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Short communication

Simultaneous determination of lithospermic acid B and its three metabolites by liquid chromatography/tandem mass spectrometry

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Abstract

A rapid and sensitive method based on liquid chromatography/tandem mass spectrometry (LC/MS/MS) was developed for the simultaneous determination of lithospermic acid B and its three main *O*-methylated metabolites in rat serum with silibinin as the internal standard. The calibration curves for LSB, and the three metabolites were linear over the ranges of 16–4096, and 8–2048 ng/ml, respectively, with coefficients of correlation >0.998. For LSB, the intra-assay coefficient of variance (CV) was less than 9.3% and the inter-assay CV was less than 8.9%. The inter-assay mean accuracy was between 92.8% and 104.7%. For the three metabolites, the intra-assay CV was less than 8.7% and the inter-assay CV was less than 9.9%. The inter-assay mean accuracy was between 92.5% and 107.9%. This quantitation method was successfully applied to a pharmacokinetic study of LSB in rats. Also, a total recovery of 5.2%was found in bile after oral administration.

Keywords: Lithospermic acid B; Metabolites; LC/MS/MS; Pharmacokinetic

1. Introduction

The traditional Chinese medicine danshen (the roots of *Salvia miltiorrhiza*) is believed to be effective in cardiovascular diseases. In recent years, the water-soluble components in danshen, especially the most common and abundant component, magnesium lithospermate B (MLB), the magnesium salt of lithospermic acid B (LSB), have been widely studied for its pharmacological activities [1,2]. Recent pharmacological studies have indicated that MLB (LSB) protects against liver damage, improves blood circulation and renal function [3–6]. As a naturally occurring polyphenol, it also has antioxidative and free radical scavenging activities [7–9]. As a result, there is great interest in the therapeutic potential of MLB (LSB) in modern society.

Earlier publications have described methods for analysis of MLB (LSB) in biological samples using high-performance liquid chromatography with UV-detection or ECD-detection or MS/MS-detection [10–12]. Zhang et al. have reported studies of metabolites of LSB in rat bile using HPLC-UV detection with the runtime of 60 min [13]. In this paper we first present a rapid and simple LC/MS/MS method for the simultaneous analysis of LSB and its three main *O*-methylated metabolites in serum, and report on a study of the intravenous administration and oral absorption in rats.

2. Experimental

2.1. Materials and chemicals

LSB (purity > 99.4%) and the internal standard silibinin (purity > 98.0%) (Fig. 1) were provided by the Department of Phytochemistry, Shanghai Institute of Materia Medica. The purity of the three *meta*-*O*-methylated metabolites M1–M3 (Fig. 1), was 98%, 95%, and 94%, respectively, verified by the supplier using HPLC methods. All other reagents were of analytical grade and used as received.

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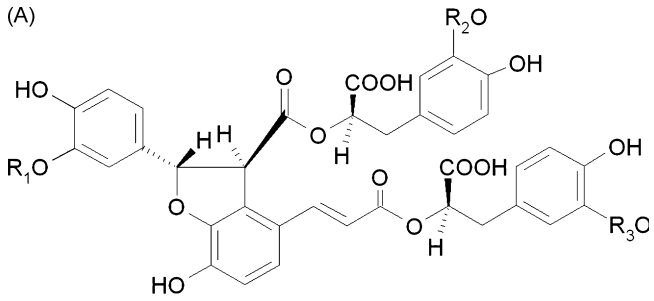
(A)



Compound	M.W.	R1	R2	R3
LSB	718	H	H	H
M1	732	CH3	H	H
M2	746	CH3	CH3	H
M3	760	CH3	CH3	CH3

(B)

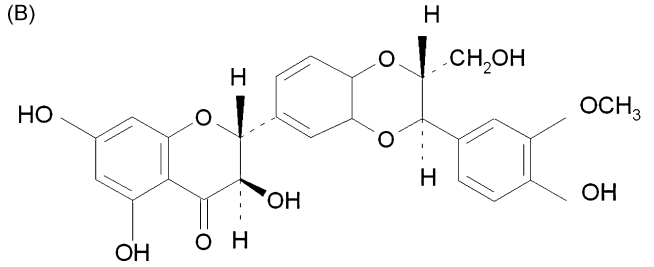


Fig. 1. Chemical structures of LSB, M1–M3 (A) and the IS silibinin (B).

