—Regular Articles—

Determination of Sophoricoside in Rat Plasma by HPLC and Its Application to Pharmacokinetic Studies

Rongning LUO, Zhangwan LI, Guangsheng QIAN, Jia LU, and Chunmei FU*

Key Laboratory of Drug Targeting and Delivery System, West China School of Pharmacy, Sichuan University, No. 17, Section 3, Southern Renmin Road, Chengdu 610041, P. R. China

(Received July 31, 2009; Accepted September 17, 2009)

A simple, sensitive, selective and reproducible reversed-phase HPLC method was developed for the determination of sophoricoside in rat plasma after intravenous administration. Naringin was successfully used as internal standard (IS) for calibration. The chromatographic separation was accomplished on a reversed-phase C_{18} column using acetonitrile-methanol-0.08% phosphoric acid (8 : 29 : 63, v/v/v) as mobile phase with a flow rate of 1.0 ml/min, with UV detection at 260 nm. Plasma samples were injected into the HPLC system after precipitating protein directly by methanol. Good linearity was achieved in the range of 0.0240～48.0 μg/ml (R^2=0.9989). The limit of detection (LOD) and limit of quantification (LOQ) of this method were 0.0075 μg/ml and 0.0240 μg/ml, respectively. The absolute recoveries of sophoricoside from plasma were 95.8%, 93.2%, 98.0% at concentrations of 0.0240, 1.92, 15.0 μg/ml. The intra-day and inter-day variabilities were 3.39%～5.78% and 2.17%～4.72%, respectively. The developed method was successfully applied to the pharmacokinetic study of sophoricoside after intravenous administration of 2.5, 10 and 20 mg/kg in rats.

Key words——sophoricoside; HPLC; pharmacokinetics; naringin

INTRODUCTION

Sophora japonica L. (Leguminosae), a well-known traditional Chinese medicinal herb, is officially listed in the Chinese Pharmacopoeia.[1] Its fruits have been used as hemostatic agent in traditional Chinese medicine. Pharmacological studies and clinical practices have demonstrated that *S. japonica* L. has anti-tumour, anti-fertility and anti-cancer activities[2,3] and the active constituents of the herb are flavonoid and isoflavonoids components.

Sophoricoside (Genistein-β-glucoside) (Fig. 1), a kind of isoflavone glycoside, containing as much as 0.73% (w/w) of the total dry weight in the fruit of *S. Japonica* L.[4] exhibits potent inhibitory effects on interleukin-5 (IL-5),[5] IL-3 and IL-6 bioactivities but very weak on granular cell macrophage colony stimulating factors bioactivity.[6,7] Jung *et al.*[8] reported the structural requirement of sophoricoside for the inhibitory activity of IL-5.

Isoflavones and glycosides have received much attention because of their health-related and clinical benefits such as anti-oxidative activities as well as triggering of natural killer cell activity[9] and of therapeutic effects in cancer, angiocardiopathy, osteoporosis and female climacteric syndrome.[10] There were many studies about metabolism of isoflavones such as genistein and genistin in China and overseas,[11–13] but there was no report about the determination of sophoricoside in biological matrices and pharmacokinetics *in vivo* to the best of our knowledge.

Wang *et al.*[14] reported a capillary electrophoresis (CE) method for the determination of sophoricoside which showed promise in the analysis of coumarin compounds in different herbal plants and its preparations. Although CE has the merits of high sensitivity, good selectivity and high efficiency, the application of CE is less widespread than HPLC because its reproducibility is still a big problem. In this study, a robust, sensitive, simple and accurate HPLC method for the determination of sophoricoside in rat plasma using naringin as IS was developed and the pharmacokinetics of sophoricoside in rat in normal physiological conditions were also investigated at three different doses of 2.5, 10 and 20 mg/kg after intravenous administration for the first time, which provide the theoretical foundation and reference for the safe and reasonable clinic exploitation of sophoricoside.

