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Pharmacokinetics and metabolism of lithospermic acid by LC/MS/MS in rats

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Abstract

The pharmacokinetics and metabolism of lithospermic acid (LA), a component isolated from *Salvia miltiorrhiza*, and its two *O*-methylated metabolites (3′-monomethyl- and 3′,3″-dimethyl-lithospermic acid), were analyzed by a rapid and specific isocratic liquid chromatography-tandem mass spectrometry (LC/MS/MS) method. Rat serum samples collected after intravenous and oral administration were analyzed for obtaining pharmacokinetic data of LA. Two *O*-methylated metabolites, namely one 3′-monomethyl- and one 3′,3″-dimethyl-lithospermic acid were detected in rat serum and bile samples after intravenous and oral administration of LA, respectively. An oral bioavailability of 1.15% was found, with the AUC_{0-t} values of 301.89 and 3.46 mg h/L for intravenous and oral administration, respectively. The total recovery from bile was 75.36% (0.46% for LA, 17.23% for M1, and 57.67% for M2) after intravenous administration, and 4.26% (0.00% for LA, 0.10% for M1, and 4.16% for M2) after oral administration. These results indicate that methylation is the main metabolic pathway of LA, and that LA is excreted into rat bile and finally into feces.
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Keywords: Lithospermic acid; Pharmacokinetics; Metabolism; Rat; Bile; LC/MS/MS

1. Introduction

Lithospermic acid (LA) is a biologically active component isolated from the aqueous extract of danshen, the dried root and rhizome of *Salvia miltiorrhiza* Bge (Labiatae) (Zhou et al., 1999). LA has also been found as an active water-soluble constituent of Lithospermum (Findley et al., 1985; Yamamoto et al., 2000). Studies have shown that LA has antioxidative and antifibrotic activities in cardiovascular diseases (Li et al., 2007; Ly et al., 2006; Parnham and Kesselring, 1985; Shigematsu et al., 1994), as well as hormone regulatory effects such as inhibition of luteinizing hormone secretion *in vitro* (Chatterton, 1981; Farnsworth et al., 1975; Wagner et al., 1970) and inhibition of endogenous gonadotropic activity in cockerels (Breneman and Zeller, 1975). More recently, LA has attracted considerable interest due to its reported potent and nontoxic anti-HIV activity, which results from inhibition of HIV-1 integrase, an intensively studied therapeutic target for the treatment of AIDS (Abd-Elazem et al., 2002; O'Malley et al., 2005). As a result, several groups have isolated LA to study its biological activity, and there is great interest in the therapeutic potential of LA in modern society.

Early methods for analysis of LA in biological samples typically involved reverse-phase high-performance liquid chromatography with UV-detection (Kelley et al., 1975, 1976). Recently a LC/MS/MS method was reported for the determination of LA and its analogs in dog serum and the study of LA distribution in rats (Li et al., 2004, 2007). However, no previous study has reported the determination of LA and its metabolites in rat serum using this LC/MS/MS-based method.

Here, we report the isolation of two major metabolites from bile samples extracted following intravenous administration of LA to rats. We identified these metabolites as *O*-methylated products, and thereafter used a rapid and simple LC/MS/MS method for the simultaneous analysis of LA and its two main

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O-methylated metabolites in serum and bile. Our findings provide important new insights into the pharmacokinetics and metabolism of LA in rats.

2. Experimental

2.1. Materials and reagents

LA (purity >98.5%) and silibinin (purity >98%, used as internal standard) (Fig. 1) were provided by the Department of Phytochemistry, Shanghai Institute of Materia Medica. The purities of the two meta-*O*-methylated metabolites M1 and M2 (Fig. 1), were 94.1 and 96.8%, respectively, as determined by HPLC methods. HPLC-grade acetonitrile was purchased from Fisher (Fair Lawn, NJ, USA). Ethyl acetate, acetone, and formic acid were purchased from Sigma–Aldrich (Germany). Deionized water was used for the preparation of all solutions. All other reagents were of analytical grade and were used as received.

2.2. Animal studies

Male drug-free (blank) rats (8 weeks old, weighing 240–260 g) were obtained from the Shanghai SLAC Laboratory Animal Inc. (Shanghai, China). The animals were acclimated to standard housing and environmental conditions (22–24 °C, 50% relative humidity, and a 12 h light/dark cycle) for 1 week. They were maintained in accordance with the Guidelines for Care and Use of Laboratory Animals at Shanghai Institute of Materia Medica, Chinese Academy of Sciences. At the end of the experiment, carbon dioxide was used for euthanasia of the animals.

