—Regular Articles—

LC-MS Determination and Pharmacokinetic Studies of Ursolic Acid in Rat Plasma after Administration of the Traditional Chinese Medicinal Preparation Lu-Ying Extract

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Sambucus chinensis L. is a native perennial herb distributed throughout China. In traditional Chinese medicine (TCM), this herb is known as Lu-Ying. Ursolic acid is the major effective constituent of Lu-Ying. A rapid, sensitive, and accurate liquid chromatography-mass spectrometry (LC-MS) method for the determination of ursolic acid in rat plasma was developed and validated. Plasma samples taken from rats that had received Lu-Ying extract orally were acidified with acetic acid and then extracted with a mixture of hexane-dichloromethane-2-propanol (20 : 10 : 1, v/v/v). Separation of ursolic acid was accomplished on a C_{18} column interfaced with a single quadrupole mass spectrometer. The mobile phase consisting of methanol and water (95 : 5, v/v) was delivered at a flow rate of 1.0 ml/min. Atmospheric pressure chemical ionization was operated in negative-ion mode. Using selected ion-monitoring mode, the deprotonated molecules $[M-H]^-$ at m/z 455 and 469 were used to quantify ursolic acid and glycyrrhetic acid (internal standard), respectively. The assay was shown to be linear over the range of 10—1000 ng/ml ($r \geq 0.9960$) with a lower limit of quantification of 10 ng/ml. The method was shown to be reproducible and reliable with intraday precision below 7.8%, interday precision below 8.1%, accuracy within $\pm 4.3\%$, and mean extraction recovery excess of 83.6%, which were all calculated from the blank plasma sample spiked with ursolic acid at three concentrations of 20, 200, and 800 ng/ml. The LC-MS method has been successfully applied to pharmacokinetic studies of ursolic acid after oral administration of Lu-Ying ethanolic extract (at a dose containing 80.32 mg/kg ursolic acid) to rats. The main pharmacokinetic parameters were: $t_{1/2}$, 4.3 h; K_e, 0.16 1/h; t_{max}, 1.0 h; C_{max}, 294.8 ng/ml; AUC_{0-t} and $AUC_{0-\infty}$, 1007.1 ng·h/ml and 1175.3 ng·h/ml, respectively.

Key words——Lu-Ying extract; ursolic acid; pharmacokinetics; liquid chromatography-mass spectrometry

INTRODUCTION

Traditional Chinese medicine (TCM) uses natural therapeutic agents under the guidance of the theory of traditional Chinese medical science and has been applied by TCM practitioners for thousands of years. *Sambucus chinensis* L. is a native perennial herb distributed throughout China. All parts of the plant can be used in TCM as Lu-Ying. Lu-Ying is one of the important folk medicines in TCM with sedative, antibacterial, antiinflammatory, and hepatoprotective activities.[1—3] Ursolic acid (Fig. 1A) is the major effective constituent of Lu-Ying. Ursolic acid has many important biological activities, such as antiinflammation, hepatoprotective, antiulcer, hypolipidemic, and antiatherosleroic.[4] Moreover, pharmacologic investigation showed that ursolic acid is one of the major active principles of Lu-Ying ethanolic extract.[5] Thus ursolic acid is used as a marker compound to characterize Lu-Ying ethanolic extract.

Pharmacokinetic studies of the active ingredients in TCM will improve the ability to illustrate their mechanisms of action and help to promote the development of TCM. There are a limited number of reports on the analysis of ursolic acid in natural plant material and Chinese medicinal preparations. The majority of the reported articles were based on TLC

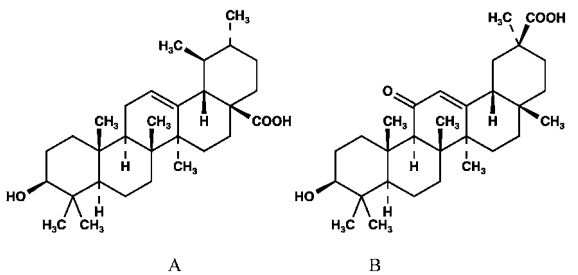

Fig. 1. Structures of Ursolic Acid (A) and Glycyrrhetic Acid (B)