*e-mail: fcm213@sina.com

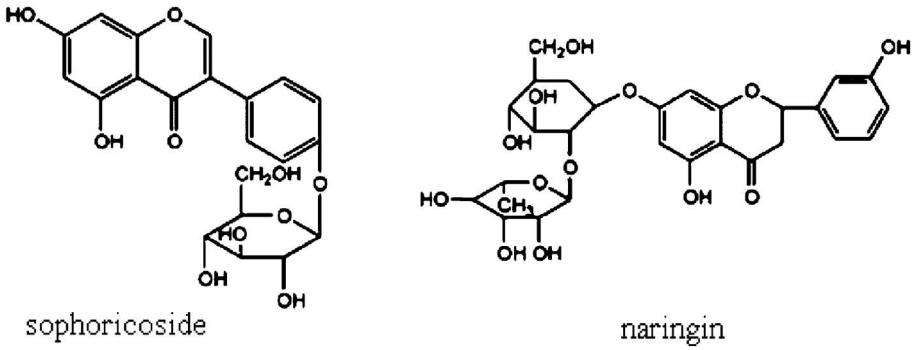

Fig. 1. Chemical Structures of Sophoricoside and Naringin

EXPERIMENTAL

Chemicals and Reagents Sophoricoside was supplied by National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Naringin (IS) was obtained from Fluka Chemie AG CH-9470 (Switzerland). Methanol and acetonitrile (HPLC grade) were obtained from Fisher Chemical Company (Canada). Water was prepared by ultra pure water system (UPA, Chongqing, China). All other chemicals were of reagent grade, and were used without further purification.

Liquid Chromatography HPLC determinations were performed with a Shimadzu model 10 Avp liquid chromatographic system (Tokyo, Japan), consisting of a LC-10AT pump and an UV/Vis detector (SPD-10A). The system was controlled by a system controller (SCL-10A) and a personal computer. The analytical column was a Diamonsil C_{18} column (150 $\times$4.6 mm, 5 μm) from Dikma Technologies. The isocratical elution with a mobile phase of acetonitrile-methanol-0.08% phosphoric acid (8 : 29 : 63, v/v/v) was used at a flow rate of 1.0 ml/min for the separation of analytes. The analytes were monitored at the UV wavelength of 260 nm.

Preparation of Calibration Standards Sophoricoside was dissolved in methanol (0.240 mg/ml) as stock solution. Working standards were prepared freshly by diluting the stock solution with methanol with concentrations from 0.0240 to 48.0 μg/ml. A working IS solution of 24.0 μg/ml was made by dissolving naringin with methanol. All solutions were stored at 4℃ before use.

Plasma standard samples for calibration were prepared daily by spiking 20 μl blank rat plasma with 40 μl working standard solutions to achieve concentrations in the range of 0.0240～48.0 μg/ml. Quality control (QC) samples were independently prepared at concentrations of 0.0240, 1.92 and 15.0 μg/ml in the same manner as the plasma sample for calibration. Then, all the calibration and QC samples were extracted after 20 μl IS solution was added to them by the method described in the sample preparation.

Sample Preparation The IS solution (20 μl) and 40 μl of methanol (protein precipitant and extraction solvent) were added to plasma samples (20 μl). Extraction was conducted by vortexing vigorously for 2 min and centrifuged at 12 000 rpm for 10 min. The supernatant was transferred to a glass tube. A 20 μl aliquot of the supernatant was injected into the HPLC system.

Recovery and Stability of Sophoricoside Samples Recoveries of sophoricoside were assessed (n=6) at 15.0, 1.92 and 0.0240 μg/ml. The recoveries were determined by comparing the peak area ratio of sophoricoside to naringin in plasma with that in methanol at the same concentration.

The ambient stability and freeze-thaw stability of sophoricoside in rat plasma were assessed using the QC samples of three different concentrations (0.0240, 1.92 and 15.0 μg/ml). The ambient stability was assessed by leaving the QC samples at room temperature for 24 h. The freeze-thaw stability was assessed over three cycles thawed at room temperature and refrozen at −20℃.

Precision and Accuracy The intra-day precision and accuracy of the assay were determined within one day by analyzing six replicates of QC samples at concentrations of 15.0, 1.92 and 0.0240 μg/ml. The inter-day precision and accuracy were estimated from the results of assays of QC samples of six different days within one week.

Pharmacokinetic Studies Male Sprague-Dawley rats (250 $\pm$ 20 g) were obtained from Laboratory

Animal Center of Sichuan University. The animal experiments in this study were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals, and approved by the Institutional Animal Care and Use Committee of Sichuan University.