2.2. Protocol for blood sample preparation

A serum sample (100 μl), together with 10 μl of the IS solution (1 μg/ml of silibinin) was put into a 2 ml polypropylene test tube, mixed with 0.2 ml of acetone/water/formic acid (70:28:2, v/v/v) solution, followed by the addition of 1 ml ethyl acetate to each tube. Vortex was used to mix the tubes for 3 min, followed by centrifugation at 16,000 × *g* for 2 min. After transferring organic layer to a clean test tube and drying under a flow of nitrogen gas at 30 °C, The residue was reconstituted in 100 μl of mobile phase. After centrifugation at 16,000 × *g* for 2 min, a volume of 10 μl of the supernatant was introduced into the LC/MS/MS system.

2.3. Instrumentation

The HPLC system utilized a LC-10ADvp HPLC pump, and a SIL-HTc system controller and autosampler (Shimadzu, Kyoto, Japan). The API-3000 triple-quadrupole mass spectrometer (Sciex, Concord, Ont., Canada) was equipped with an ESI source and the whole machine was controlled by Analyst™ 1.3 software (Perkin-Elmer Sciex Instruments). A 5 μm CAPCELL PAK C18 column (100 mm × 2 mm, Shiseido, Japan) was used at room temperature to separate the samples with a Phenomenex C18 guard column as percolating column. The samples were kept at 4 °C in the autosampler before injecting into the HPLC column. The mobile phase comprised 60% (v/v) water (containing 0.5% (v/v) formic acid) and 40% (v/v) acetonitrile and pumped at a flow of 0.25 ml/min for an isocratic elution. The mass spectrometer was operated in the negative mode. Quantification was performed using multiple reaction monitoring (MRM) of the transitions of *m/z* 717 → 519 for LSB, *m/z* 731 → 533 for M1, *m/z* 745 → 547 for M2, *m/z* 759 → 547 for M3, and *m/z* 481 → 301 for silibinin, respectively.

2.4. Construction of standard curves and quality control (QC) samples

Standard stock solutions of LSB, M1–M3, and silibinin prepared in methanol at 1.0 mg/ml, were serially diluted with water to be working solutions. Blank serum of 90 μl was mixed with 10 μl of the working solutions, 10 μl of the IS working solutions (silibinin, 1 μg/ml). The resultant concentrations were 4096, 2048, 1024, 512, 256, 128, 64, and 16 ng/ml for LSB, and 2048, 1024, 512, 256, 128, 64, 32, and 8 ng/ml for M1–M3. The calibration of serum samples was extracted and prepared under the same conditions as the test samples. The nominal serum concentrations of QC samples were 32, 1600 and 3200 ng/ml for LSB, and 16, 800 and 1600 ng/ml for every metabolite.

2.5. Validation procedures

Validation was tested through intra- and inter-day accuracy and precision of the method on quality control (QC) samples. Intra-day variability was detected on six different rat QC samples through the same calibration curve, and the calibration curves were made at four times in different days to test inter-day variability. The precision of the method was performed through the coefficient of variation (CV), the ratio of the standard deviation to the mean calculated concentration, while the accuracy of the assay was determined by the ratio of the mean to the true value. The lower limit of quantification (LLOQ) was defined as the lowest calibrator with an inter-day coefficient of variation, usually below 20%.

The absolute recoveries at three different serum concentrations of LSB, M1–M3, and internal standard, were determined by comparing the peak areas of analyte or internal standard obtained from samples through the complete extraction procedure with those from the same amount of compounds but injecting directly. Correspondingly, extracted QC samples were placed in the autosampler (4 °C) and analyzed at times 0, 2, 4, 6 and 12 h to test the stability. Also, the matrix effect was studied by analyzing standards of every analyte injected directly in mobile phase, and standards spiked into extracts of blank serum.