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analysis[6—9] and HPLC,[10—12] whereas few articles were based on GC-MS[13] or LC-MS[14] determinations. However, to our knowledge, there is still no report on the determination of ursolic acid in biological samples. We developed and validated a rapid, sensitive, and accurate LC-MS method for the determination of ursolic acid in rat plasma after oral administration of Lu-Ying extract (the concentration of ursolic acid in Lu-Ying extract was 5.02 mg/ml), to obtain an overview of its pharmacokinetic profile.

EXPERIMENTAL

Materials and Reagents Lu-Ying was purchased from Zhongxin Co. Ltd. (Anhui, China) and identified by Professor Qishi Sun (Department of Pharmacognosy, Shenyang Pharmaceutical University, Shenyang, China). Ursolic acid (98.2% pure) was ordered from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Glycyrrhetic acid (98.5% pure, Fig. 1B) used as internal standard (IS) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Methanol was of chromatographic grade. All the other reagents were of analytical grade. Distilled water, prepared from demineralized water, was used throughout the study.

Animals Male Wistar rats (200—220 g) were purchased from the Experimental Animal Center of Shenyang Pharmaceutical University. They were kept in an environmentally controlled breeding room for 3 days before starting the experiments. They were fed with food and water *ad libitum* and fasted overnight before drug administration. All procedures involving animals were in accordance with the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of People's Republic of China.

LC-MS Instrumentation and Analytical Conditions The assay was performed on a Shimadzu LCMS-2010A system (Shimadzu, Co., Ltd., Kyoto, Japan) consisting of an LC connected to a single quadrupole MS analyzer with an atmospheric pressure chemical ionization (APCI) interface usable in either positive-ionization or negative-ionization mode. An LCMSsolution 3.0 workstation was used for LC-MS control and signal acquisition.

The LC separation was carried out on a Hypersil C_{18} column (150×4.6 mm i.d.; 5 μm) protected by a guard C_{18} column (5 μm), both from Zhonghuida Co. (Dalian, China). The mobile phase consisting of methanol and water (95 : 5, v/v) was delivered at a flow rate of 1.0 ml/min. The column temperature was maintained at 25℃.

The optimum operating parameters of the APCI interface in negative mode were: interface temperature 400℃, heat block temperature 200℃, CDL temperature 250℃, nebulizing gas (N_2) 2.5 l/min, drying gas (N_2) 2.0 l/min, and detector voltage 1.40 kV. Quantification was achieved using selected ion-monitoring (SIM) mode of ion at m/z 455 for ursolic acid and ion at m/z 469 for IS.

Preparation of Lu-Ying Extract Lu-Ying 250 g was refluxed together three times with 75% ethanol for 1 h. After removing ethanol under reduced pressure, the residue was dissolved in 100 ml of water to obtain the Lu-Ying ethanolic extract with a Lu-Ying concentration of 2.5 g/ml.

Preparation of Calibration Curve and Quality Control Samples The standard stock solution was prepared by dissolving 12.5 mg of ursolic acid in 25 ml of methanol to obtain a nominal concentration of 500 μg/ml. The IS stock solution was prepared by dissolving 10.0 mg of glycyrrhetic acid in 25 ml of methanol to obtain a nominal concentration of 400 μg/ml. All stock solutions were kept at 4℃ and were found to be stable for at least 1 month.

Ursolic standard samples (10, 20, 40, 80, 200, 500, and 1000 ng/ml) were prepared by spiking 500 μl of blank rat plasma with the respective amounts of the standard stock solution prepared above. Quality control (QC) samples (20, 200, and 800 ng/ml) were independently prepared in the same manner. A solution containing 32 μg/ml of IS was also prepared using methanol.