The rats were allowed to freely access to food and water under the controlled temperature at 25±1℃ and fasted overnight before use. 0.5 ml of sophoricoside solubilized in N,N-dimethylformamide/isotonic Na chloride (60∶40, v/v) were given to the rats through the tail vein at three different doses (2.5, 10, 20 mg/kg, respectively) by mathematical conversion of doses between different species (from human to rat) according to the Chinese Pharmacopeia. Serial blood samples (0.3 ml) were obtained from fossa orbitalis into heparinized tubes. Plasma samples were collected at 0, 10, 30, 60, 120, 180, 300 min for low dose after drug administration. They were collected at 0, 10, 60, 120, 180, 300, 420 min for middle dose and at 0, 10, 60, 120, 180, 300, 420, 480 min for high dose, respectively. Plasma samples were obtained following centrifugation at 4000 rpm for 20 min and were stored at −20℃ until analysis.

The pharmacokinetic parameters were determined by using PK software Drug and Statistics (Version 2.1.1, China). The pharmacokinetic parameters estimated in this study were as follows: the area under the plasma concentration-time curve from 0 to ∞ ($AUC_{0-\infty}$), elimination half-life ($t_{1/2}$), volume of distribution (Vd), clearance (Cl) and mean residence time (MRT).

RESULTS AND DISCUSSION

Selection of IS Due to the high accuracy requirement for biological samples analysis, assay method with a suitable IS is often adopted. An ideal IS should have similar chromatographic and spectral properties as the compounds to be analyzed. Several substances had been tried such as baicalin, jasminoidin and naringin in this study. Naringin was chosen at last since it presented a good peak shape, satisfactory resolution under the HPLC conditions applied. In addition, the suitability of naringin as IS was further proven by recovery studies (95.8%, RSD =1.33%, n=6).

HPLC Method Validation In the current study, an HPLC method was optimized and validated for the quantitative determination of sophoricoside in rat plasma. Typical chromatograms of blank plasma, spiked plasma and plasma sample are shown in Fig. 2. The chromatogram showed a clear and good separation of the peaks, and the retention times were 10.6 min for sophoricoside and 8.4 min for IS, respectively. The resolution of naringin to sophoricoside was 3.21 and no interfering peaks derived from endogenous substance were observed in the blank plasma at the retention time of the analytes of interest.

The calibration curve for sophoricoside exhibited a good linear response within the range of concentrations from 0.0240～48.0 μg/ml (y=0.0448x−0.0067, R^2=0.9989, y is the peak area ratio of sophoricoside to the IS, and x is the plasma concentration of sophoricoside). The LOD for this method defined as signal-to-noise ratio of 3∶1 was 0.0075 μg/ml and the LOQ defined as the lowest concentration

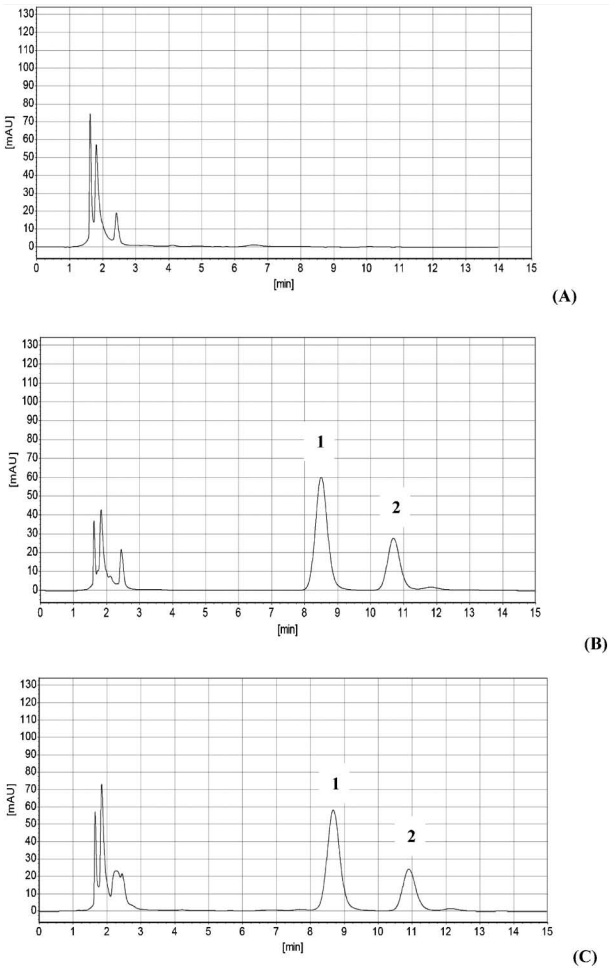

Fig. 2. Chromatograms of Sophoricoside in Rat Plasma
(A) blank plasma of rats, (B) plasma spiked with sophoricoside (15.0 μg/ml) and naringin (24.0 μg/ml), (C) plasma sample 2 min after 20 mg/kg intravenous administration of sophoricoside. 1, naringin; 2, sophoricoside.

of spiked plasma samples that can be determined with sufficient precision and accuracy, *i.e.*, relative standard deviation (RSD) less than 20% and relative error (RE) of −20 to 20%. The LOQ was estimated as 0.0240 μg/ml.

