Arch Pharm Res Vol 27, No 12, 1202-1206, 2004

Archives of Pharmacal Research
http://apr.psk.or.kr

Liquid Chromatography-Tandem Mass Spectrometry for the Determination of Lithospermic Acid B in Rat Serum

Hui-Hyun Kim, Hye Young Ji, Hye Won Lee, Youn-Chul Kim, Dong Hwan Sohn, and Hye Suk Lee

College of Pharmacy and Phytofermentation Research Center, Wonkwang University, Iksan 570-749, Korea

(Received October 11, 2004)

A rapid, sensitive and selective liquid chromatography-tandem mass spectrometric (LC-MS/MS) method for the determination of lithospermic acid B (LSB) in rat serum was developed. LSB and internal standard, 7-hydroxy-3-phenyl-chromen-4-one (HPC) were extracted from rat serum with methyl-tert-butyl ether at acidic pH and analyzed on a Luna C_8 column with the mobile phase of acetonitrile-ammonium formate (10 mM, pH 6.5) (50:50, v/v). The analytes were detected using a negative electrospray ionization tandem mass spectrometry in the multiple-reaction-monitoring mode. The standard curve was linear (r^2 = 0.997) over the concentration range of 10.0-500 ng/mL. The coefficient of variation and relative error for intra- and inter-assay at three QC levels were 1.1~6.2% and -10.3~-2.7%, respectively. The recovery of LSB from serum sample ranged from 73.2 to 79.5%, with that of HPC (internal standard) being 75.1 %. The lower limit of quantification for LSB was 10 ng/mL using 50 μL of serum sample.

Key words: Lithospermic acid B, Rat serum, LC-MS/MS

INTRODUCTION

Salvia miltiorrhiza Radix (Dansham in Korean and Danshen in Chinese) has been widely used in Chinese folk medicine for the treatment of coronary heart diseases, renal diseases, myocardial infarction and hypertension. Seven phenolic compounds isolated from *Salvia miltiorrhiza* as active components have strong anti-oxidative activity against oxidative damage to liver microsomes, hepatocytes and erythrocytes and lithospermic acid B (LSB, Fig. 1) is a major component (Zupko *et al.*, 2001).

LSB possesses the various biological activities, that is, anti-fibrotic effect due to inhibition of collagen hydroxylation (Shigematsu, 1994), hepatoprotection (Hase *et al.*, 1997), the hypotensive effects in rats *via* endothelium-dependent vasodilator effect (Kamata *et al.*, 1993, 1994), the inhibition of HIV-1 replication (Abd-Elazem *et al.*, 2002) and the amelioration effect of cephaloridine, adenine- and ischemia/reperfusion-induced renal injury in rats (Yokozawa *et al.*, 1991, 1997; Kang *et al.*, 2004).

There was no method reported for the determination of LSB in biological fluids. A sensitive, simple, fast and reliable bioanalytical method is required in order to evaluate the pharmacokinetic disposition of LSB. The purpose of this paper was to describe and validate a LC-MS/MS method using liquid-liquid extraction for the determination of LSB in rat serum. The pharmacokinetics of LSB in male Sprague-Dawley rats were also reported.

Correspondence to: Hye Suk Lee, Drug Metabolism and Bioanalysis Laboratory, College of Pharmacy, Wonkwang University, Shinyongdong, Iksan 570-749, Korea
Tel: 82-63-850-6817, Fax: 82-63-851-2013
E-mail: hslee@wonkwang.ac.kr