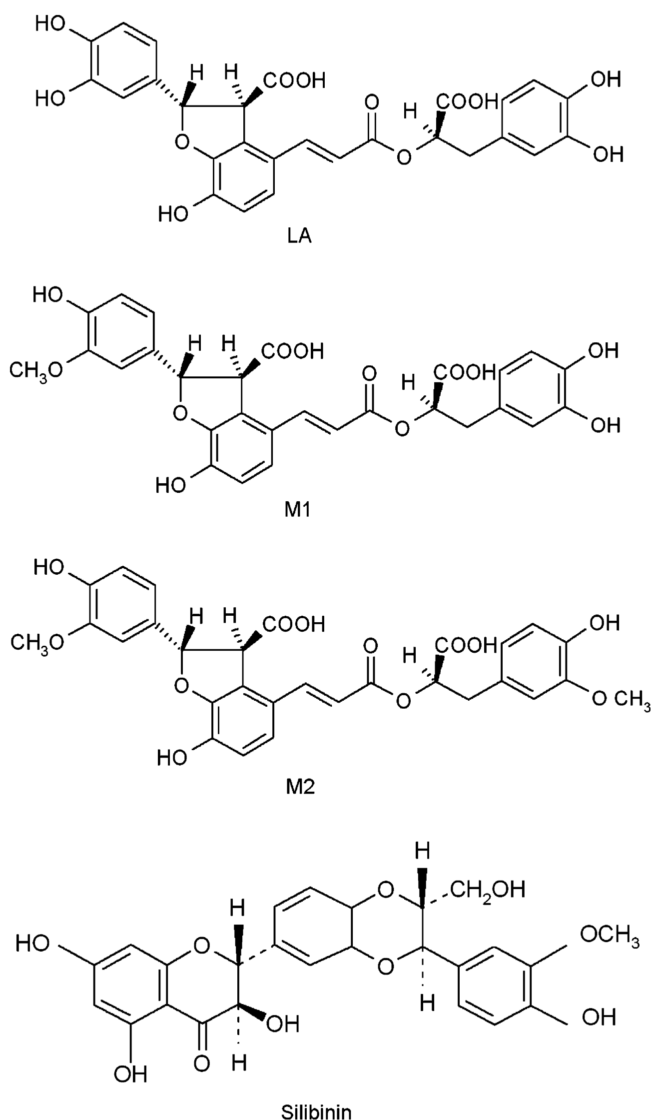

Fig. 1. Chemical structure of LA, M1, M2 and silibinin.

2.3. Isolation of biliary metabolites

Twelve rats received 20 mg/kg LA each by intravenous injection. Bile fistulas were cannulated from each rat using PE-10 polyethylene tubing. Bile was collected after dosing for 12 h. Bile (125 mL) was applied to a Diaion HP-20 column containing a porous polymer resin and aromatic-type absorbents based on a crosslinked polystyrenic matrix (70 mm × 10 mm, Mitsubishi Chemical Industries Co., Japan). Fraction M1 (37.5 mg) was acquired by washing the column with 480 mL of water, and fraction M2 (21.1 mg) was obtained by eluting the column with MeOH–H_2O (1:9; 310 mL). The two fractions obtained from this first-step column chromatography were further purified by application to C-18 reverse-phase column (50 mm × 8 mm; Nacalai Tesque Inc., Japan) followed by elution with MeOH–H_2O (460 mL of 1:19 for M1 and 320 mL with 1:9 for M2). The purities of M1 and M2 were 94.1 and 96.8%, respectively.

M1, pale yellow powder, $[\alpha]_D^{25}$ + 314° (c = 0.035, MeOH). UV (MeOH) λ_{max} (log ε): 312 (4.23), 289 (4.20) 255 nm (4.25); IR (KBr) ν_{max}: 3427, 2962, 1691, 1610, 1379, 1265, 1120, 1032, 810 cm^{-1}; ^{1}H NMR, see Table 1; ^{13}C NMR, see Table 2; ESI-MS (positive ion mode) m/z: 575 $[M+Na]^+$; HR–ESI–MS m/z: 575.1139 (calculated for $[C_{28}H_{24}O_{12}+Na]^+$: 575.1165).

M2, pale yellow powder, $[\alpha]_D^{25}$ + 99° (c = 0.089, MeOH); UV (MeOH) λ_{max} (log ε): 310 (4.05), 287 (4.02) 255 nm (4.08); IR(KBr) ν_{max}: 3404, 2941, 1693, 1605, 1522, 1379, 1265, 1175, 1128, 1034, 814 cm^{-1}; ^{1}H NMR, see Table 1; ^{13}C NMR, Table 2; ESI-MS (positive ion mode) m/z: 589 $[M+Na]^+$; HR–ESI–MS m/z: 589.1332 (calculated for $[C_{29}H_{26}O_{12}+Na]^+$: 589.1322).