2.6. Application to pharmacokinetic study

The animals were maintained in accordance with the Guidelines for Care and Use of Laboratory Animals at Shanghai Institute of Materia Medica, Chinese Academy of Sciences. The LC/MS/MS procedure was used to investigate the serum concentration–time profiles of LSB and its main metabolites after an intravenous infusion of 50 mg/kg LSB to six rats.

Blood (0.3 ml) was sampled at 0.08, 0.17, 0.33, 0.5, 0.75, 1, 1.5, 2, 3, 5, and 8 h after administration. Centrifugation at $3000 \times g$ for 10 min (Biofuge pico, Heraeus, Germany) was used to distill serum from blood. All serum samples were frozen and stored at −80 °C until analysis. Samples tested above LSB of 4096 ng/ml were diluted with blank serum, and then re-analyzed.

For oral bioavailability study, bile in additional six rats was collected after oral dosing of 150 mg/kg LSB. Bile fistulas in rats were cannulated with PE-10 polyethylene tubing. The bile was collected into successive vials on ice at 0–2, 2–6, and 6–12 h after dosing and stored at −80 °C until analysis. The bile samples were diluted with water and extracted for analysis under the same conditions as the serum samples. Statistical analysis was performed using statistical program SPSS. All data were expressed as mean ± S.D.

3. Result and discussion

3.1. LC/MS/MS detection

The representative MRM chromatography obtained for blank serum and sample serum spiked with LSB, M1–M3, and silibinin was shown in Fig. 2(A) and (B). Formic acid in the mobile phase was used to sharpen the peak and result good signal-to-noise ratios. LSB, M1–M3, and silibinin had short retention times about 2.0, 2.2, 2.6, 3.1, and 2.9 min, respectively. Thus 4.5 min was enough time to analyse the entire sample.