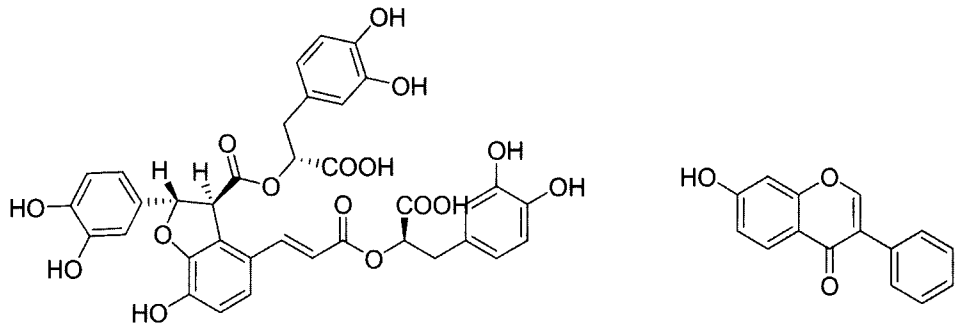
MATERIALS AND METHODS

Materials and reagents

LSB used for this study was isolated from the roots of *Salvia miltiorrhiza* as previously described (Kang *et al.*, 2004). 7-Hydroxy-3-phenyl-chromen-4-one (HPC, internal standard, Fig. 1) was obtained from Samchundang Pharm. Co. (Seoul, Korea). Acetonitrile and dichloromethane (HPLC grade) were obtained from Burdick & Jackson Inc. (Muskegon, MI, USA) and the other chemicals were of HPLC grade or the highest quality available.

Preparation of calibration standards and quality control samples

Primary stock solutions of LSB and HPC (1 mg/mL) were prepared in water and acetonitrile. Working standard solutions of LSB were prepared by diluting each primary solution with water. The working solution for internal



Lithospermic acid B

7-Hydroxy-3-phenyl-chromen-4-one (HPC)

Fig. 1. Chemical structures of lithospermic acid B and 7-hydroxy-3-phenyl-chromen-4-one (HPC, internal standard)

standard (200 ng/mL) was prepared by diluting an aliquot of stock solution with acetonitrile. All LSB and HPC solutions were stored at *ca* 4°C in polypropylene bottles in the dark when not in use.

Rat serum calibration standards of LSB (10.0, 20.0, 50.0, 100, 200 and 500 ng/mL) were prepared by spiking appropriate amount of the working standard solutions into a pool of ten lots of drug-free rat serum. Quality control (QC) samples at 15.0, 75.0 and 400 ng/mL were prepared in bulk by adding 250 μL of the appropriate working standard solutions (0.30, 15 and 8 μg/mL) to drug-free rat serum (4750 μL). The QC samples were aliquoted (50 μL) into polypropylene tubes and stored -20°C until analysis.

Sample preparation

50 μL of blank serum, calibration standards and QC samples were mixed with 10 μL of internal standard working solution and 25 μL of 1M HCl to adjust pH of samples to 2. The samples were extracted with 1000 μL of methyl-*tert*-butylether in 1.5 mL-polypropylene tubes by vortex-mixing for 5 min at high speed and centrifuged at 5000 *g* for 10 min at 4°C. The organic layer was pipette transferred and evaporated to the dryness under nitrogen at 35°C. The residues were dissolved in 35 μL of 40% acetonitrile by vortex-mixing for 2 min, transferred to injection vials, and 5 μL were injected onto LC-MS/MS system.

LC-MS/MS analysis

For LC-MS/MS analysis, the chromatographic system consisted of a Nanospace SI-2 pump, a SI-2 autosampler and a S-MC system controller (Shiseido, Tokyo, Japan). The separation was performed on a Luna C_8 column (3 μm, 2 mm i.d.×100 mm, Phenomenex, Torrance, CA, USA) using a mixture of acetonitrile-ammonium formate (10 mM, pH 6.5) (50:50, v/v) at a flow rate of 0.2 mL/min. The column and autosampler tray temperature were 30°C and 4°C, respectively. The analytical run time was 3.5 min. The eluent was introduced directly onto the tandem quadrupole mass spectrometer (Quattro LC, Micromass UK Ltd, UK) through the negative ionization electrospray interface. The ion source and desolvation temperature were held at 120°C and 350°C, respectively. The optimum cone voltages for ionization of LSB and HPC were 45 V and 38 V, respectively. Multiple-reaction-monitoring (MRM) mode using specific precursor/product ion transitions was employed for the quantification. The molecular ions of LSB and HPC were fragmented at collision energy of 20 eV and 35 eV using argon as collision gas. Detection of the ions was performed by monitoring the transitions of *m/z* 717 to *m/z* 519 for LSB and *m/z* 237 to *m/z* 117 for HPC. Peak areas for all components were automatically integrated using MassLynx Version 3.5 (Micromass UK Ltd, UK).