Sample Preparation Aliquots (500 μl) of plasma were acidified with 20 μl of acetic acid, with addition of IS 50 μl (32 μg/ml). Then the mixture was extracted with 3 ml of hexane-dichloromethane-2-propanol (20 : 10 : 1, v/v/v). The resulting mixture was shaken mechanically for 3 min. After centrifugation at 3000 ***g*** for 10 min, the supernatant was transferred to a clean test tube and evaporated to dryness in a water bath at 40℃ under a stream of nitrogen. The residue was reconstituted in 100 μl of the mobile phase with vortexing for 60 s and then the centrifugation procedure was repeated. A 5-μl aliquot of the supernatant was injected into the LC-MS system.

Method Validation The spiked standard sam-

ples at seven concentrations over the concentration range (10—1000 ng/ml) were prepared in triplicate and analyzed in three separate analytical runs. Standard curves were constructed using weighted ($1/x^2$) linear least-squares regression analysis of the observed peak area ratios of ursolic acid and the IS. The unknown sample concentrations were calculated from the linear regression equation of the peak area ratio against concentrations of the calibration curve.

QC samples at three concentrations (20, 200, and 800 ng/ml), and another sample at 10 ng/ml were analyzed to assess the accuracy and precision of the proposed methodology. Six replicates were analyzed in each of three analytical runs. The accuracy was expressed by the relative error (RE), and the precision was evaluated by the relative standard deviation (RSD). The intra- and interday accuracy was required to be within 20% at the lower limit of quantification (LLOQ) and within 15% for other concentrations. The precision was required to be less than 20% at the LLOQ and less than 15% at other concentrations.

Recovery of the liquid-liquid extraction procedure was evaluated at low (20 ng/ml), medium (200 ng/ml), and high (800 ng/ml) concentrations for ursolic acid, and at 3.2 μg/ml for the IS. It was determined by comparing the mean peak areas ($n=6$ at each concentration) obtained from plasma samples spiked before extraction to those from plasma samples spiked after extraction.

The stability of ursolic acid in the processed samples during storage at −20℃ was studied at low (20 ng/ml), medium (200 ng/ml), and high (800 ng/ml) concentrations. The concentration of ursolic acid after 14-day storage was compared with the initial concentration as determined for freshly prepared samples. The freeze-thaw stability was determined after three freeze and thaw cycles. In each cycle, the QC samples were stored at −20℃ for 24 h and thawed unassisted at room temperature. When completely thawed, the sample was refrozen within 24 h. The cycle was repeated two times and then the samples were analyzed after the third cycle.

Application of the LC-MS Method and Pharmacokinetic Study In a previous study, we studied the effects of Lu-Ying extract (at three dosages of Lu-Ying 20, 40, and 80 g/kg) against carbon tetrachloride (CCl_4)-induced hepatic damage in rats. The results indicated that the dose of 40 g/kg yielded significant hepatoprotective effects. Therefore to study the pharmacokinetic profile of ursolic acid, the Lu-Ying extract at the dosage of 40 g/kg was administered to rats by oral gavage. Blood samples (1 ml) were collected from the abdominal vein before (0 h) and at 0.25, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0, 10.0, and 12.0 h after dosing, then immediately transferred into heparinized tubes and centrifuged at 3000 ***g*** for 10 min. The plasma obtained was stored frozen at −20℃ until analysis. Because a large volume of blood sample was required, 24 Wistar rats were divided into four groups of 6 animals each. Blood was collected from each group at three time points.