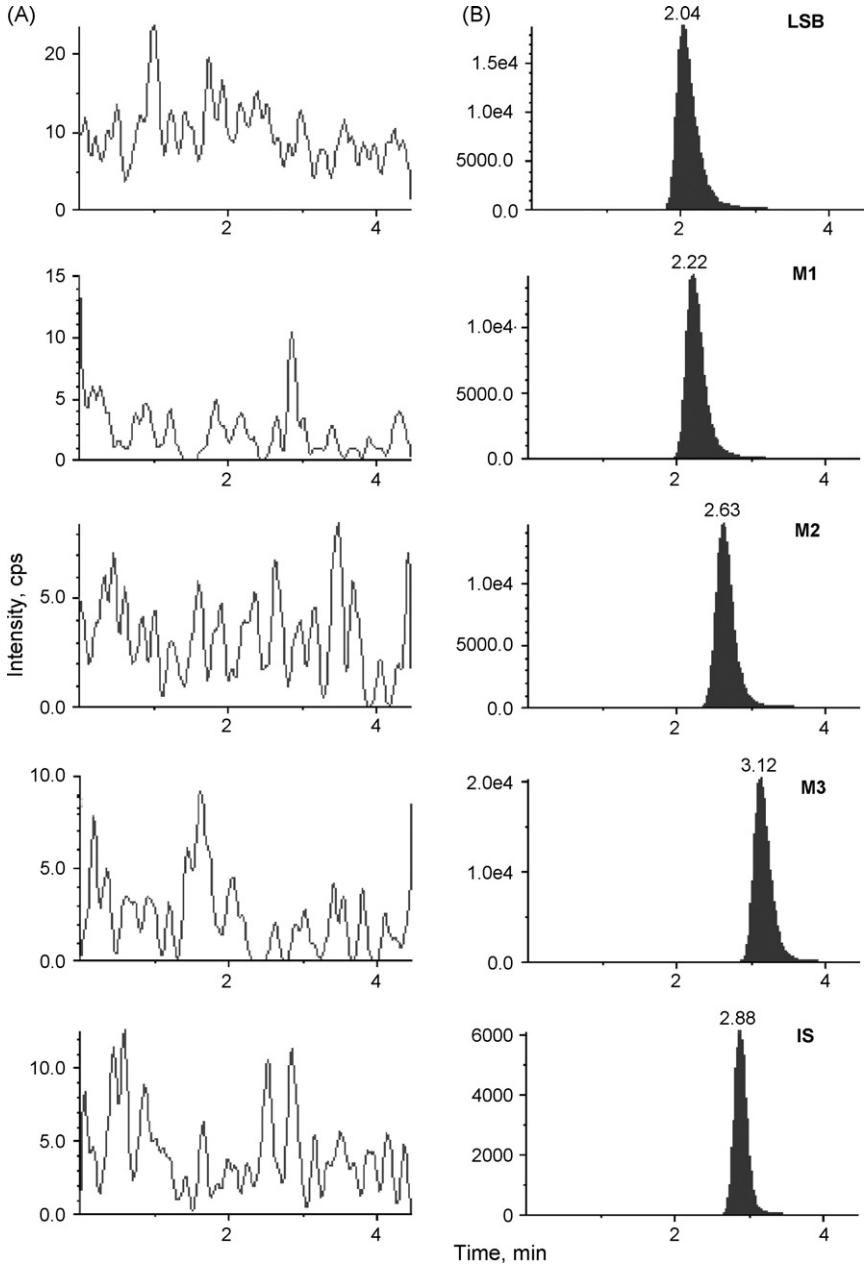


Fig. 2. Chromatograms for LSB, M1–M3 and IS in rat serum. (A) Blank serum sample; (B) serum sample spiked with LSB (512 ng/ml), M1 (256 ng/ml), M2 (256 ng/ml), M3 (256 ng/ml) and IS (100 ng/ml).

Table 1
Statistical calculation of calibration standards for assay linearity validation for LSB, M1–M3 in rat serum ($n = 4$)

Standard level	1	2	3	4	5	6	7	8
LSB								
Theoretical concentration (ng/ml)	16	64	128	256	512	1024	2048	4096
Calculated concentration (ng/ml)	17.6	65.3	116.1	233.4	493.0	1052.2	2052.9	4114.2
CV (%)	1.6	4.1	7.2	3.2	3.8	4.0	2.7	5.8
Accuracy (%)	110.2	102.0	90.7	91.2	96.3	102.8	100.2	100.4
M1								
Theoretical concentration (ng/ml)	8	32	64	128	256	512	1024	2048
Calculated concentration (ng/ml)	8.9	30.6	58.8	118.2	242.8	517.3	1017.1	2077.3
CV (%)	4.4	2.4	7.0	0.5	5.8	4.5	2.9	6.7
Accuracy(%)	110.9	95.8	91.8	92.4	94.8	101.0	99.3	101.4
M2								
Theoretical concentration (ng/ml)	8	32	64	128	256	512	1024	2048
Calculated concentration (ng/ml)	8.6	31.2	64.7	127.7	244.6	515.4	984.7	2095.2
CV (%)	3.0	7.3	3.8	0.3	3.3	3.8	2.3	2.2
Accuracy (%)	107.0	97.4	101.1	99.8	95.5	100.7	96.2	102.3
M3								
Theoretical concentration (ng/ml)	8	32	64	128	256	512	1024	2048
Calculated concentration (ng/ml)	7.4	32.7	69.9	135.6	259.0	517.7	998.8	2051.7
CV (%)	3.3	2.5	1.9	2.6	2.5	1.8	1.8	4.2
Accuracy (%)	92.9	102.2	109.2	105.9	101.2	101.1	97.5	100.2

3.2. Linearity and LLOQ

The linearity of the calibration curve was evaluated from four consecutively prepared batches. Standard curves exhibited excellent linearity in the range of 16–4096 ng/ml for LSB, and 8–2048 ng/ml for metabolites, and all of the coefficients of correlation (r^2) were more than 0.998. The mean back-calculated concentrations of the standards were between 90.7% and 110.2% of the theoretical concentrations for LSB and between 92.4% and 110.9% for the metabolites (Table 1). The CVs were determined to be $\leq$7.3% for all the analytes. The LLOQ, for which the CVs were just below 20%, were 4, 4, 4, and 4 ng/ml for LSB, M1–M3, respectively.

