LC-MS Method for Determination and Pharmacokinetic Study of Chimaphilin in Rat Plasma after Oral Administration of the Traditional Chinese Medicinal Preparation Lu Xian Cao Decoction

YuanYuan ZHANG,* a XiaoHui CHEN,a ShengYing QIN,a ChulSa KIM,b Shin-ichi TEBAYASHI,b and KaiShun Bi*b,a

a School of Pharmacy, Shenyang Pharmaceutical University; Wenhua Road 103, Shenyang 110016, Liaoning Province, P.R. China; and b Department of Bioresources Science, Faculty of Agriculture, Kochi University; B200 Monobe, Nankoku 783–8502, Japan. Received May 17, 2006; accepted August 2, 2006

Lu xian cao (Hebra pyrolae) is a traditional Chinese medicine used for the treatment of several major diseases. Chimaphilin has been demonstrated one of the major active components in Lu xian cao by modern pharmacological studies. In this study, a liquid chromatography-mass spectrometry method for the determination of chimaphilin in rat plasma was developed and validated. The separation was carried out on a C18 column using methanol and water as mobile phase after the plasma sample was extracted with diethyl ether. Atmospheric pressure chemical ionization in negative ion mode and selected ion monitoring method were developed to determine [M]- at 186 and 210 for chimaphilin and benzil (internal standard), respectively. The lower limit of quantitation was 10 ng/ml and the calibration curve was linear (r>0.9964) over the concentration range 10—1000 ng/ml. The method was demonstrated reproducible and reliable with intra-day precision <11.5%, inter-day precision <7.6%, accuracy in the range of 88.4—113.0%, and mean extraction recovery excess of 83.0%, which were all calculated from the quality control samples at concentrations of 20, 100, and 500 ng/ml. The method was successfully applied to pharmacokinetic study of chimaphilin in rat plasma following oral administration of a 30-mg/kg dose of chimaphilin in Lu xian cao decoction to male Wistar rats.

Key words Lu xian cao decoction; chimaphilin; pharmacokinetics

Pyrola decorata H. ANDRES is a perennial herbaceous plant growing throughout China. The dried whole plant of Pyrola decorata H. ANDRES is recognized as “Lu xian cao” in Chinese and officially listed in the Chinese pharmacopoeia. Lu xian cao has been used as tonics, sedatives, hemostatics, anti-inflammatory, and analgesics against rheumatoid arthritis in China since the antiquity.1—4)

Chimaphilin (Fig. 1A) is one of the major components in Pyrola plants. So far, many studies concerning the pharmacological activities of this component have been reported. It was demonstrated that chimaphilin was one of the anti-inflammatory and analgesics isolated from the methanol extract of Pyrola rotundifolia.5) During the evaluation of histamine release inhibitory effects of 17 plants, significant inhibitory activity was shown in the ethyl acetate fraction of Pyrola incarnata and chimaphilin was further isolated from this plant as an active component.5) Chimaphilin also contributed to both inotropic and platelet aggregation inhibitory activity of Pyrola plants.6) In addition, the cytotoxic active component in Pyrola incarnata against L1210 and K562 cells isolated by bioassay-guided method gave rise to chimaphilin.6) Thus chimaphilin plays an important role in the bioactivity of Lu xian cao; using chimaphilin as one of the marker compounds to characterize Lu xian cao is reasonable.

However, to the best of our knowledge, no report on quantitation methods for chimaphilin in either crude drugs or traditional Chinese medicine (TCM) preparations has been published. Moreover, the relationship between chimaphilin’s biological activity and its kinetic action in vivo is still unknown. In the present study, quantitative determination and pharmacokinetic study of chimaphilin in rat plasma after orally administering the decoction of Lu xian cao was first reported using benzil (Fig. 1B) as internal standard (IS) by liquid chromatography-mass spectrometry (LC-MS) method. The validation assays indicated that the method is simple and performs well in terms of selectivity, linearity, precision, and accuracy.