2.4. Serum pharmacokinetic study

For the intravenous administration experiment, LA was dissolved in a saline solution containing 0.9% sodium chloride, and administrated to six rats at 20 mg/kg LA. Blood samples (0.3 mL) were collected at 0.05, 0.133, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 5, 8, 12 and 24 h after dosing. Blood samples were stored at 4 °C for 2 h, and serum was obtained by centrifugation at 4000 × g for 10 min. All serum samples were frozen and stored at −80 °C until analysis.

For the oral absorption experiment, six rats were orally dosed with 20 mg/kg LA each, blood samples (0.3 mL) were collected at 0.167, 0.333, 0.5, 0.75, 1, 1.5, 2, 3, 5, 8, 12, and 24 h after dosing, and serum was obtained as described above.

For analysis, 100 μL of serum was mixed with 10 μL of the internal standard (I.S.) solution (4.0 μg/mL of silibinin) in a 2 mL polypropylene test tube, and then 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. Each tube was vortexed for 3 min for extraction, followed by centrifugation for 3 min at 16,000 × g. The organic layer was transferred to a clean test tube and dried under a flow of nitrogen gas at 35 °C. The residue was reconstituted in 100 μL of the mobile phase and the solution was centrifuged at 16,000 × g for 3 min. Ten μL of the supernatant was introduced into the LC/MS/MS system for analysis. In cases where serum samples were found to have high concentrations of LA, the sample was diluted with blank serum to an LA concentration below 4096 ng/mL, and then re-analyzed.

The pharmacokinetic parameters were calculated using the Drug and Statistics version 2.0 software package (Anhui Provincial Center for Drug Clinical Evaluation, China). We used a two-compartment model and a weighing function of $1/C^2$ for data fitting and parameter estimation. All data were expressed as mean ± S.D. The bioavailability of LA after oral administration was calculated based on the AUC_{0-t} values according to the equation ($AUC_{p.o.}/AUC_{i.v.}$) × 100%.

2.5. Total recovery in bile

Prior to experiments, 12 male drug-free rats (body weight 240–260 g) were fasted overnight but with access to water. The rats were anesthetized with diethyl ether, and PE-10 polyethylene tubing was used to cannulate the bile fistulas.

Table 1
^{1}H NMR data for M1, M 2 and LA (400 MHz)

H	M1			M2			LA		
	δ_H (J in Hz)[a]			δ_H (J in Hz)[b]			δ_H (J in Hz)[c,d]		
5	6.81	d	8.4	6.68	d	7.8	6.9	d	8.3
6	7.18	d	8.4	7.06	d	8.2	7.3	d	8.5
7	7.67	d	16	8	d	15.6	7.85	d	16
8	6.28	d	16.2	6.12	d	15.8	6.37	d	15.9
2′	7.02	d	1.8	6.89	brs		6.86	d	2
5′	6.83	d	8.2	6.78	d	7.8	6.82	d	8.1
6’	6.89	dd	2.0, 8.0	6.73	d	8	6.81	dd	1.8, 8.1
7′	5.88	d	5	5.93	d	5.6	5.98	d	4.6
8′	4.23	d	5.2	3.99	d	5.3	4.53	d	4.7
2″	6.98	d	1.9	6.8	brs		6.89	s	
5″	6.76	d	8.3	5.59	d	7.7	6.73	d	8
6″	6.69	dd	1.9, 8.2	5.61	d	7.9	6.67	dd	2.0, 8.1
7″	2.99	dd	8.9, 14.1	2.8	m		3.01	dd	8.6, 14.3
	2.86	dd	2.6, 13.8	–			3.1	dd	4.1, 14.3
				3.1					
8″	4.81[e]			4.88	d	10.1	5.18	dd	4.1, 8.5
3′-OMe	3.76	s		3.77	s				
3″-OMe				3.62	s				

[a] CD_3OD.
[b] DMSO-d_6.
[c] CD_3COCD_3.
[d] O’Malley et al.(2005).
[e] overlapped by the exchangeable D_2O.