The plasma concentrations of ursolic acid at different time points were expressed as mean ± SD, and the mean concentration-time curve was plotted. All data were analyzed using noncompartmental analysis using the TopFit 2.0 software package (Thomae GmbH, Germany). The maximum plasma concentration (C_{max}) and the time to reach the peak concentration (t_{max}) were obtained directly from the observed values. The apparent elimination rate constant (K_e) was calculated using fitting mean data at four terminal points of the plasma concentration profile with a log-linear regression equation using the least-squares method. The $t_{1/2}$ was calculated as $0.693/K_e$. The area under the plasma concentration-time curve from zero to the time of the final measurable sample (AUC_{0-t}) was calculated using the linear-trapezoidal rule up to the last sampling point with detectable levels (C). The area under the plasma concentration-time curve from zero to infinity ($AUC_{0-\infty}$) was calculated using the trapezoidal rule with extrapolation to infinity with K_e. The mean residence time (MRT) was calculated as the ratio of the area under the first moment curve ($AUMC_{0-t}$) to AUC_{0-t}. The apparent total body clearance (CL_{tot}/F) after oral administration was calculated using the equation $CL_{tot}/F = \text{Dose}/AUC_{0-t}$. The volume of distribution (V_z/F) after oral administration was calculated using the equation $V_z/F = \text{Dose}/C_0$, where F is the unknown fraction of drug available to the systemic circulation.

RESULTS AND DISCUSSION

Method Development Structurally, ursolic acid is a pentacyclic triterpenic substance. Its chemical structure lacks UV-absorbing chromophores, which limits the chromatographic determination of ursolic acid by HPLC using UV or fluorescence detection.

Furthermore, a preliminary derivatization process is necessary for the analysis of ursolic acid by GC. Application of LC-MS is appropriate because it does not depend on the presence of a particular chromophore in the molecule. On the other hand, taking into consideration the lower levels of ursolic acid in plasma, LC-MS is the first choice for our purpose.

Liquid-liquid extraction is more effective in producing a clean sample and avoiding the introduction of nonvolatile materials to the column and MS system. Clean samples are essential for minimizing ion suppression and matrix effects on LC-MS analyses. Thus liquid-liquid extraction was used for the sample preparation in this method. Various organic solvents, such as ethyl ether, ethyl acetate, dichloromethane, and their mixtures in different combinations and ratios were evaluated. Finally, a mixture of hexane-dichloromethane-2-propanol (20 : 10 : 1, v/v/v) was found to be optimal for producing a clean blank plasma chromatogram and yielding the highest recovery for ursolic acid and the IS.

To improve the recovery of ursolic acid by suppressing its ionization, after testing both hydrochloric acid and acetic acid for suitability, acetic acid was used as acidic modifier to adjust the pH of plasma samples. The addition of acetic acid to plasma also denatured and precipitated proteins, which helped to release the bound organic components, including ursolic acid, to the extract solvent.

Optimization of LC-MS Conditions Based on our experience, APCI was preferred to electrospray ionization (ESI) to quantify ursolic acid in rat plasma due to its lower level of background noise. Since ursolic acid and the IS both contain a carboxyl group, the possibility of using positive- or negative-ion detection was first evaluated. By comparison, the negative-ionization mode could offers higher sensitivity than the positive-ionization mode. With negative APCI,