MATERIALS AND METHODS

Materials and Reagents Lu xian cao was purchased from Tongrentang TCM shop (Shenyang, China) and identified by Professor Qishi Sun (Department of Pharmacognosy, Shenyang Pharmaceutical University, Shenyang, China). Chimaphilin was isolated from Lu xian cao by the author at the Faculty of Agriculture, Kochi University (Kochi, Japan). The structural characterization was done by 1H-NMR, 13C NMR, and IR assays and all spectra coincided well with the data reported for Chimaphilin.9) Benzil was from Beijing Chemical Reagents Company (Beijing, China). HPLC-grade methanol was obtained from Caledon Laboratories Ltd. (Georgetown, Canada). All other reagents were of analytical grade.

Animals Male Wistar rats (220—250 g) were obtained from the Laboratory Animal Center of Shenyang Pharmaceutical University. All animal handing procedures were in ac-
cordance with the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of People’s Republic of China. The rats were not fed except for water for 12 h before drug administration.

**Instruments and Analytical Conditions** The assay was performed on a Shimadzu LC-MS 2010A system (Kyoto, Japan) consisting of LC-10ADvp HPLC connected to a single quadrupole MS analyzer with interface usable for either atmospheric pressure chemical ionization (APCI) or electrospray ionization (ESI) sources. The LC separation was carried out on a Cromosil C18 column (150×4.6 mm, 5 μm), which was preceded by a guard column (C18, 5 μm) both from Zhonghuida Co. (Dalian, China). The temperatures of column oven and auto-sampler tray were set at room temperature and 14 °C, respectively. The mobile phase consisting of methanol–water (75 : 25) was delivered at a flow rate of 0.8 ml/min.

Samples were ionized by APCI probe in negative ion mode under the following source conditions: nebulizing gas flow: 2.5 l/min; interface temperature: 400 °C; heat block temperature: 200 °C; CDL temperature: 250 °C; detector voltage: 1.5 kV; drying gas flow: 2.0 l/min. The full-scan mass spectra of chimaphilin and the IS are shown in Fig. 2.

The [M] ions at m/z 186 and 210 were chosen in the selected ion monitoring (SIM) acquisition for the quantitation of chimaphilin and the IS, respectively. Peak areas for all analytes were automatically integrated by LC-MS Solution Version 3.0 (© 1997—2002 Shimadzu Corp).

**Preparation of Lu Xian Cao Decoction** Lu xian cao, which contained 0.167% of chimaphilin (w/w) 37.4 g, was extracted three times by refluxing with 70% ethanol (1 : 15, w/v) for 2 h. After evaporating the solvent under reduced pressure, the residue was re-dissolved in 25 ml of water to obtain the Lu xian cao decoction with a chimaphilin concentration of 2.5 mg/ml.

**Preparation of Calibration Curve and Quality Control (QC) Samples** Stock solutions of chimaphilin (400 μg/ml) and the IS (100 μg/ml) were prepared by dissolving suitable amounts of each pure substance in methanol.

“Individual working solutions” for chimaphilin (40, 80, 200, 400, 800, 2000, 4000 ng/ml) were prepared by diluting the stock solution above with methanol. The IS working solution (1000 ng/ml) was also prepared by diluting the IS stock solution with methanol. All solutions were stored at 4 °C and protected from daylight.

Calibration standards containing 10, 20, 50, 100, 200, 500, and 1000 ng/ml of chimaphilin and QC samples containing 20 (low), 100 (medium), and 500 ng/ml (high) of chimaphilin were prepared by spiking 200 μl of the control rat plasma with 50 μl of the respective working solutions prepared above. The IS working solution (50 μl) was added to each standard sample just prior to sample processing. The spiked plasma samples (calibration standards and QCs) were extracted on each analytical run along with the unknown samples.

**Drug Administration and Blood Sampling** Lu xian cao decoction was administered orally at a chimaphilin dose of 30 mg/kg, namely, the administration volume was 12 ml/kg. Blood samples (about 500 μl) were collected in heparinized tubes at 0, 0.25, 0.5, 1, 1.5, 2, 3, 4, 5, 7, 9, and 12 h after administration. The blood samples were immediately centrifuged at 3000 r/min for 10 min and the plasma fractions were stored frozen at −20 °C until LC-MS assay.