Table 2
^{13}C NMR data for M1, M2 and LA (400 MHz)

C	M1 (δ_c[a], m)	M2 (δ_c[b], m)	LA (δ_c[c,d], m)
1	124.9(s)	123.0(s)	123.5(s)
2	130.5(s)	130.6(s)	128.6(s)
3	148.6(s)	146.6(s)	146.8(s)
4	144.0(s)	142.8(s)	144.2(s)
5	117.6(d)	115.6(s)	117.6(d)
6	122.6(d)	119.1(d)	121.4(d)
7	144.2(d)	142.8(d)	142.4(d)
8	117.2(d)	115.8(d)	115.6(d)
9	169.8(s)	166.2(s)	168.3(s)
1′	135.1(s)	133.4(s)	133.3(s)
2′	111.1(d)	110.1(d)	113.4(d)
3′	149.1(s)	147.5(s)	144.2(s)
4′	146.6(s)	146.0(s)	142.4(s)
5′	118.3(d)	115.4(d)	116.3(d)
6′	119.8(d)	118.0(d)	118.1(d)
7′	90.4(d)	88.2(d)	88.9(d)
8′	61.1(d)	59.7(d)	59.0(d)
9′	180.1(s)	173.6(s)	178.8(s)
1″	131.9(s)	130.3(s)	130.3(s)
2″	119.0(d)	113.4(d)	116.9(d)
3″	144.4(s)	147.0(s)	144.0(s)
4″	145.6(s)	144.4(s)	142.7(s)
5″	117.3(d)	115.1(d)	116.3(d)
6″	122.3(d)	121.3(d)	121.6(d)
7″	38.5(t)	37.2(t)	37.2(t)
8″	78.4(d)	75.7(d)	76.6(d)
9″	178.3(s)	172.3(s)	176.9(s)
3′-OMe	57.3(q)	55.7(q)	
3″-OMe		55.3(q)	

[a] CD_3OD.
[b] DMSO-d_6.
[c] D_2O.
[d] Kelley et al. (1976).

Bile was collected into successive vials on ice during 0–2, 2–6, and 6–12 h after a single intravenous ($n = 6$) or oral administration ($n = 6$) of 20 mg/kg LA, respectively, and the obtained samples were stored at −80 °C until analysis. The bile samples were diluted with water, and LA and its metabolites were extracted and analyzed as described above for the serum samples.

2.6. LC/MS/MS analysis

Serum and bile samples were analyzed by LC/MS/MS using a system composed of an HPLC apparatus (Shimadzu, Japan) with a 5-μm CAPCELL PAK C18 column (50 mm × 2 mm; Shiseido, Japan) coupled to a Perkin-Elmer SCIEX API-3000 triple-quadruple mass spectrometer (Applied Biosystems, Foster city, CA, USA) equipped with an electrospray ionization source. The mobile phase for the HPLC column was consisted of 60% water (containing a mass faction of 0.5% formic acid) and 40% acetonitrile, and was pumped at a flow-rate of 0.25 mL/min for isocratic elution. The mass spectrometer was operated at low mass resolution for both Q1 and Q3 in the multiple reactions monitoring (MRM) mode. The turbo source temperature and the ion spray potential were set to 450 °C and −4500 V, respectively. The collision energies were 12, 20, 14, and 28 eV for LA, M1, M2, and ‘I.S.’, respectively.

An internal standard calibration method using matrix-matched standards was used for the quantitative analyses. LA, M1, M2, and silibinin were acquired from Department of Phytochemistry, Shanghai Institute of Materia Medica. Standard and quality control (QC) samples were prepared as previously described (Li et al., 2004). The resultant serum concentrations were 4096, 2048, 1024, 512, 256, 128, 32, and 8 ng/mL each for LA, M1, and M2. QC samples were prepared at nominal concentrations of 64, 1600 and 3200 ng/mL for LA and each metabolite. Quantification was based on the ratios of the peak areas of each compound against that of ‘I.S.’

3. Results and discussion

3.1. LC/MS/MS detection

The ionization and fragmentation patterns of LA, M1, and M2 were studied using electrospray ionization tandem mass spec-

trometry. Fig. 2 shows the MS/MS spectra of LA, M1, M2, and silibinin ('I.S.'). The dominating ions of LA, M1, M2 were charged molecular ions ($[M-H]^-$) at m/z 493, 507, and 522 instead of singly charged ones at m/z 537, 551, and 565, that based on the m/z 481 → 301 transition was specific for 'I.S.'

Short retention times were observed for LA (1.16 min), M1 (1.28 min), M2 (1.50 min) and 'I.S.' (1.76 min), with the end result that the short run-time was only 3 min. Fig. 3 shows representative extracted ion chromatograms for LA, M1, M2, and 'I.S.' There was very little background noise, and a stable baseline was maintained throughout.

3.2. Method validation

The standard curves exhibited excellent linearity over a range of 8–4096 ng/mL for all three analytes, and all of the curves had coefficients of correlation (r) greater than 0.998. The typical regression equation for LA was $Y = 0.00298X + 0.000721$ ($r = 0.9990$, X: 8–4096 ng/mL), that for M1 was $Y = 0.00245X - 0.00565$ ($r = 0.9987$, X: 8–4096 ng/mL), that for M2 was $Y = 0.00392X - 0.00185$ ($r = 0.9994$, X: 8–4096 ng/mL). The CVs were determined to be <9.4% and the recoveries were 92–107%. The lower limit of detection of the method was 1 ng/mL (S/N > 3).