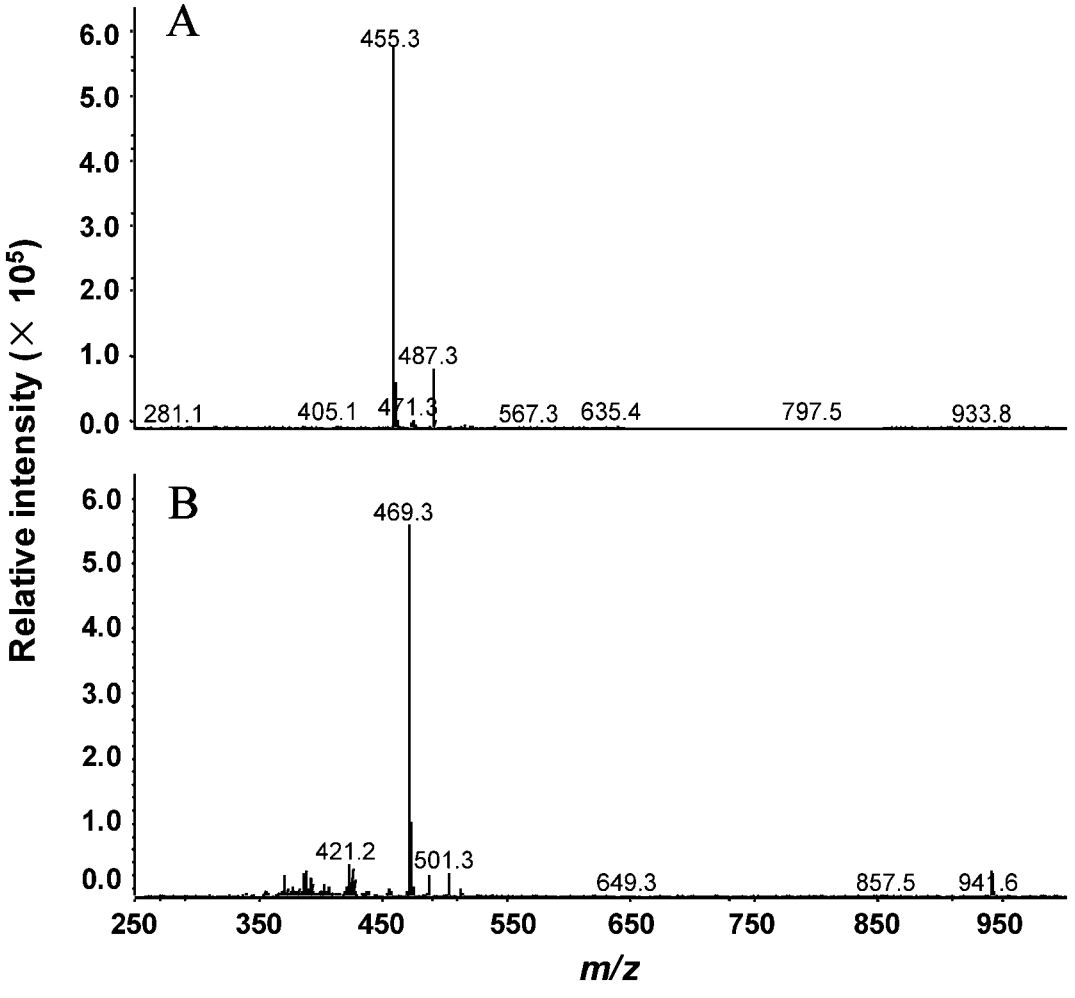

Fig. 2. Full-scan Mass Spectra of Ursolic Acid (A) and Glycyrrhetic Acid (B)

