noise ratio (S/N) was 22.9.

*Qualitative matrix effect and recovery*

The matrix factors of the analytes and IS were studied through the comparison of the analyte/IS ratio in post-extraction and pure solution samples at low, medium, and high concentrations. The recovery results are presented in Supplemental Table S2.

*Carryover*

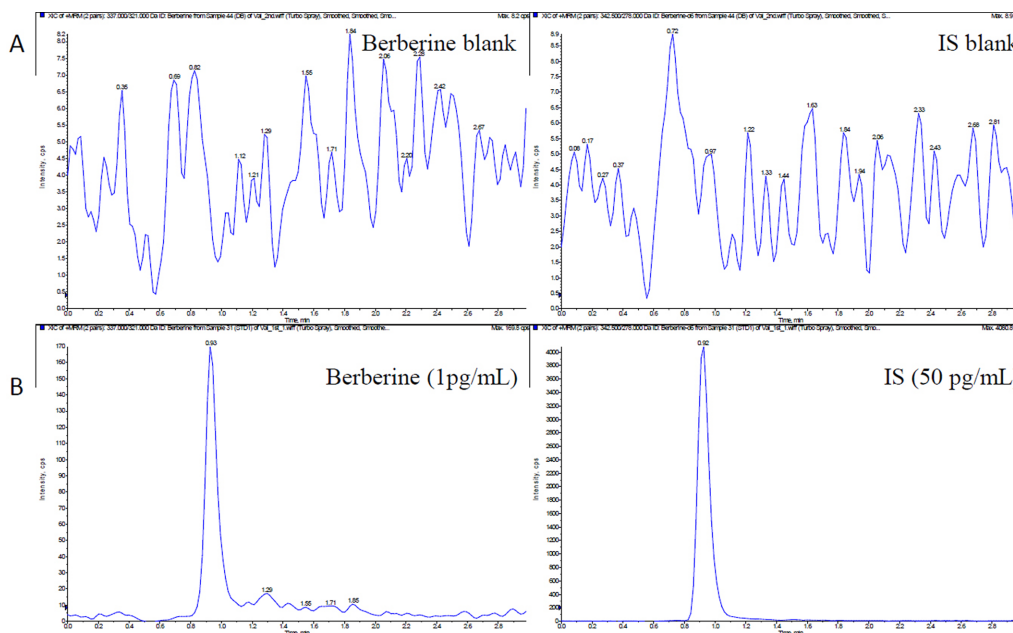
The carryover effect was evaluated by instantly injecting the blank samples after injection of samples at the upper limit of quantification. There was no residual signal in the blank samples.

*Linearity*

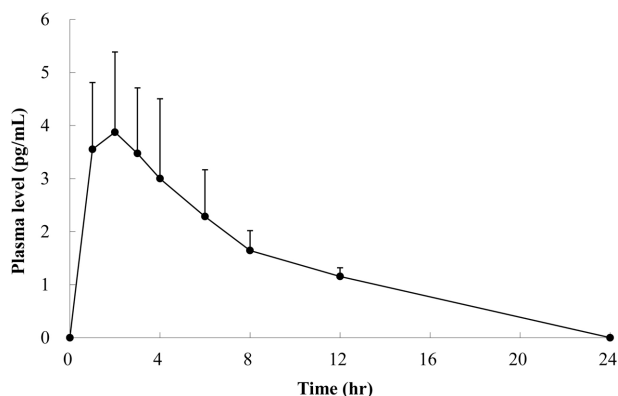
The linearity was tested within the concentration range of 1–50 pg/mL. To ensure high accuracy within the tested concentration range, a weighting factor of  $1/x^2$  was used in the linear regression analysis. The linearity of the developed method was determined based on the  $r^2$  values, which were all above 0.9985.

*Precision and accuracy*

The within- and between-run precision and accuracy



**Figure 2.** Multiple reaction monitoring chromatogram of (A) blank human plasma, and (B) berberine and the internal standard.



**Figure 3.** Concentration of berberine in human plasma ( $n = 12$ ).

were assessed through the analysis of five replicates of the QC samples at four concentrations on three consecutive days. The results are presented in Supplemental Table S3.

#### Batch size

To confirm the analysis pattern in the continuous sample analysis, this validation parameter was evaluated for the number of samples equal to the number of actual sample analysis batches to check the accuracy and precision. The results for a 60-sample batch were within the criteria, where 50% of the quality control samples at each concentration were within 15% accuracy, and 67% of the total quality control samples had to be within 15% accuracy.

#### Stability

The stabilities of the analyte under different storage conditions (stock solution stability, processed sample stability, short term stability, and freeze/thaw stability) are presented in Supplemental Table S4. No significant degradation was detected under the storage conditions described, as the calculated concentrations deviated by less than 15.0% relative to the reference nominal concentrations.

#### Dilution integrity

To demonstrate the validity of the dilution, the dilution integrity quality control samples (DQC) were diluted with the biological samples and assayed repeatedly five times for each dilution factor. The accuracy and precision of the five-fold dilution were 100.0% and 4.63%, respectively, and that of the two-fold dilution were 101.5% and 2.93%, respectively.

#### Application

This method was applied for the detection and quantitation of berberine in plasma samples obtained from volunteers at 1, 2, 3, 4, 6, 8, 12, and 24 h after the oral administration of the syrup ( $n = 12$ ). The average  $C_{max}$  of

**Table 1.** PK parameters of berberine.

Parameters	Berberine		
	$(n = 6)$		
	Mean	SD	CV
$C_{max}$ (pg/mL)	3.9	1.3	31.5
$AUC_t$ (hr·pg/mL)	21.6	8.7	37.6
$AUC_{inf}$ (hr·pg/mL)	32.2	8.6	26.8
$T_{1/2\beta}$ (hr)	5.4	2.2	43.5
CL/F (mL/hr)	1069719.2	412521.7	38.6
$T_{max}$ (hr)	[1.0 ~ 2.0]		

#### Abbreviations

$C_{max}$ : Maximum (peak) plasma drug concentration  
 $AUC_{inf}$ : Area under the plasma concentration-time curve from time zero to infinity  
 CL/F: Apparent total clearance of the drug from plasma after oral administration  
 $T_{1/2\beta}$ : Terminal elimination half-life (two-compartmental model)  
 $T_{max}$ : Time to reach  $C_{max}$  following drug administration

the 12 subjects was 3.87 pg/mL, which was approximately four times higher than the lower limit of quantification (Figure 3). Results of berberine pharmacokinetic parameters of are shown in Table 1.

## Conclusions

A sensitive LC-MS/MS method for the quantitation of berberine in human plasma was developed and validated. To the best of our knowledge, no other studies have reported a lower limit of quantitation of 1 pg/mL. The method was confirmed to be sensitive, accurate, and precise for application in the pharmacokinetic studies of modern natural products containing CR extract in which berberine, one of main bioactive compounds, was present at trace levels.

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## Notes

†Electronic Supplementary Information (ESI) available: [https://drive.google.com/file/d/1CAAdJdgNVtbDOM\\_iKe mRpw7NxDR4ufdAe/view?usp=sharing](https://drive.google.com/file/d/1CAAdJdgNVtbDOM_iKe mRpw7NxDR4ufdAe/view?usp=sharing)

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