3.3. Precision and accuracy

The precision and accuracy of the assay were presented in Table 2. For LSB, the intra-day precision (CV) was less than 9.3% for each of the three QC samples; and the accuracy was in the range 98.0–102.0%. The inter-day precision (CV) was less than 8.9% for all the QC samples, and the corresponding accuracy was about 92.8–104.7%. For the three metabolites, the intra-day precision (CV) was less than 8.7% for each of the three QC samples; and the accuracy was in the range 92.5–109.8%. The inter-day precision (CV) was less than 9.9% for all the QC samples, and the corresponding accuracy was about 92.5–107.9%.

Table 2
Accuracy and precision of the LC/MS/MS method in determinations of LSB, M1–M3 in rat serum

Compound	Nominal concentration (ng/ml)	Intra-day ($n = 6$)			Inter-day ($n = 4$)		
		Found concentration (ng/ml)	CV (%)	Accuracy (%)	Found concentration (ng/ml)	CV (%)	Accuracy (%)
LSB	32	31.4	9.3	98.0	30.0	6.0	93.7
	1600	1632.2	7.8	102.0	1674.7	8.9	104.7
	3200	3026.2	6.7	94.6	2970.6	6.4	92.8
M1	16	17.5	6.3	109.3	16.6	8.3	103.9
	800	796.8	8.7	99.6	799.7	5.6	100.0
	1600	1498.2	5.5	93.6	1542.8	9.9	96.4
M2	16	17.3	3.1	1080	16.9	2.6	105.6
	800	788.3	5.8	98.5	760.0	7.6	95.0
	1600	1488.2	2.2	93.0	1518.3	5.9	94.9
M3	16	17.6	7.2	1098	17.3	3.7	107.9
	800	766.0	6.7	95.7	770.2	3.8	96.3
	1600	1479.3	2.6	92.5	1480.3	2.2	92.5

Table 3
Oral recovery of LSB, M1–M3 in rat bile after oral administration of 150 mg/kg LSB

Time after administration (h)	Ratio to LSB administration (%)				
	LSB	M1	M2	M3	Total
0–2	0.29 ± 0.12	1.53 ± 0.99	1.36 ± 0.94	0.37 ± 0.19	3.56 ± 2.23
2–6	0.05 ± 0.01	0.30 ± 0.11	0.56 ± 0.08	0.40 ± 0.08	1.31 ± 0.27
6–12	0.02 ± 0.02	0.08 ± 0.07	0.14 ± 0.09	0.13 ± 0.05	0.38 ± 0.21
0–12	0.36 ± 0.12	1.91 ± 0.99	2.07 ± 0.97	0.91 ± 0.30	5.24 ± 2.36

3.4. Extraction recovery and analyte stability

The extraction recoveries of LSB were in the range of 72–75% at concentrations of 32, 1600, and 3200 ng/ml, and M1–M3 were each in the range of 75–81%, 82–87% and 89–91% at concentrations of 16, 800, and 1600 ng/ml. Correspondingly, the extraction recovery of the IS was about 93%. Stability of LSB, M1–M3 of processed samples was evaluated. The autosampler temperature (4 °C) storage at 2, 4, 6 and 12 h before analysis had little effect on the quantification. Previous report indicated that LSB was unstable at high temperature, so the sample storage at 4 °C was necessary for analysis [14]. The matrix effect was examined by comparing peak areas of standard and internal standard for samples spiked after extraction from serum with those obtained by injecting neat standard and IS directly. The result for LSB, M1–M3 and IS were above 89.5%, 91.5%, 92.3%, 96.9% and 95.0%, respectively, suggesting ion suppression by endogenous components was low.

3.5. Pharmacokinetic studies

After intravenous infusion of 50 mg/kg LSB to six rats, serum concentrations of LSB and its main metabolites were determined by the described LC/MS/MS method. Fig. 3 shows the mean serum concentration–time curves of LSB, M1–M3 after intravenous infusion ($n = 6$).

