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Liquid chromatography/tandem mass spectrometry for the determination of magnesium lithospermate B in beagle dog serum

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Abstract

A sensitive and specific isocratic liquid chromatography/tandem mass spectrometry (LC/MS/MS) method was developed for the quantification of magnesium lithospermate B (MLB) in beagle dog serum with silibinin as internal standard. The serum samples were treated by special liquid–liquid extraction, and the analytes were determined using electrospray negative ionization mass spectrometry in the selective monitoring mode, with sufficient sensitivity to allow analysis of dog serum samples generated following administration of a clinically relevant dose. The calibration curve for MLB was linear over the range 8–2048 ng/ml with coefficients of correlation >0.999. The intra- and inter-day precisions (CV) of analysis were <7%, and accuracy ranged from 90 to 106%. This quantitation method was successfully applied to a pharmacokinetic study of i.h. administration of MLB with dosages of 3, 6, 12 mg/kg in beagle dogs.

Keywords: LC/MS/MS; Pharmacokinetics; MLB; Beagle dogs

1. Introduction

Magnesium lithospermate B (MLB, Fig. 1) is a biologically active component isolated from the aqueous extract of danshen [1]. The Chinese traditional medicine, danshen, is the dried root and rhizome of Salvia miltiorrhiza Bge (Labiatae). MLB has been found to have strong antioxidative and free radical scavenging effect [2–5]. Recent pharmacological studies have indicated that MLB protects against renal dysfunction, liver damage, and lung fibrosis [6–12]. As a result, there is great interest in the therapeutic potentials of MLB.

Earlier publications describe methods for analysis of MLB in biological samples using high-performance liquid chromatography with UV-detection [13] or ECD-detection [14]. In this paper we present a selective and simple LC/MS/MS method for analysis of MLB in serum, with the capacity to analyze a wide range of concentrations, and for the study of the pharmacokinetics of MLB in beagle dogs.

2. Experimental

2.1. Materials and reagents

MLB and its corresponding internal standard silibinin (Fig. 1) were provided by Department of Phytochemistry, Shanghai Institute of Materia Medica. The purity of these compounds was above 99.8%, which was verified by the supplier using HPLC methods. Acetonitrile (HPLC grade) was purchased from Fisher (Fair Lawn, NJ, USA). Ethyl acetate, acetone, and formic acid (HPLC grade) were purchased from

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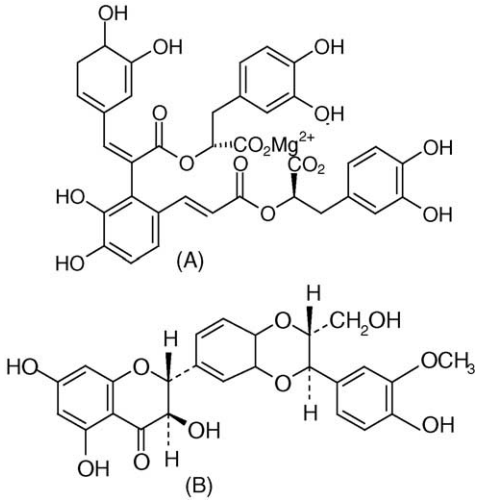

Fig. 1. Chemical structure of MLB (A) and silibinin (B) (internal standard).

Sigma-Aldrich (Germany) and the other reagents were of the highest quality available.

2.2. Preparation of calibration standards and quality control samples

Standard stock solutions of MLB and silibinin were prepared in methanol at 1.0 mg/ml and serially diluted to a concentration of working solution with water. All the stock and working solutions were stored at −20 °C. The working solutions of MLB were prepared from the stock solutions by dilution with water. Blank serum (90 μl) was mixed with 10 μl of the working solutions, 10 μl of the IS working solutions (silibinin, 2 μg/ml), and 16 μl of the 25% formic acid. The resultant serum concentrations were 2048, 1024, 512, 256, 128, 64, 32, 16, 8 ng/ml. The serum samples for calibration were extracted with ethyl acetate and prepared under the same conditions as the test samples. Calibration graphs were constructed using a linear regression of the test compound/IS peak area ratio (Y) to nominal serum concentrations of the test compound (X, ng/ml) with weighting of reciprocal concentration ($1/X$).