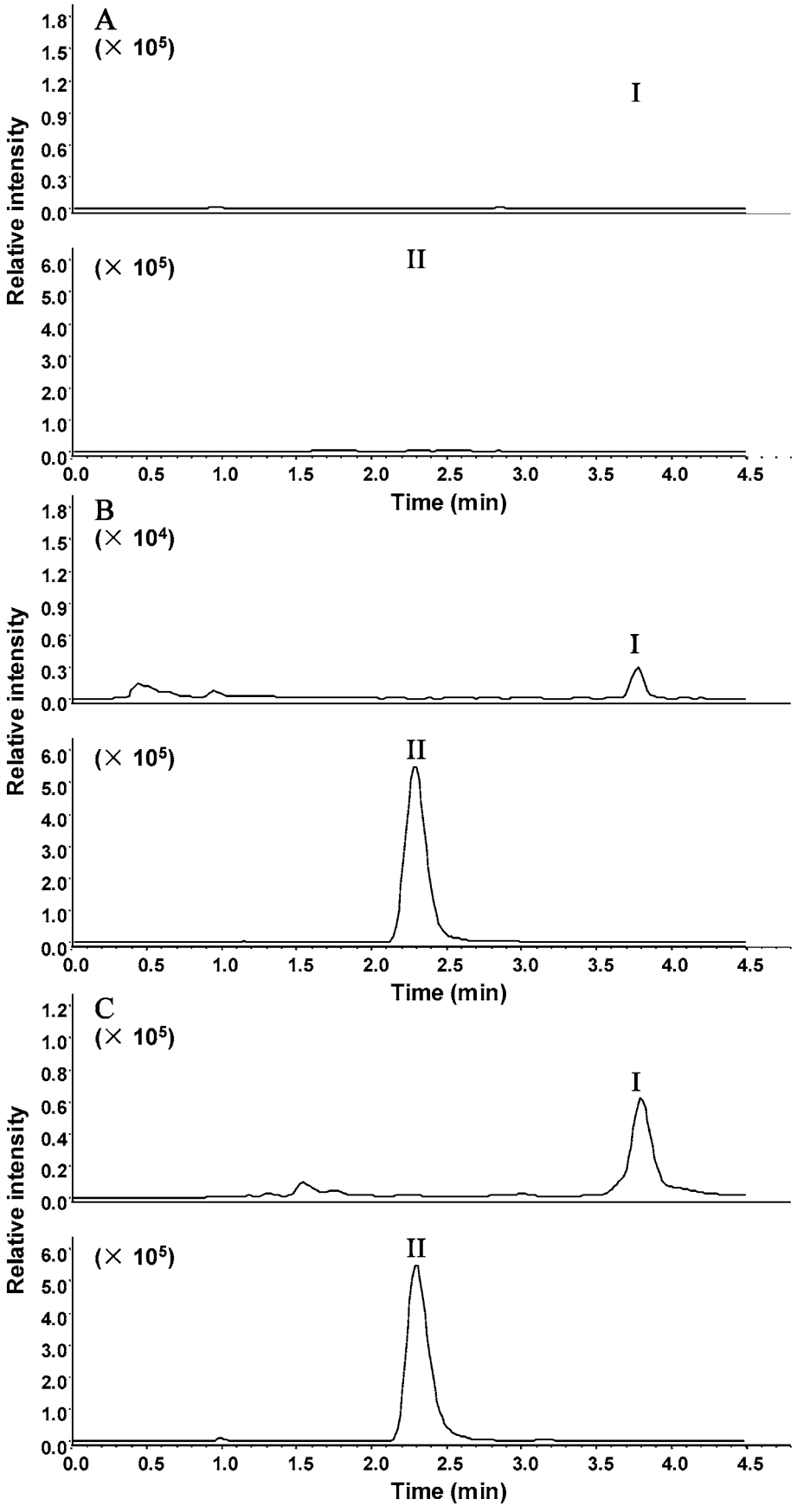

Fig. 3. Representative SIM Chromatograms for the Determination of Ursolic Acid in Rat Plasma

(A): Blank rat plasma sample, (B): Blank plasma sample spiked with ursolic acid (I, 10 ng/ml) and internal standard (II, 3.2 μg/ml), (C): Plasma sample from a rat 1.5 h after oral administration of Lu-Ying extract (I, 278.6 ng/ml and II, 3.2 μg/ml).

ursolic acid and the IS formed predominately deprotonated molecules $[M-H]^-$ in full-scan mass spectra (Fig. 2). The ions at m/z 455 and 469 were chosen in the SIM acquisition for ursolic acid and the IS, respectively.

Selectivity Representative SIM chromatograms of blank plasma, plasma spiked with 10 ng/ml of ursolic acid and 3.2 μg/ml of the IS, and a rat plasma sample 1.5 h after administration of Lu-Ying extract (ursolic acid, 278.6 ng/ml and IS, 3.2 μg/ml) are presented in Fig. 3. The retention times of ursolic acid and the IS were 2.3 and 3.8 min, respectively. As shown by analysis of blank plasma from each of six rats, the assay was free of interference from compounds in the biomatrix. Matrix effects were evaluated by comparing the peak areas of analyte in extracted samples of blank plasma spiked after extraction with the corresponding areas obtained by direct injection of standard solutions. No coeluting ''unseen'' endogenous species interfered with the ionization of the analyte and IS.