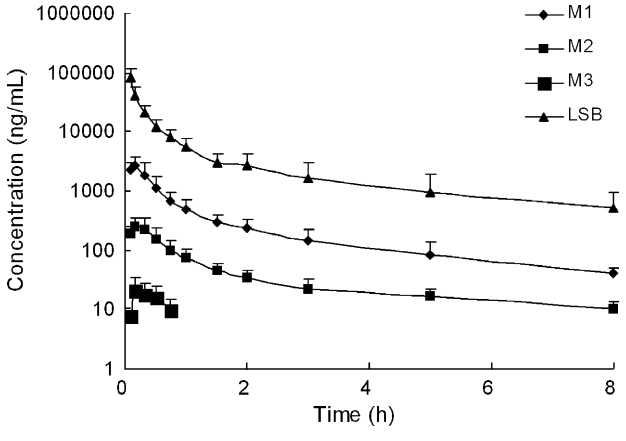


Fig. 3. Profiles of mean serum concentration of LSB, M1–M3 vs. time after intravenous infusion of 50 mg/kg LSB in rats.

For oral bioavailability study, after oral dosing of 150 mg/kg LSB in six rats, the percentage of mean excretion into bile over 12 h for LSB, M1–M3 were 0.36%, 1.91%, 2.07%, and 0.91%, respectively (Table 3). Total recovery in bile after oral administration was 5.24%. Also, Zhang et al. found a considerable amount of 65% after the oral dose of 100 mg/kg LSB in the gastrointestinal tract, indicating poor absorption of LSB from the rat intestine [11]. The extremely low bioavailability in serum also indicated significant first-pass effect of oral administration.

4. Conclusions

The LC/MS/MS-based method described here proved to be with good sensitivity, selectivity, and rapid speed of analysis. It was a validated method to guarantee a reliable determination of LSB and its three main metabolites in rat serum and bile which was then successfully applied to a pharmacokinetic study in rats.

References

[1] R. Kasimu, K. Tanaka, Y. Tezuka, Z.N. Gong, J.X. Li, P. Basnet, T. Namba, S. Kadota, Chem. Pharm. Bull. (Tokyo) 46 (1998) 500–504.
[2] L.N. Li, Pure Appl. Chem. 70 (1998) 547–554.
[3] K. Hase, R. Kasimu, P. Basnet, S. Kadota, T. Namba, Planta Med. 63 (1997) 22–26.
[4] T. Yokozawa, H. Oura, T.W. Lee, G. Nonaka, I. Nishioka, Nephron 57 (1991) 78–83.
[5] T. Yokozawa, T.W. Lee, H. Oura, G. Nonaka, I. Nishioka, Nephron 60 (1992) 460–465.
[6] T. Yokozawa, E. Dong, Z.W. Liu, T. Shibata, M. Hasegawa, H. Watanabe, H. Oura, Exp. Toxicol. Pathol. 49 (1997) 337–341.
[7] K.P. Fung, J. Wu, L.H. Zeng, H.N. Wong, C.M. Lee, P.M. Hon, H.M. Chang, T.W. Wu, Life Sci. 53 (1993) 189–193.
[8] T. Yokozawa, H.Y. Chung, E. Dong, H. Oura, Exp. Toxicol. Pathol. 47 (1995) 341.
[9] X.J. Wu, Y.P. Wang, W. Wang, W.K. Sun, Y.M. Xu, L.J. Xuan, Acta Pharmacol. Sin. 21 (2000) 855–858.
[10] H. Zhang, C. Yu, J.Y. Jia, S.W.S. Leung, Y.L. Siow, R.Y.K. Man, D.Y. Zhu, Acta Pharmacol. Sin. 23 (2002) 1163–1168.
[11] Y. Zhang, T. Akao, N. Nakamura, C.L. Duan, M. Hattori, X.W. Yang, J.X. Liu, Planta Med. 70 (2004) 138–142.
[12] X. Li, C. Yu, W. Sun, G. Liu, J. Jia, Y. Wang, Rapid Commun. Mass Spectrom. 18 (2004) 2878–2882.
[13] Y. Zhang, T. Akao, N. Nakamura, M. Hattori, X.W. Yang, C.L. Duan, J.X. Liu, Drug Metab. Dispos. 32 (2004) 752–757.
[14] Y.X. Guo, Z.L. Xiu, D.J. Zhang, H. Wang, L.X. Wang, H.B. Xiao, J. Pharm. Biomed. Anal. (2006) (Epub).