**Plasma Sample Preparation** According to the literature chimaphilin is sensitive to sunlight, therefore during the sample pretreatment all tubes were wrapped with aluminum
paper and processed under dark conditions to avoid exposure to light. Plasma sample (200 μl) was spiked with 50 μl of the IS working solution and 50 μl of methanol, and then extracted with 2 ml of diethyl ether. The resulting mixture was vortexed for 5 min. After centrifugation (3000 r/min, 5 min), the organic phase was transferred to another vial and evaporated to dryness under a stream of nitrogen at room temperature. The residue was dissolved in 100 μl of methanol, extracted by ultrasonic for 30 s, mixed by vortexing for 1 min, and then centrifuged at 14000 r/min for 10 min. An aliquot of 5 μl of the supernatant was injected into the LC-MS system.

Method Validation Specificity was assessed by comparing the chromatograms of blank rat plasma with the corresponding spiked plasma. Linear calibration curves were obtained with correlation coefficients >0.99 using a 1/x² weighted least-squares linear regression model. The plot was made of peak area ratio of chimaphilin to the IS against chimaphilin concentration. The lower limit of quantitation (LLOQ) was at the lowest concentration level among the linear range. Accuracy and precision were assessed by determining five replicates of QC samples at three concentration levels on 3 separate analytical runs. The accuracy was expressed by comparing the calculated concentrations using calibration curves to known concentrations. The precision was evaluated by the relative standard deviation (RSD). The accuracy required to be within 80—120% at LLOQ and within 85—115% for QC samples. The precision was required to be <20% at LLOQ and <15% for QC samples. Absolute recovery was determined by comparing the mean peak areas (n=6 at each concentration) obtained from the plasma samples spiked before extraction to those after extraction. The stability of chimaphilin and the IS in methanol was assessed by placing the working solutions under ambient conditions. The stability of plasma samples was studied by storing the QC samples at −20°C. The concentration of the analyte was compared with the initial concentration as determined for freshly prepared samples.

Application to Pharmacokinetic Study To demonstrate the reliability of the proposed method for pharmacokinetic study, chimaphilin concentrations in plasma samples of 6 male Wistar rats were determined. Experimental data and pharmacokinetic parameters were expressed as mean±S.D. The concentration–time curve was plotted and all data were processed by computer program TopFit 2.0 (Godecke, Schering, Thomaue). Due to the existence of double peak, statistical moment theory instead of compartment model was used to calculate the pharmacokinetic parameters such as mean residence time (MRT) and area under the curve (AUC0→t). The maximum concentration (Cmax) and time to Cmax (Tmax) were obtained from the observed data directly. The elimination rate constant (K) was calculated by fitting individual data for three terminal points of the plasma concentration profile with a log-linear regression equation using the least-squares method. The area under the concentration–time curve from zero to infinity (AUC0→∞) was calculated as follows: AUC0→∞=AUC0→t+C/K, where C is the last measurable plasma concentration. MRT0→∞ was calculated as the ratio of the area under the first moment curve (AUMC0→∞) to AUC0→∞. The apparent body clearance (CL/F) after oral administration was calculated using the equation CL/F=Dose/AUC0→t.

RESULTS AND DISCUSSION

LC-MS Optimization The LC-MS conditions were investigated to optimize for sensitivity, speed, and peak shape. The possibility of using ESI or APCI source under negative ion mode was evaluated at the early stage of method development. It was found that APCI mode could offer higher sensitivity and better linearity for the analytes than ESI mode. Moreover, higher flow rate of 0.8 ml/min could be used in APCI mode than in ESI mode (usually <0.3 ml/min), which reduced the chromatographic separating time and provided sharper peak shape. Therefore the former was chosen as the ionization mode. The optimization of the mobile phase involved the combination and composition of the elution solvents. Methanol was chosen as the organic solvent because it was less toxic and cheaper than acetonitrile while providing the same sensitivity for the analytes as acetonitrile. It was also found that high organic solvent contents (>70%) in the mobile phase could decrease the background noise and provide stable MS signal throughout the analytical run. Therefore methanol–water (75:25) was used as the mobile phase with acceptable run time 8.5 min.