Method validation

Batches, consisting of triplicate calibration standards at each concentration, were analyzed on three different days to complete the method validation. In each batch, QC samples at 15.0, 75.0, and 400 ng/mL were assayed in sets of six replicates to evaluate the intra- and inter-day precision and accuracy. The percentage deviation of the mean from true values, expressed as relative error (RE), and the coefficient of variation (CV) serve as the measure of accuracy and precision. The lower limit of quantitation (LLOQ) was set at a level where the following criteria were met: signal-to-noise ratio ≥ 5.0 with RE ≤ ±20% and CV ≤ 20%.

The absolute recoveries of LSB were determined by comparing the peak area of six extracted samples at the concentrations of 15.0, 75.0, and 400 ng/mL with the mean peak area of recovery standards. Three replicates of each of the recovery standards were prepared by adding LSB and internal standard to blank rat serum extracts.

Animal study

The developed LC-MS/MS method was used in a pharmacokinetic disposition study after i.v. injection of LSB to male Sprague-Dawley rats (7-8 weeks of age, body weight 225±15 g, Biogenomics, Seoul, Korea). Animals were kept in plastic cages with free access to standard rat diet (Biogenomics, Seoul, Korea) and water. The animals were maintained at a temperature of 22-24 °C with a 12 h light/dark cycle and relative humidity of 50±10%. The rats were anesthetized by ether and cannulated with polyethylene tubing (0.58 mm i.d. and 0.96 mm o.d., Natume CO., Tokyo, Japan) in the left femoral and right jugular veins. After a 1-day recovery period, LSB dissolved in saline was injected intravenously at a dose of 10 mg/kg into the left femoral vein of the rats (n=4). Venous blood samples sere collected at 2, 5, 10, 15, 30 min, and 1, 2, 4, 6, 8, and 10 h after i.v. injection. Blood samples were immediately centrifuged at 3000×g for 5 min and harvested serum samples were stored at -20°C until analysis. The volume of the serum samples used in the analysis was 50 μL.

RESULTS AND DISCUSSION

LC-MS/MS

The electrospray ionization of LSB and HPC produced the abundant pseudomolecular ions ([M-H]$^-$) at *m/z* 717 and 237, respectively under negative ionization conditions, without any evidence of fragmentation. [M-H]$^-$ ions from LSB and HPC were selected as the precursor

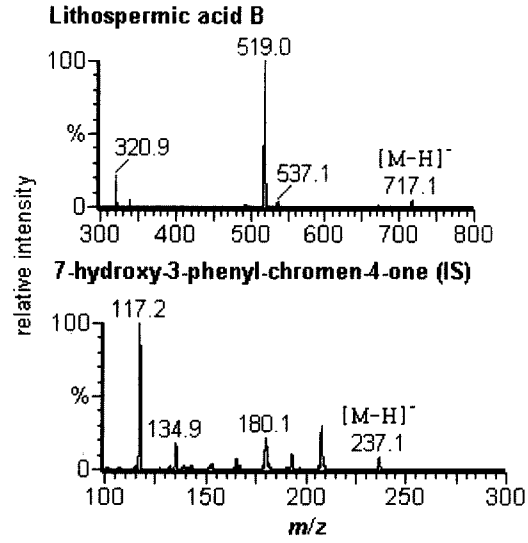


Fig. 2. Product ion mass spectra of (a) lithospermic acid B and (b) HPC (internal standard)

ion and subsequently fragmented in MS/MS mode to obtain the product ion spectra yielding useful structural information (Fig. 2). The fragment ions at *m/z* 519 [the loss of 1-carboxy-2-(3,4dihydroxy-phenyl)-ethoxy group from [M-H]$^-$ ion] and *m/z* 117 (the loss of 7-hydroxyphenyl and ketone group from [M-H]$^-$ ion) were produced as the prominent product ions for LSB and HPC, respectively. The quantification of the analytes was performed using the MRM mode due to the high selectivity and sensitivity of MRM data acquisitions, where the precursor and