For all concentration QC samples, the intra-and inter-day precision (CV) of the assay was determined to be <11.8% and the accuracy was 87.0–107.6% (Table 3). The recovery was determined at three different serum concentrations and 'I.S.' (400 ng/mL) by comparing the pre- and post-extraction spikes for the QC samples. The results showed that the extraction recoveries of LA, M1, and M2 were in the range of 83–89%. The stability of the analytes in serum at the autosampler temperature (4 °C) was good, and the CVs were determined to be <6.0% and the recoveries were 98–105%. Analysis of matrix effects revealed that no significant signal suppression was observed due to matrix effect during the ionization process.

3.3. Metabolite identification

LA, along with its two metabolites, were detected in rat bile samples. Using Diaion HP-20, the metabolites were purified from pooled bile obtained from rats injected intravenously with

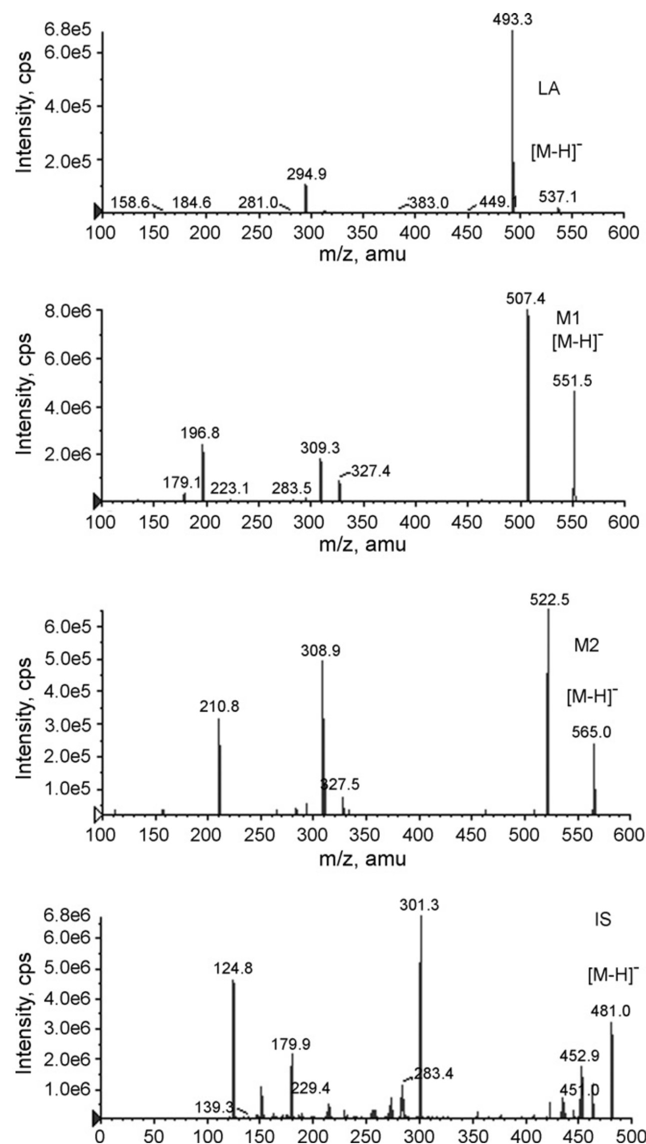

Fig. 2. Product ion mass spectra of $(M-H)^-$ of LA, M1, M2, and silibinin ('I.S.').

Table 3
Accuracy and precision of the LC/MS/MS method for determination of LA, M1, and M2 in rat serum

Compound	Nominal conc. (ng/mL)	Intra-day ($n = 6$)			Inter-day ($n = 5$)		
		Found conc. (ng/mL)	CV (%)	Accuracy (%)	Found conc. (ng/mL)	CV (%)	Accuracy (%)
LA	64	57.87	11.81	90.43	59.52	7.94	93.00
	1600	1489.81	6.11	93.11	1626.49	6.99	101.66
	3200	2989.67	4.95	93.43	3178.49	9.37	99.33
M1	64	55.68	7.24	87.00	55.81	8.32	87.21
	1600	1494.73	5.54	93.42	1650.24	0.56	103.14
	3200	2992.00	4.30	93.50	3166.65	3.86	98.96
M2	64	56.50	7.72	88.28	61.77	1.58	96.52
	1600	1437.99	7.02	89.87	1720.85	1.84	107.55
	3200	2941.54	5.13	91.92	3237.79	6.52	101.18

Fig. 3. Chromatograms of LA, M1, M2 and silibinin ('I.S.') in rat serum. (A) Blank serum sample; (B) Serum sample spiked with LA, M1, M2 and 'I.S.' at 256 ng/mL each; (C) Serum sample 8 h after intravenous administration of 20 mg/kg LA to a rat. Peaks a, b, c, and d represent LA, M1, M2 and 'I.S.', respectively.