2.3. Extraction procedures

The serum sample (100 μl) and 10 μl of the IS solution (2.0 μg/ml of silibinin) were added to a 2 ml polypropylene test tube, mixed with 16 μl formic acid-water (1:3, v/v). Then 0.2 ml of acetone–water (70:30, v/v) solution was added using an Eppendorf repeater pipette, followed by the addition of 1ml ethyl acetate to each tube. Extraction was performed by vortex, mixing the tubes for 10 min at 2500 × g, followed by centrifugation for 2 min at 16,000 × g. The organic layer was transferred to a clean test tube and dried under a flow of nitrogen gas at 30 °C. The residue was reconstituted in 100 μl of water (contained with 25% acetone). After centrifugation at 16000 × g for 2 min, a volume of 10 μl of the supernatant was introduced into the LC/MS/MS system.

2.4. LC/MS/MS analysis

The LC/MS/MS system consisted of an HPLC system including a vacuum degasser, a quaternary pump and an autosampler (Shimadzu, Japan) coupled to Perkin–Elmer SCIEX API-3000 triple-quadrupole mass spectrometer (Sciex, Concord, ON, Canada) equipped with TurboIonSpray source. The LC/MS/MS system was controlled by Analyst™ software. LC separations were performed on a 5-μm CAPCELL PAK C18 column (50 mm × 2 mm i.d., Shiseido, Japan) with a mobile phase consisted of 56% water (containing a mass fraction of 0.5% formic acid) and 44% acetonitrile at a flow of 0.25 ml/min, and a Phenomenex C18 guard column was used before the analytical column. The samples were kept at 4 °C in the autosampler, and 10 μl of sample was injected onto the HPLC column.

A negative TurboIonSpray mode was selected to get higher sensitivity than that in positive ion mode. The operating parameters of the ion source, including the compound-dependent and the source-dependent ones, were optimized to obtain the best performance from the mass spectrometry for the analysis of MLB and silibinin. The source-dependent parameters of MLB and silibinin consisted of the flow rates of the nebulizer gas, the curtain gas, the collision gas, the ionspray voltage, and the temperature of heated gas, with optimum values of 12, 10, 12 l/min, −4500 V, 400 °C, respectively. The compound-dependent parameters were also tuned for the test compound to achieve the highest instrument response (Table 1). The mass spectrometer was operated at low mass resolution for both Q1 and Q3 in multiple reactions monitoring (MRM) mode. The precursor-to product ion transitions of m/z 717.2 → 519.2 for MLB, and m/z 481.0 → 300.9 for silibinin, were monitored.

2.5. Method validation

Four QC samples prepared at nominal concentrations of 8, 10, 600 and 1200 ng/ml of MLB validated the method

Table 1
The compound parameters of MLB and silibinin

Compounds	Q1 m/z (amu)	Q3 m/z (amu)	Time (ms)	Parameter				
				DP (V)	FP (V)	EP (V)	CE (V)	CXP (V)
MLB	717.2	519.2	200	−36	−215	−9	−27	−15
Silibinin(IS)	481.0	300.9	200	−40	−230	−10	−28	−15

DP: declusering potential; FP: focusing potential; EP: entrance potential; CE: collision energy; CXP: collision cell exit potential.

presented here to determine the intra-day and inter-day accuracy and precision. Quantification was based on the ratios of the peak areas of the MLB against that of internal standard. Validation was performed through establishing inter- and intra-day accuracy and precision of the method on quality control (QC) samples. Intra-day variability was tested on six different dogs' serum QC samples using the same calibration curve and inter-day variability was tested on five different days using calibration curves obtained daily. The precision of the method at each QC concentration was expressed as a coefficient of variation (CV) by calculating the standard deviation as a percentage of the mean calculated concentration, while the accuracy of the assay was determined by expressing the percentage of the mean with reference to the true value. The limit of detection (LOD) was defined as the lowest concentration at which the analytical assay can reliably differentiate analyte LC peaks from background levels ($S/N > 3$). The lower limit of quantification (LLOQ) was defined as the lowest calibrator with an inter-day coefficient of variation <20%.