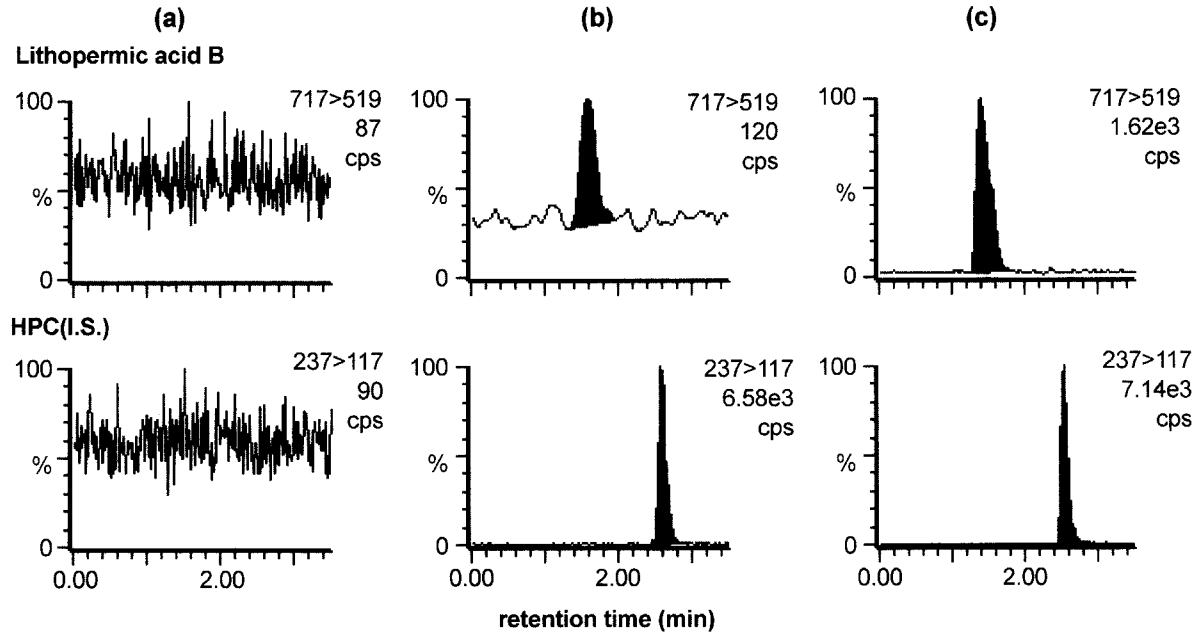


Fig. 3. MRM LC-MS/MS chromatograms of (a) a blank rat serum, (b) rat serum sample spiked with 10.0 ng/mL of lithospermic acid B and (c) a rat serum sample obtained 120 min after i.v. injection of lithospermic acid B (10 mg/kg).

Table I. Calculated concentrations of lithospermic acid B in calibration standards prepared in rat serum (n = 9)

statistical variable	theoretical concentration (ng/mL)						slope	r^2
	10.0	20.0	50.0	100	200	500		
Mean (ng/mL)	11.7	21.8	51.3	96.4	209	510	0.00102	0.997
CV (%)	4.5	8.4	5.4	7.7	7.1	6.8	12.7	
RE (%)	17.0	9.0	2.6	-3.6	4.5	2.0		

product ions are monitored. Two pairs of MRM transitions were selected: *m/z* 717→519 for LSB and *m/z* 237→117 for HPC (internal standard).

Fig. 3 shows the representative MRM chromatograms obtained from the analysis of blank rat serum, rat serum sample spiked with 10.0 ng/mL of LSB and a rat serum sample obtained 120 min after i.v. injection of LSB (10 mg/kg). The analysis of blank rat serum samples from ten different sources did not show any interference at the retention times of LSB (1.5 min) and HPC (2.5 min) (Fig. 3a), confirming the specificity of the present method.