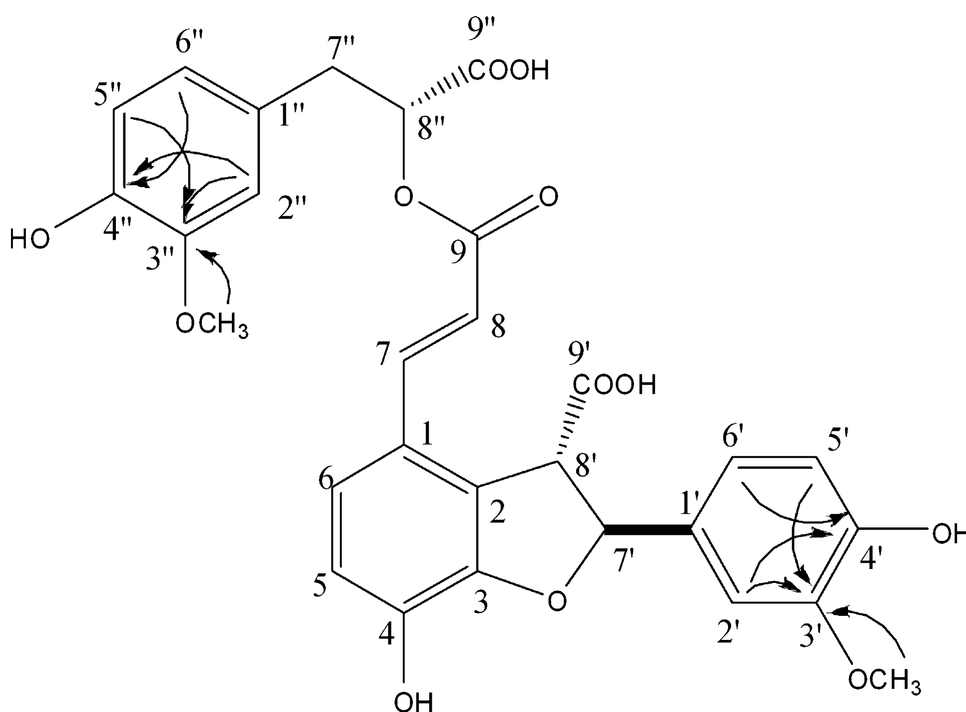

Fig. 4. The selective HMBC correlations of M2.

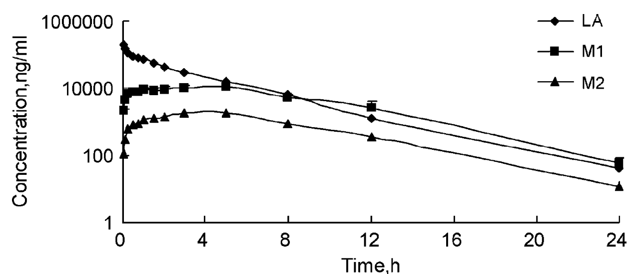

Fig. 5. Profiles of mean serum concentration of LA, M1, and M2 vs. time after intravenous administration of 20 mg/kg LA to rats ($n = 6$).

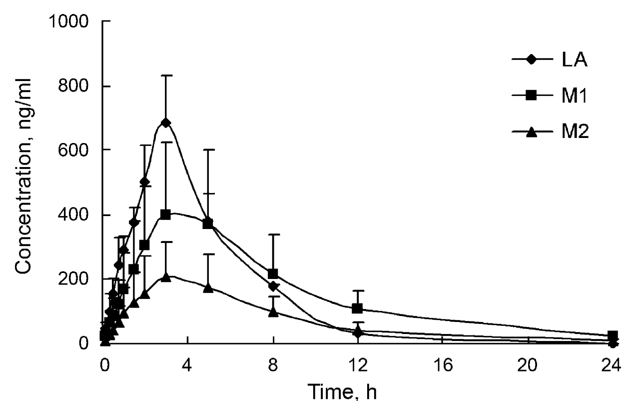

Fig. 6. Profiles of mean serum concentration of LA, M1, and M2 vs. time after oral administration of 20 mg/kg LA to rats ($n = 6$).