The absolute recoveries at four different serum concentrations of MLB (8, 10, 800, 1600 ng/ml) and internal standard (200 ng/ml), were determined by comparing the peak areas for MLB or internal standard from samples obtained through the complete extraction procedure with those obtained from direct injection of the same amount of the test compounds dissolved in acetone–water (1:3, v/v). Samples spiked with 8, 10, 800, 1600 ng/ml MLB were placed in the autosampler (4 °C) and analyzed at times 0, 2.5, 4, 8 h to test the stability. Freeze-thaw cycle's stability was done after three times of cycles.

2.6. Dog study

The LC/MS/MS procedure developed was used to investigate the serum concentration–time profile of MLB after an i.h. administration of 3, 6, 12 mg/kg MLB to six beagle dogs. Blood (1 ml) was removed by venepuncture prior to dosage and at 0.083, 0.167, 0.25, 0.33, 0.5, 0.75, 1, 1.5, 2, 3, 4, 5, and 6 h thereafter. Blood was processed for serum by centrifugation at 3000 × *g* for 10 min. All serum samples were frozen and stored at −20 °C until analysis. Samples that were found to contain concentrations above 2048 ng/ml were diluted five times with blank serum and then re-analyzed.

The pharmacokinetic parameters were calculated by Drug and Statistics version 1.0 (Anhui, China) program. We used a two-compartment model and a weighting function of 1/CC for data fitting and parameter estimation. All data were expressed as mean ± S.D.

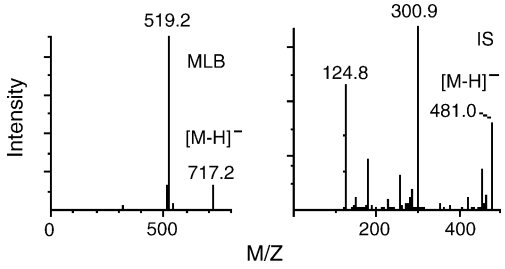

Fig. 2. Product ion mass spectra of $(M–H)^-$ of MLB and silibinin (IS).

3. Result and discussion

3.1. Chromatography and mass spectrometry

MLB and silibinin formed predominantly deprotonated molecules $(M–H)^-$ in the mobile phase containing formic acid using the TurboIon Spray source. The stable product ions for MLB and silibinin were found at *m/z* 519.2 and 300.9, respectively (Fig. 2 displays the MS/MS spectra of MLB and silibinin). The MS/MS parameters were optimized to maximize the response for the MLB precursor/product ion combination of *m/z* 717.2 → 519.2 in the negative ion mode. The isocratic elution was used and a flow rate of 0.25 ml/min was used. Lower formic acid concentrations in the mobile phase resulted in lower signal-to-noise ratios. Very short retention times for both MLB (1.29 min) and silibinin (1.40 min) were found. Consequently, the short run-time of 2.5 min was achieved. There was very little background noise, and a stable baseline was maintained throughout. Fig. 3 shows representative extracted ion chromatograms for blank and sample serum.

3.2. Linearity of calibration curves and lower limit of quantitation

Standard curves exhibited excellent linearity in the range 8–2048 ng/ml, with all coefficients of correlation (r^2) greater than 0.999. the typical regression equation for MLB was: $Y = 0.00385X - 0.0153$ ($r = 0.9994$, X: 8−2048 ng/ml), The CVs were determined to be <8.3% and the recoveries were 92–105%. Table 2 shows the slopes, intercepts, and correlation coefficients obtained for typical calibration curves.

Table 2
Accuracy and precision of the calibration curve in validation method to determine MLB in beagle dog serum. ($n = 4$)

Statistical variable	Theoretical concentration (ng/ml)									Slope	r^2
	8.0	16.0	32.0	64.0	128	256	512	1024	2048		
Mean (ng/ml)	7.4	14.7	33.1	66.5	135	267	516	1016	2034	0.00385	0.999
CV (%)	5.7	8.3	5.9	5.3	5.8	6.1	6.3	3.9	2.3		
RE (%)	−7.5	−8.4	3.3	3.9	5.1	4.1	0.7	−0.8	−0.7		