In order to optimize the sample preparation method, a single-step liquid-liquid extraction with different solvents (include chloroform, petroleum ether, ethyl ether, ethyl acetate) and precipitating protein directly with different solvents (include methanol, ethanol, acetonitrile) and the volume ratio of sample to solvent had been investigated to compare recoveries of sophoricoside from rat plasma sample. Methanol precipitating protein was adopted because it had high absolute recovery without any significant interference and was simple relatively. From the experiment, we found that the recoveries were the same generally when the volume ratios of plasma sample to methanol 1∶2, 1∶3 and 1∶4 (v/v) were used to precipitate protein. Considering the level of cleaness and diluted volume, 1∶3 (v/v) was adopted to precipitate protein. This method was simple and small volume of plasma sample was required. The mean absolute recoveries of sophoricoside at three concentrations were 95.8%, 93.2% and 98.0%, respectively. The results are shown in Table 1.

The intra-day and inter-day precision calculated by replicate assays of sophoricoside in rat plasma were less than 6% over a wide range of sophoricoside concentration. The accuracy during replicate assays varied between 94% and 103%. Precision and accuracy studies indicated that the developed HPLC method was reproducible and accurate (Table 2).

Stability investigation (Table 3) demonstrated that the concentrations of sophoricoside in processed samples had no significant difference to nominal values within 24 h at 25℃ and between the three freeze-thaw cycles (room temperature to −20℃). These indicated that the analyte in rat plasma was stable for up to 24 h at 25℃ with the maximal loss of 2.9% and was stable over at least three freeze-thaw cycles with no significant loss (≤10.4%).

Pharmacokinetic Application The plasma concentrations of sophoricoside after intravenous administration in rats were quantitatively determined by the developed HPLC method. The pharmacokinetic parameters (Table 4) were determined by fitting pharmacokinetic models to the plasma concentration-time profiles (Fig. 3) for each rat after intravenous dosing. From the results of kinetic analysis, a one-compartment model was proposed. From Table 4, it can be seen that with the increase of dose, $AUC_{0\to\infty}$ increased proportionally, while MRT, $t_{1/2}$ and Cl kept almost the same. Thus, the composite pharmacokinetics was first order process in the range of dose examined.

CONCLUSIONS

A sensitive, specific, accurate and reproducible reversed-phase HPLC method for the determination

Table 1. Recovery of Sophoricoside from Rat Plasma (n=6)

Nominal concentration (μg/ml)	Measured concentration* (μg/ml)	Recovery* (%)	RSD (%)
0.0240	0.0230±0.001	95.8±4.3	3.59
1.92	1.79±0.063	93.2±3.5	3.26
15.0	14.7±0.354	98.0±2.4	2.36

* mean±S.D.

Table 2. Intra- and Inter-day Precision and Accuracy of Sophoricoside in Rat Plasma (n=6)

	Nominal concentration (μg/ml)	Measured concentration* (μg/ml)	Accuracy (RE%)	Precision (RSD%)
Intra-day	0.0240	0.0239±0.001	99.6	5.78
	1.92	1.88±0.068	97.9	3.55
	15.0	15.4±0.509	103	3.39
Inter-day	0.0240	0.0225±0.001	93.8	4.72
	1.92	1.91±0.056	99.5	2.94
	15.0	15.1±0.326	101	2.17

* mean±S.D.

Table 3. Assessment of Stability in Rat Plasma

Condition	Conentration (μg/ml)		
	0.0240	1.92	15.0
Freeze-thaw stability (−20℃)*			
Cycle 1	96.3	97.7	98.3
Cycle 2	90.2	87.2	90.3
Cycle 3	89.6	89.7	92.7
Short-term stability (25℃)*			
Time=2.0 h	98.4	99.3	99.8
Time=4.0 h	99.1	98.7	100
Time=8.0 h	97.6	100	99.8
Time=24.0 h	97.1	102	99.3

* Expressed as the mean percentage change from time zero (*nominal* concentration).