LA. The UV and IR spectra of the two metabolites were similar to those of LA, suggesting that they possessed the same functional groups. ESI-MS showed that M1 and M2 had molecular ion peaks at m/z 575 and 589 $[M + Na]^+$, respectively, which were 14 and 28 mass units higher, respectively, than that of LA, indicating that the two metabolites might correspond to monomethyl-, and dimethyl-lithospermic acid, respectively.

The ^{1}H NMR spectra of M1 and M2 showed signals corresponding to one and two methoxyl groups, respectively. Detailed studies including the 2D-NMR spectral analysis showed that these metabolites were identical in structure apart from the differences in their methoxy groups. As shown in Fig. 4, M2 showed long-range correlations between methoxy protons (δ_H 3.77 and 3.62) and phenolic carbons at δ_c 147.5 (C-3′) and 147.0 (C-3″), respectively, on the HMBC spectra. Therefore, the structure of M2 was determined to be 3′,3″-*O*-dimethyl-LA. Similarly, from the long-range correlations between methoxy protons and phenolic carbons (δ_H 3.76 and δc 149.1) on the HMBC spectra, the structure of M1 was identified as 3′-*O*-monomethyl-LA.

Since no glucuronide or sulfate conjugates of LA were found in rat bile samples, we propose that methylation appeares to be the major metabolic pathway of LA in rat.

3.4. Pharmacokinetic study

After intravenous or oral administration of 20 mg/kg LA to rats ($n = 6$ each), the serum concentrations of LA and its main metabolites were determined by the described LC/MS/MS method. Figs. 5 and 6 show mean serum concentration-time curves of LA, M1, and M2 after intravenous and oral administration, respectively. The corresponding pharmacokinetic parameters (C_{max}, T_{max}, $T_{1/2}$, $AUC_{0–t}$, and $AUC_{0–\infty}$) are given in Table 4. Following oral dosing, the serum concentration of M1 and M2 were very low. Comparison of the oral and intravenous administration data allowed us to calculate that the oral bioavailability was fairly low, at only 1.15%.

Table 4
Pharmacokinetic parameters of 20 mg/kg LA after intravenous and oral administration to rats ($n = 6$; mean ± S.D.)

Parameter	Intravenous administration	Oral administration
C_{max} (mg/L)	NA	0.69 ± 0.15
T_{max} (h)	NA	3.00 ± 0.00
$AUC_{0–t}$ (mg h/L)	301.89 ± 57.57	3.46 ± 0.60
$AUC_{0–\infty}$ (mg h/L)	302.02 ± 57.59	3.59 ± 0.64
$MRT_{0–t}$ (h)	2.70 ± 0.21	4.30 ± 0.17
$MRT_{0–\infty}$ (h)	2.71 ± 0.21	4.58 ± 0.24
$T_{1/2\alpha}$ (h)	0.21 ± 0.20	2.67 ± 3.01
$T_{1/2\beta}$ (h)	2.15 ± 0.14	3.08 ± 2.74
F (%)	1.15	

NA = not available.

After intravenous administration of LA (20 mg/kg), the percentages of mean excretion into bile over 12 h for LA, M1, and M2 were 0.46, 17.23, and 57.67%, respectively (Table 5), and total recovery in bile was 75.36%. Notably, low amounts of LA (0.00%), M1 (0.10%), and M2 (4.16%) were recovered in bile following oral administration of 20 mg/kg LA, indicating that LA was only poorly absorbed from the rat intestine and metabolized in liver.

Table 5
Total recovery from bile after intravenous and oral administration

Time (h)	Oral administration				Intravenous administration			
	LA	M1	M2	Total	LA	M1	M2	Total
0–2	0	0	0.80 ± 0.61	0.80 ± 0.62	0.43 ± 0.38	14.32 ± 7.26	29.29 ± 5.22	44.04 ± 9.87
2–6	0	0.02 ± 0.01	2.12 ± 1.67	2.14 ± 1.68	0.02 ± 0.02	2.58 ± 1.58	19.02 ± 1.69	21.62 ± 1.63
6–12	0	0.08 ± 0.13	1.23 ± 1.11	1.32 ± 1.24	0	0.34 ± 0.34	9.36 ± 2.51	9.70 ± 2.68
0–12	0	0.10 ± 0.13	4.16 ± 3.30	4.26 ± 3.44	0.46 ± 0.40	17.23 ± 8.12	57.67 ± 5.23	75.36 ± 7.94

4. Conclusion

LA and its two main *O*-methylated metabolites in rat serum and bile samples were successfully determined by a rapid and sensitive LC/MS/MS assay that may be used for future pharmacokinetic studies. Here, our pharmacokinetic results showed that LA had a low bioavailability of 1.15% after oral administration. Subsequently, only 4.26% of the dose was excreted into bile after oral administration, whereas 75.36% was found in bile after intravenous administration.