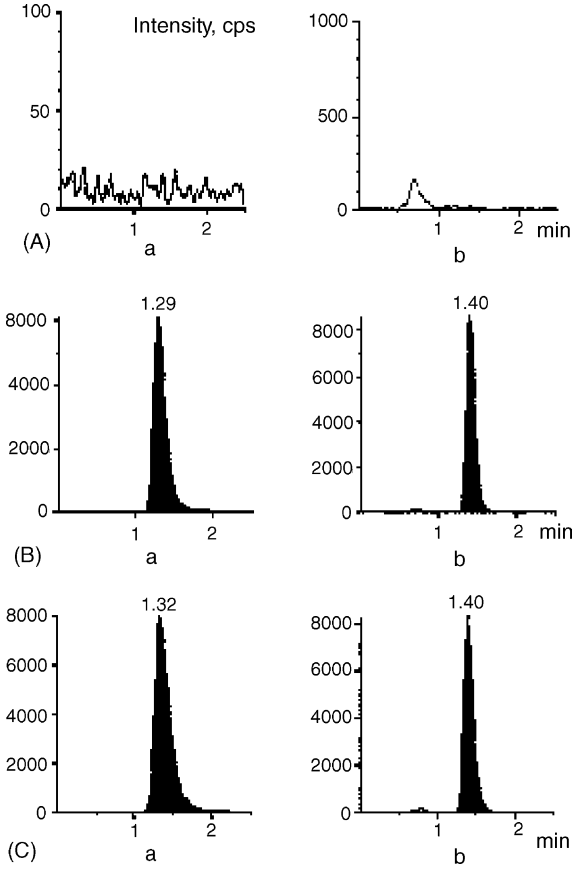

Fig. 3. Chromatograms of MLB and silibinin (IS) in beagle dog serum. (A) Blank serum sample; (B) serum sample spiked with MLB 128 ng/ml and IS; (C) serum sample 1 h after i.h. administration of MLB 6 mg/kg to a beagle dog. Peak a, b refer to MLB and IS, respectively.

The lower limit of detection of the method was 1.5 ng/ml ($S/N > 3$). The lower limit of quantification was 8 ng/ml.

3.3. Precision and accuracy

The resulting assay precision and accuracy data are presented in Table 3. The intra-day precision (CV) of the assay was less than 5.7% for each concentration on three QC samples. Assay accuracy was in the range 90–98%. The inter-day precision (CV) of the assay was less than 6.8% for all the QC samples. Assay accuracy was in the range 100–106%. At this concentration, the intra- and inter-day CVs were determined to be <6.8% and the accuracy was 90–106%.

Table 4
Stability of serum samples ($n = 6$)

Statistical variable	Theoretical concentration (ng/ml)			
	8	10	800	1600
Short-term temperature stability (8 h at 4 °C)				
Mean (ng/ml)	9.1	10.2	750	1448
CV (%)	4.5	6.9	1.8	1.6
RE (%)	13.3	2.2	−6.3	−9.5
Three freeze and thaw stability				
Mean (ng/ml)	8.8	9.7	758	1462
CV (%)	7.9	2.8	3.6	1.4
RE (%)	9.8	−3.2	−5.2	−8.6

3.4. Extraction recovery and analyte stability

The extraction recoveries of MLB were determined by comparing the peak area of each analyte in serum samples that had been spiked with the analyte prior to extraction, with those for samples to which the analyte had been added post-extraction. The results showed that the extraction recoveries of MLB were in the range of 68–75% at concentrations of 8, 10, 800, and 1600 ng/ml, respectively. Formic acid was essential in the extraction process to get a high extraction recovery. The extraction recovery of the IS was 74.2%. Acetone–water (1:3, v/v) was used to reconstitute the analytes, which make them stable enough in a long testing time. The stability of the analytes in serum at autosampler (4 °C) was good, and the Freeze-thaw cycle's stability was excellent, the CVs were determined to be <7.9% and the recoveries were 90–103% (Table 4).

3.5. Pharmacokinetic studies of MLB

After i.h. administration of 3, 6, and 12 mg/kg MLB to 6 beagle dogs, serum concentrations of MLB were determined by the described LC/MS/MS method. Fig. 4 shows mean serum concentration–time curves of MLB after i.h. administration ($n = 6$). The corresponding pharmacokinetic parameters (C_{max}, T_{max}, $T_{1/2}$, AUC_{0-t} and $AUC_{0-\infty}$) are presented in Table 5. After i.h. administration of MLB at the doses of 3, 6, and 12 mg/kg, the C_{max} values for MLB were estimated to be of 1568, 4895, and 6528 ng/ml, respectively. The mean AUC_{0-t} values were 1647, 3756, and 7318 $\mu g\,h\,l^{-1}$, respectively.