The choice of the IS was also practised during the process of method development. Osthole was once attempted as the IS due to its similar structure and chemical property to chimaphilin. However, osthole formed predominately prototated molecule [M+H]+ at 245 in full scan mass spectra while chimaphilin showed no peak at positive ion mode. The continuous switch of detecting events (chimaphilin detection in negative mode, osthole detection in positive mode) decreased the sensitivity and made more background noise. Benzil was found suitable as the IS for its stable ionization under negative APCI mode and adequate recovery at the condition of extraction.

Several organic solvents such as diethyl ether, ethyl acetate, n-hexane, and their mixtures in different combinations and ratios were investigated as the liquid–liquid extraction solvents. A good linearity and adequate recovery values were obtained using 2 ml of diethyl ether as the extraction solvent. Additionally, low boiling point of diethyl ether made the evaporation process faster at room temperature.

Specificity Typical chromatograms of blank plasma, blank plasma spiked with chimaphilin and the IS, and rat plasma sample after administration of Lu xian cao decoction are shown in Fig. 3. No significant interference from endogenous substances with the analytes was observed. The retention times for chimaphilin and the IS were approximately 6.7 and 5.9 min, respectively.

Calibration Curve The spiked standard plasma samples at 10, 20, 50, 100, 200, 500, and 1000 ng/ml of chimaphilin were prepared to construct the corresponding calibration curves. Satisfactory linearity was observed over the concentration range 10—1000 ng/ml. A typical regression equation for calibration curve was as follows: Y=1.95×10⁻³X−8.80×10⁻³ (r=0.9973).

Precision, Accuracy, and LLOQ Table 1 presents the results of intra- and inter-day precisions and accuracy for chimaphilin from QC samples. In this assay, the intra- and inter-day precisions ranged from 3.9 to 11.5% and from 4.9 to 7.6%, respectively, for each QC level. The accuracy was within 88.4—113.0%. The results, calculated using one-way
ANNOVA, indicated that the values were within the acceptable range and the method was precise and accurate.

Five replicates of standard samples at concentrations of 10 ng/ml were determined on 3 separate analytical runs. The intra- and inter-day precisions were 13.5 and 11.1%, respectively. The accuracy was 86.1—112.8%. The results indicated that the LLOQ of 10 ng/ml was achieved.

**Extraction Recovery** The recoveries of chimaphilin at concentrations of 20, 100, and 500 ng/ml were 88.1±2.3, 85.4±2.6, and 83.0±1.9%, respectively. The recovery of the IS at concentration of 250 ng/ml was 90.5±1.2%. We can see from the results that the recovery values of chimaphilin and the IS were similar and stable at the extraction condition.

**Stability** Being protected from daylight, the solutions of chimaphilin and the IS showed good stability at room temperature for ≥15 d after preparation, and there was no evidence that they aroused chemical reactions. Chimaphilin and the IS were also stable within 15 d of storage at −20°C with the relative errors (RE) <5.3% in all QC samples examined.

**Pharmacokinetic Analysis** The mean concentration versus time profile is shown in Fig. 4. The pharmacokinetic parameters are listed in Table 2. From the concentration-time curve, it can be observed that quick absorption and re-absorption and smooth excretion of chimaphilin exist after orally administering the Lu xian cao decoction to rats. Although it has been reported that enterohepatic circulation occurs during the absorption of chimaphilin’s analogue phylloquinone (vitamin K₃) due to its fat-soluble property, since no studies on the physiological disposition of chimaphilin are available, the factors contributing to the double peak phe-
nomenon in the plasma concentration–time curve and re-absorption of chimaphilin will be further investigated.

CONCLUSION

Although many previous studies have reported that chimaphilin plays an important role in the bioactivities of Lu xian cao, there are no reports on analytical methods for chimaphilin until now. In this paper, we established for the first time a rapid and simple LC-MS method for determination of chimaphilin in rat plasma. Since the method proved to be of good linearity, selectivity, precision, and accuracy, it was successfully used for the pharmacokinetic study of chimaphilin after oral administration of Lu xian cao decoction. The features of chimaphilin’s pharmacokinetics could be applied as references for physiological disposition or other pharmacological studies of chimaphilin.

REFERENCES