Method validation

Calibration curves were obtained over the concentration range of 10 to 500 ng/mL of LSB in rat serum (r^2 = 0.997). Linear regression analysis with a weighting of 1/concentration gave the optimum accuracy of the corresponding calculated concentrations at each level (Table I). The low CV value for the slope indicated the repeatability of the method (Table I).

Table II shows a summary of intra- and inter-batch precision and accuracy data for QC samples containing LSB. Both intra-and inter-assay CV values ranged from 1.1 to 6.2 % at three QC levels. The intra- and inter-assay RE values for LSB were -10.3 to -2.7% at three QC levels. These results indicated that the present method has an acceptable accuracy and precision.

The lower limit of quantitation (LLOQ) was set at 10 ng/mL for LSB using 50 μL of rat serum. Representative chromatogram of an LLOQ is shown in Fig. 3b and the signal-to-noise ratio for LSB is about 8 at 1.00 ng/mL. CV and RE at the LLOQ level were 6.5% and 8.0%, respectively (Table II).

The extraction recoveries of LSB from spiked rat serum were determined at the concentrations of 15.0, 75.0 and 400 ng/mL in six replicates. The recoveries of LSB ranged from 73.2 to 79.5%, with that of HPC (internal standard) being 75.1±6.0% (Table III). The one-step liquid-liquid extraction with methyl-*tert*-butyl-ether at pH 2 has been successfully applied to the extraction of LSB from rat serum.

Application of method

This method has been successfully used to the pharmacokinetic study of LSB in rats. After i.v. injection of 10 mg/kg of LSB, average serum concentration-time curve declined in a polyexponential fashion (Fig. 4). The pharmacokinetic parameters of LSB were determined by non-

Table II. Precision and accuracy of lithospermic acid B in quality control samples

	Intra-batch (n=6)				Inter-batch (n=18)		
QC (ng/mL)	10.0	15.0	75.0	400	15.0	75.0	400
Mean (ng/mL)	10.8	14.5	67.3	368	14.6	69.5	384
CV (%)	6.5	1.6	5.5	6.2	1.1	4.8	2.7
RE (%)	8.0	-3.3	-10.3	-8.0	-2.7	-7.3	-4.0

Table III. Absolute recoveries of lithospermic acid B and 7-hydroxy-3-phenyl-chromen-4-one (internal standard) from spiked rat serum

Concentration (ng/mL)	Recovery (%, mean ± SD, n=6)	
	lithospermic acid B	7-hydroxy-3-phenyl-chromen-4-one (IS)
15.0	73.2 ± 8.5	–
75	79.5 ± 3.9	–
400	73.5 ± 4.0	–
20	-	75.1 ± 6.0

– : Not assayed

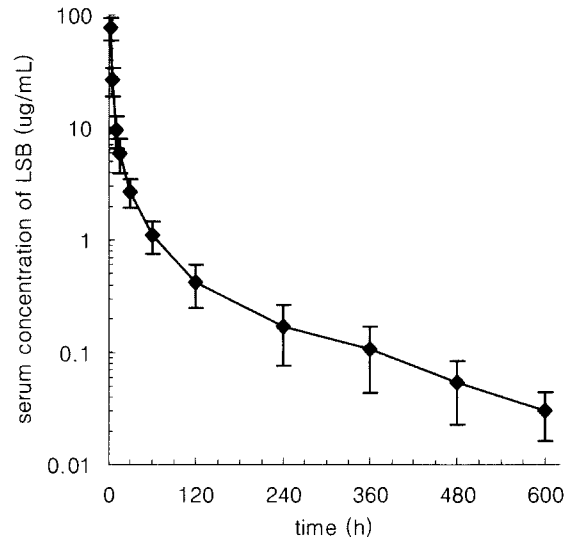


Fig. 4. Mean serum concentration-time plot of lithospermic acid B after an i.v. injection of lithospermic acid B (10 mg/kg) to four male rats. Each point represents the mean ± S.D.

compartmental analysis (WinNonlin Scientific Consultants, NC, USA). LSB exhibited a high distribution volume (V_z, 3627±2932 mL/kg), and a moderate systemic clearance (*Cl*, 13.1±4.3 mL/kg/min) and apparent elimination half-life ($t_{1/2lz}$, 2.4±1.2 h). The area under the plasma concentration-time curve (AUC) for LSB calculated using the trapezoidal rule extrapolated to infinite time was 763 ± 199 μg·min/mL.