Calibration and Validation The representative regression equation for the calibration curve was $Y=1.332\times10^{-3}+3.134\times10^{-4}x$ over the range of 10—1000 ng/ml with a correlation coefficient of 0.9960.

The intra- and interday precision and accuracy results are shown in Table 1. The intra- and interday precision was less than 7.8 and 8.1%, respectively, and the accuracy was within (4.3% for QC samples. The intraday and interday precision and accuracy of a sample of ursolic acid concentration at 10 ng/ml were 11.4%, 8.9%, and 9.2%, respectively. The values obtained were lower than the limits required for biological sample analysis. The results indicated that the LLOQ of 10 ng/ml was achieved.

The results showed that the extraction recoveries of ursolic acid from rat plasma were 84.3±2.6, 81.2±2.1, and 85.3±3.1% at concentrations of 20, 200, and 800 ng/ml, respectively. The mean recovery was 83.6%. The recovery of the IS was 80.4±2.4% at the concentration (3.2 μg/ml), used in the method.

Table 1. Intra- and Interday Precision and Accuracy of the LC-MS Method to Determine Ursolic Acid in Rat Plasma

Concentration (ng/ml)		RSD (%)		RE (%)
Added	Found	Intraday	Interday	
10.0	10.9	11.4	8.9	9.2
20.0	20.7	7.8	8.1	3.5
200.0	192.5	4.3	5.2	−3.8
800.0	834.6	3.9	4.6	4.3

After storage at −20℃ for 14 days, the corresponding relative errors were 2.3, −3.1, and −3.6 % for spiked samples of 20 ng/ml, 200 ng/ml, and 800 ng/ml, respectively. Results of the stability experiments indicated that ursolic acid storage at −20 ℃ was stable at least for 2 weeks in rat plasma. After three freeze-thaw cycles, the corresponding relative errors from the same three concentrations were 3.2, −4.1, and −2.8%, respectively, which indicated that ursolic acid was stable in rat plasma after three freeze-thaw cycles.

Application of the Method in Pharmacokinetic Studies The developed and validated LC-MS method was used to determine ursolic acid in rat plasma after oral administration of Lu-Ying extract at a dose containing ursolic acid 80.32 mg/kg. Using the method with the degree of sensitivity (LLOQ of ursolic acid, 10 ng/ml), pharmacokinetics studies of ursolic acid in rats were successfully performed. If 100 μl of plasma was used, the profile would remain stable until around 6 h after administration. To determine ursolic acid in plasma obtained from rats more than 6 h after administration exceeding 6 h, a large quantity (500 μl) of plasma would be necessary and only the mean plasma concentration-time curve profile was determined. The mean plasma concentration-time curve profile is illustrated in Fig. 4 and its pharmacokinetic parameters are shown in Table 2. The pharmacokinetic results suggest that the absorption of ursolic

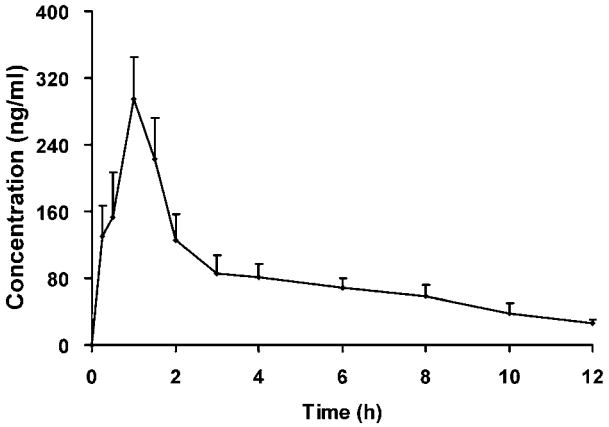

Fig. 4. Plasma Concentration-time Curve of Ursolic Acid in Rats after Oral Administration of Lu-Ying Extract (at a Dose Containing Ursolic Acid 80.32 mg/kg)
Each point and bar represent mean±S.E. (n=6).