Table 3
Accuracy and precision of the LC/MS/MS method to determine MLB in beagle dog serum

Concentration (ng/ml)	Intra-day ($n = 6$)		Inter-day ($n = 5$)	
	CV (%)	Accuracy (relative bias, %)	CV (%)	Accuracy (relative bias, %)
8	5.7	−7.6	6.8	4.1
10	2.9	−2.4	5.9	0.1
800	2.1	−7.6	3.6	3.8
1600	1.1	−9.7	6	6.3

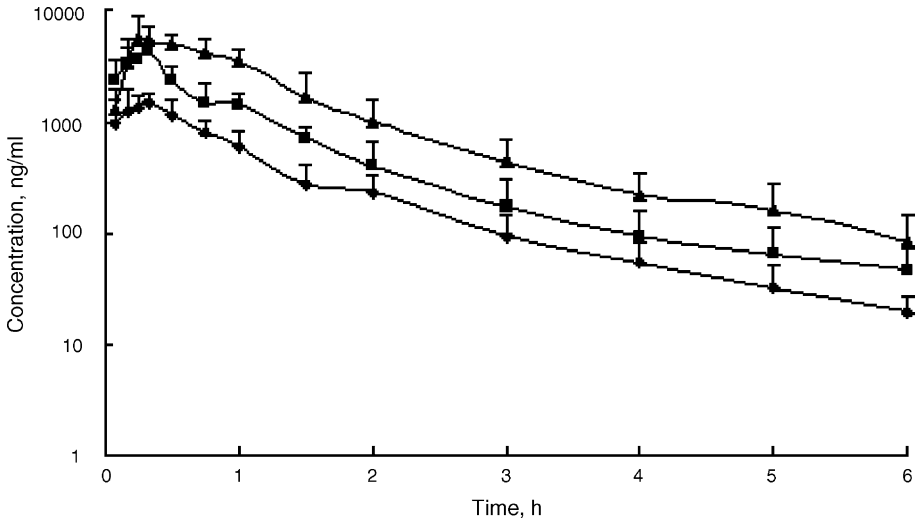

Fig. 4. Profiles of mean serum concentration of MLB vs. time after i.h .administration of MLB to beagle dogs. ((♦), 3 mg/kg; (■), 6 mg/kg; (▲), 12 mg/kg).

Table 5
Pharmacokinetic parameters of MLB after i.h. administration to beagle dogs

Parameter	Dose/mg/kg		
	3	6	12
T_{max} (h)	0.31 ± 0.07	0.29 ± 0.07	0.43 ± 0.30
C_{max} (μg/l)	1568 ± 467	4895 ± 618	6528 ± 2382
AUC $_{(0–tn)}$ (μg/l h)	1647 ± 564	3756 ± 784	7318 ± 2468
AUC $_{(0–\infty)}$ (μg/l h)	1648 ± 564	3759 ± 783	7321 ± 2466
MRT $_{(0–\infty)}$ (h)	1.13 ± 0.07	1.00 ± 0.20	1.22 ± 0.22
k_{10} (1/h)	1.39 ± 0.80	1.27 ± 0.40	1.08 ± 0.56
k_{12} (1/h)	0.26 ± 0.24	0.31 ± 0.29	0.28 ± 0.23
k_{21} (1/h)	0.55 ± 0.39	0.53 ± 0.53	0.75 ± 0.34
V/F (l/kg)	11.19 ± 14.54	7.78 ± 4.02	13.77 ± 20.43
$T_{1/2}$ α (h)	0.44 ± 0.17	0.47 ± 0.18	0.48 ± 0.18
$T_{1/2}$ β (h)	3.31 ± 3.26	2.92 ± 1.74	2.18 ± 2.22
CL/F (l/h/kg)	1.78 ± 1.14	2.17 ± 1.06	3.48 ± 3.17

$n = 6$. Mean ± S.D.

4. Conclusions

The LC/MS/MS-based method described here has been proved to be sensitive, selective and rapid for the determination of MLB in serum samples. The optimized method was validated to guarantee a reliable quantification of MLB in dog serum. It was successfully applied to a pharmacokinetic study of MLB after i.h. administration of 3, 6, and 12 mg/kg MLB.

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