Table 4. Pharmacokinetic Parameters of Sophoricoside in Rats after Intravenous Administration ($n=6$)

Parameters	Estimated (2.5 mg/kg)	Estimated (10 mg/kg)	Estimated (20 mg/kg)
$t_{1/2}$ (min)	33.375±19.786	43.148±37.269	42.291±35.463
$AUC_{0\to\infty}$ (μg·min/ml)	101.248±31.162	253.951±94.352	434.808±34.128
Vd (l/kg)	1.495±0.925	1.367±1.086	2.292±1.256
Cl (l/min/kg)	0.030±0.008	0.042±0.028	0.041±0.005
MRT (min)	80.271±20.439	85.69±23.039	91.779±16.083

All the values presented above are means±S.D.

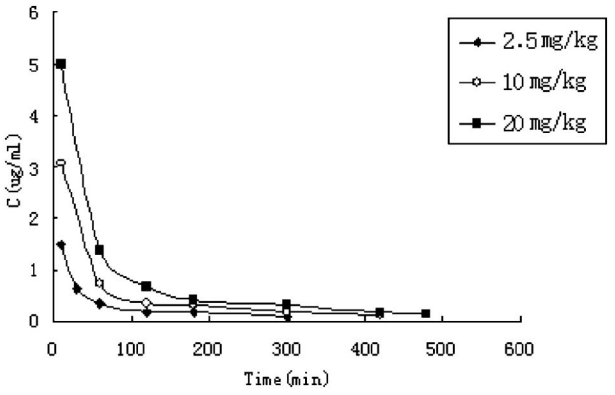

Fig. 3. Mean Plasma Concentration-time Curves after Intravenous Administration of Sophoricoside to Rats

of sophoricoside in rat plasma was developed which had been successfully applied to the study of pharmacokinetics of sophoricoside in rat for the first time. This method can be available for large number of biological samples very efficiently. Further studies are ongoing in our laboratory to further characterize the absorption and metabolism of sophoricoside.

REFERENCES

1) China Pharmacopoeia Committee, ''Pharmacopoeia of People's Republic of China,'' the First Division of 2005 Edition, China Chemical Industry Press, Beijing, 2005, pp. 60.
2) Wang J. H., Wang Y. L., Lou F. C., *J. Chin. Pharm. Univ.*, **6**, 471–473 (2001).
3) Ma L., Lou F. C., *Chin. J. Nat. Med.*, **4**, 151–153 (2006).
4) Tian Z. M., Wan M. X., Wang Z. L., Wang B., *Drug Develop. Res.*, **61**, 6–12 (2004).
5) Min B., Oh S. R., Lee H. K., Takatsu K., Chang I. M., Min K. R., Kim Y., *Planta Med.*, **65**, 408–412 (1999).
6) Yun J., Lee C. K., Chang I. M., Takatsu K. S., Hirano T. S., Min K. R., Lee M. K., Kim Y. S., *Life Sci.*, **67**, 2855–2863 (2000).
7) Kim B. H., Chung E. Y., Ryu J., Jung S., Ming K. R., Kim Y., *Arch. Pharm. Res.*, **26**, 306–311 (2003).
8) Jung S. H., Cho S. H., Dang T. H., Lee J., *Eur. J. Med. Chem.*, **38**, 537–545 (2003).
9) Zhang Y., Song T. T., Cunnick J. E., Murphy P. A., Hendrich S., *J. Nutr.*, **129**, 399–405 (1999).
10) Wang J. H., Li M. H., Wang Y. L., Lou F. C., *Zhong Cao Yao*, **33**, 586–588 (2002).
11) Chen C., Bakhiet R. M., *Mech. Ageing Dev.*, **127**, 344–348 (2006).
12) Tsai T. H., *J. Chromatogr. A*, **1073**, 317–322 (2005).
13) Kwon S. H., Kang M. J., Huh J. S., Ha K. W., Lee J. R., Lee S. K., Lee B. S., Han I. H., Lee M. S., Lee M. W., Lee J., Choi Y. W., *Int. J. Pharm.*, **337**, 148–154 (2007).
14) Wang W. P., Tang J. H., Wang S. M., Zhou L., Hu Z. D., *J. Chromatogr. A*, **1148**, 108–114 (2007).