Table 2. Pharmacokinetic Parameters of Ursolic Acid in Rats after Oral Administration of Lu-Ying Extract (at a Dose Containing Ursolic Acid 80.32 mg/kg)

C_{max} (ng/ml)	t_{max} (h)	K_e (l/h)	$t_{1/2}$ (h)	AUC_{0-t} (ng·h/ml)	$AUC_{0-\infty}$ (ng·h/ml)	MRT_{0-t} (h)	CL_{tot}/F (ml/min·kg)	V_z/F (l/kg)
294.8	1.0	0.16	4.3	1007.1	1175.3	4.1	1130.1	424.1

acid was rapid, with the peak concentration occurring 1.0 h after oral administration of Lu-Ying extract. The ursolic acid concentration was lower than the LLOQ 12 h postdose. Although the oral administration dose of Lu-Ying extract contained ursolic acid 80.32 mg/kg, its concentrations in rat plasma were extremely low. This implies that ursolic acid has high binding activity in organs and low blood distribution; another possibility was the low bioavailability of ursolic acid because it is metabolized by the gut wall and liver on one hand, and on the other hand, it is poorly absorbed by the intestine.

CONCLUSIONS

A simple and reliable LC-MS method has been developed and validated for the determination of ursolic acid in rat plasma after oral administration of Lu-Ying extract. The method offers sensitivity, with LLOQ of 10 ng/ml, and specificity, without interferences from endogenous substances. This is the first report of pharmacokinetic studies of ursolic acid in rat plasma after oral administration of Lu-Ying extract. The pharmacokinetic results provide a firm basis for evaluating the clinical efficacy of Lu-Ying extract.

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REFERENCES

1) ''Zhong Guo Yao Cai Xue,'' Chinese Scientific and Technological Medicinal Publishing House, Beijing, 1996, p. 1601.
2) ''Zhong Yao Da Ci Dian,'' Shanghai People's Publishing House, Shanghai, 1977, p. 2456.
3) ''Zhong Guo Zhong Yao Zi Yuan Zhi Yao,'' Scientific Publishing House, Beijing, 1994, p. 1207.
4) Liu J., *J. Ethnopharmacol.*, **49**, 57–68 (1995).
5) Chen W., Li K.-Q., Xiong X.-J., *Nanchang Da Xue Xue Bao*, **25**, 165–167 (2001).
6) Yuan Z.-Y., Deng Z.-H., *Zhong Yao Cai*, **22**, 534–535 (1999).
7) Zhao C.-X., Zhang G.-J., *Zhong Guo Zhong Yao Za Zhi*, **24**, 415–416 (1999).
8) Cui J., Gong J.-M., *Zhong Cheng Yao*, **18**, 400–402 (1999).
9) Wang X., Guan J., *Zhong Guo Zhong Yao Za Zhi*, **24**, 675–676 (1999).
10) Yang X.-T., Cheng L.-L., Mi K., *Shanghai Zhong Yi Yao Da Xue Xue Bao*, **15**, 53–55 (2001).
11) Zhang S.-M., Gu H., Chen J.-M., *Fen Xi Hua Xue*, **28**, 129–131 (2000).
12) Hao R.-X., Yan C.-P., Sun X.-F., *Yao Wu Fen Xi Za Zhi*, **14**, 30–32 (1994).
13) Yang L.-H., Zhao L.-H., Zhu D.-N., *Zhong Guo Zhong Yao Za Zhi*, **24**, 744–745 (1999).
14) Ladislav N., Mohammed E., Hoda H., *J. Pharm. Biomed. Anal.*, **31**, 961–968, (2003).