In summary, a sensitive and reliable LC-MS/MS method for the analysis of LSB in rat serum has been successfully developed and validated. To extract LSB in the serum, a liquid-liquid extraction with methyl-*tert*-butyl-ether after acidic treatment to pH 2 was used. This assay method demonstrated the acceptable sensitivity, precision, accuracy, selectivity and recovery with a small sample volume (50 μL) and a relatively short analysis time. This method was successfully applied to a pharmacokinetic study of LSB in rats, where limited sample volumes were available.

ACKNOWLEDGEMENT

This research was supported by a grant (PF 0320401-00) from Plant Diversity Research Center of 21st Century Frontier Research Program funded by Ministry of Science and Technology of Korean government and Spela Ltd.

REFERENCES

Abd-Elazem, I. S., Chen, H. S., Bates, R. B., and Huang, R. C., Isolation of two highly potent and non-toxic inhibitors of human immunodeficiency virus type 1 (HIV-1) integrase from *Salvia miltiorrhiza*. *Antiviral Res.*, 55, 91-106 (2002).

Hase, K., Kasimu, R., Basnet, P., Kadota, S., and Namba, T., Preventive effect of lithospermate B from *Salvia miltiorhiza* on experimental hepatitis induced by carbon tetrachloride or D-galactosamine/lipopolysaccharide. *Planta Med.*, 63, pp. 2226 (1997).

Kamata, K., Iizuka, T., Nagai, M., and Kasuya, Y., Endothelium-dependent vasodilator effects of the extract from *Salvia miltiorrhizae* radix. A study on the identification of lithospermic acid B in the extracts. *General Pharmacol.*, 24, 977-981 (1993).

Kamata, K., Noguchi, M., and Nagai, M., Hypotensive effects of lithospermic acid B isolated from the extract of *Salvia miltiorrhiza* Radix in the rat. *General Pharmacol.*, 25, 69-73 (1994).

Kang, D. G., Oh, H., Sohn, E. J., Hur, T. Y., Lee, K. C., Kim, K. J., Kim, T. Y., and Lee, H. S., Lithospermic acid B isolated from *Salvia miltiorrhiza* ameliorates ischemia/reperfusion-induced renal injury in rats. *Life Sci.*, 75, 1801-1816 (2004).

Shigematsu, T., Tajima, S., Nishikawa, T., Murad, S., Pinnell, S. R., and Nishioka, I., Inhibition of collagen hydroxylation by lithospermic acid B magnesium salt, a novel compound isolated from *Salvia miltiorrhizae* Radix. *Biochem. Biophys. Acta*, 1200, 79-83 (1994).

Yokozawa, T., Chung, H. Y., Lee, T. W., Oura, H., Nonaka, G., and Nishioka, I., Potentiating effect of converting enzyme inhibitor captopril to the renal responses of magnesium lithospermate B in rats with adenine-induced renal failure. *Chem. Pharm. Bull.*, 39, 732-736 (1991).

Yokozawa, T., Dong, E., Liu, Z.W., Shibata, T., Hasegawa, M., Watanabe, H., and Oura, H., Magnesium lithospermate B ameliorates cephaloridine-induced renal injury. *Exp. Toxicol. Pathol.*, 49, 337-341 (1997).

Zupko, I., Hohmann, J., Redei, D., Falkay, G., Janicsak, G., and Mathe, I., Antioxidant activity of leaves of Salvia species in enzyme-dependent and enzyme-independent systems of lipid peroxidation and their phenolic constituents. *Planta Med.*, 67, 366-368 (2001).