Two metabolites of LA were successfully isolated from rat bile, and both were characterized as *meta*-*O*-methylated products by spectroscopic analysis. Thus, our metabolism study indicated that LA was rapidly and extensively metabolized to its methylated metabolites, further suggesting that methylation might be the major metabolic pathways of LA in rats. This unique metabolic fate of LA may be related to its pharmacological activities *in vivo*. Future work will be required to fully elucidate the biological fate of LA, the bioavailability of M1 and M2, and the relevant pharmacological responses following the metabolism of LA in liver cells.

References

Abd-Elazem, I.S., Chen, H.S., Bates, R.B., Huang, R.C., 2002. 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.

Breneman, W.R., Zeller, F.J., 1975. Lithosperm inhibition of anterior pituitary hormones. Biochem. Biophys. Res. Commun. 65, 1047–1053.

Chatterton, R.T., 1981. Inhibition of progestational activity for fertility regulation. Res. Front Fertil. Regul. 1, 1–14.

Farnsworth, N.R., Bingel, A.S., Cordell, G.A., Crane, F.A., Fong, H.H., 1975. Potential value of plants as sources of new antifertility agents I. J. Pharm. Sci. 64, 535–598.

Findley, W.E., Hollstein, U., Besch, P.K., 1985. Effect of purified lithospermic acid and its oxidation product on luteinizing hormone release in vitro. Biol. Reprod. 33, 309–315.

Kelley, C.J., Harruff, R.C., Carmack, M., 1976. The polyphenolic acids of *Litbospermum ruderale*. II. Carbon-13 nuclear magnetic resonance of lithospermic and rosmarinic acids. J. Org. Chem. 41, 449–455.

Kelley, C.J., Mahajan, J.R., Brooks, L.C., Neubert, L.A., Breneman, W.R., Carmack, M., 1975. Polyphenolic acids of *Litbosperinum ruderale* Dougl. cx Luhm. (Boraginaceae). I. Isolation and structure determination of lithospermic acid. J. Org. Chem. 40, 1804–1815.

Li, X.C., Yu, C., Lu, Y.L., Lu, J., Xu, W., Xuan, L.J., Wang, Y.P., 2007. Pharmacokinetics, tissue distribution, metabolism, and excretion of depside salts from *Salvia miltiorrhiza* in rats. Drug Metab. Dispos. 35, 234–239.

Li, X.C., Yu, C., Sun, W.K., Liu, G.Y., Jia, J.Y., Wang, Y.P., 2004. Simultaneous determination of magnesium lithospermate B, rosmarinic acid, and lithospermic acid in beagle dog serum by liquid chromatography/tandem mass spectrometry. Rapid Commun. Mass Spectrom. 18, 2878–2882.

Ly, T.N., Shimoyamada, M., Yamauchi, R., 2006. Isolation and characterization of rosmarinic acid oligomers in *Celastrus hindsii* Benth leaves and their antioxidative activity. J. Agric. Food Chem. 54, 3786–3793.

O'Malley, S.J., Tan, K.L., Watzke, A., Bergman, R.G., Ellman, J.A., 2005. Total synthesis of (+)-lithospermic acid by asymmetric intramolecular alkylation via catalytic C–H bond activation. J. Am. Chem. Soc. 127, 13496–13497.

Parnham, M.J., Kesselring, K., 1985. Rosmarinic acid. Drugs Future 10, 756–757.

Shigematsu, T., Tajima, S., Nishikawa, T., Murad, S., Pinnell, S.R., Nishioka, I., 1994. Inhibition of collagen hydroxylation by lithospermic acid magnesium salt, a novel compound isolated from *Salviae miltiorrhizae* Radix. Biochim. Biophys. Acta 1200, 79–83.

Wagner, H., Horhammer, L., Frank, U., 1970. Lithospermic acid, the antihormonally active principle of *Lycopus europaeus* L. and *Symphytum officinale*. 3. Ingredients of medicinal plants with hormonal and antihormonal-like effect. Arzneimittelforschung 20, 705–713.

Yamamoto, H., Inoue, K., Yazaki, K., 2000. Caffeic acid oligomers in *Lithospermum erythrorhizon* cell suspension cultures. Phytochemistry 53, 651–657.

Zhou, C., Luo, H., Niwa, M.J., 1999. Studies on isolation and identification of water-soluble constituents of *Salvia miltiorrhiza*. Chin. Pharm. Univ. 30, 411–416.