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Simultaneous determination of six phenolic constituents of danshen in human serum using liquid chromatography/tandem mass spectrometry

Xiaochuan Li [a,b,*], Chen Yu [c], Yongbao Cai [c], Gangyi Liu [c], Jingying Jia [c], Yiping Wang [a]

[a] *Department of Pharmacology, Shanghai Institute of Materia Medica, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 201203, China*
[b] *Graduate School of the Chinese Academy of Sciences, China*
[c] *Central laboratory, Shanghai Xuhui Central Hospital, Shanghai 200002, China*

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Abstract

The six phenolic constituents are water-soluble components extracted from the Chinese medical herb danshen, the dried roots of Salvia miltiorrhiza Bunge (Labiatae). An liquid chromatography/tandem mass spectrometry (LC/MS/MS)-based method has been developed for the simultaneous quantification of six phenolic constituents of danshen (magnesium lithospermate B (MLB), rosmarinic acid (RA) and lithospermic acid (LA), caffeic acid (CAA), protocatechuic aldehyde (3,4-dihydroxybenzaldehyde, Pal), 3,4-dihydroxyphenyllactic acid (danshensu)) in human serum with chloramphenicol 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 multiple reaction monitoring (MRM) mode, with sufficient sensitivity to allow analysis of human serum samples generated following administration of a clinically relevant dose. Good linearity over the range 8–2048 ng/mL for six phenolic constituents was observed. The intra- and inter-day precisions (CV) of analysis were <13%, and the accuracy ranged from 88 to 116%. This quantitation method was successfully applied to a pharmacokinetic study of i.v. drip infusion of Danshen injection fluid in human.

Keywords: Magnesium lithospermate B; Rosmarinic acid; Lithospermic acid; Caffeic acid; Protocatechuic aldehyde; Danshensu; Danshen; Pharmacokinetics

1. Introduction

Danshen is the dried roots of the medicinal plant Salvia miltiorrhizae. It is a widely used and extensively studied oriental herb used for the treatment of coronary heart disease, hepatitis, menstrual disorders, menostasis, blood circulation diseases and other cardiovascular diseases. Recently, studies have confirmed many of its traditional properties and also uncovered some additional properties including anticoagulant and antibacterial activities and beneficial effects in patients with chronic renal failure.

In recent years, the water-soluble components of Danshen has attracted increasing attention because of their effectiveness in improving the renal function of rats with adenine-induced renal failure, as an antioxidant for the removal of free radicals and their potential in treating Alzheimer's disease [1–4]. In a series of studies, Tanaka et al. [2] isolated the active components that exhibit an improving effect on renal functional parameters, namely marked reduction of glomerular filtration rate following adenine ingestion by injection of the components. Zhou et al. [5] used macropore resin separation to isolate rosmarinic acid (RA),

* Corresponding author. Tel.: +86 21 5403 0254; fax: +86 21 5403 0254.
E-mail address: xchli@sibs.ac.cn (X. Li).

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prolithospermic acid, lithospermic acid (LA), magnesium lithospermate B (MLB), ammonium potassium lithospermate B and magnesium salvianolate E oligomers of caffenic acids.

Earlier publications have described methods for analysis of MLB in biological samples using high-performance liquid chromatography with UV-detection [6] or ECD-detection [7]. Nakazawa and Ohsawa [8] have reported metabolism of RA in rats by using HPLC equipped with photodiode array detection techniques (the limit of detection (LOD) for RA was 0.366 μg/mL). Baba et al. [9] have measured RA and its related metabolites in plasma and urine by LC–MS method. The poor sensitivity and lack of specificity of the above methods demand developing a more advanced analytical procedure that can be the basis of a method that is amendable to measurement of a broad range of phenolic constituents at low-level concentrations in serum.

Though we have reported a simultaneous determination of magnesium lithospermate B, rosmarinic acid, and lithospermic acid in dog serum using liquid chromatography/tandem mass spectrometry (LC/MS/MS) method [10], a quantitative method of broad range of phenolic constituents is needed in the clinical study of i.v. drip infusion of Danshen injection fluid. In this paper we present a selective and simple LC/MS/MS method that is capable of determining six phenolic constituents in a very short run-time and its application to analyze these components in human serum.

2. Experimental

2.1. Chemicals

All solvents used were of HPLC grade. Acetonitrile were obtained from Fisher (Fair Lawn, NJ, USA) and methanol from Caledon Laboratories (Georgetown, ON., Canada). Ethyl acetate, acetone, and formic acid (HPLC grade) were purchased from Sigma–Aldrich (Germany). Double-distilled water was made in this laboratory.

Magnesium lithospermate B, rosmarinic acid and lithospermic acid, caffeic acid (CAA), protocatechuic aldehyde (Pal), danshensu and their corresponding internal standard (Fig. 1) were provided by the Department of Phytochemistry, Shanghai Institute of Materia Medica. The purity of these compounds was above 99.0%, which was verified by the supplier using HPLC methods. Danshen injection fluid (250 mL) was obtained from Treeful Pharmaceutical (Shanghai, China), which contains MLB (≥65 mg), RA (≥160 mg), LA (≥3 mg), CAA (≥5 mg), Pal (≥3 mg), and danshensu (≥90 mg).

2.2. Liquid chromatography/tandem mass spectrometry

The quantification of the analytes was performed by LC/MS/MS in the multiple reaction monitoring (MRM) mode. The LC/MS/MS system consisted of a HPLC system (including a vacuum degasser, a quaternary pump and an autosampler; Shimadzu, Japan) coupled to Perkin-Elmer

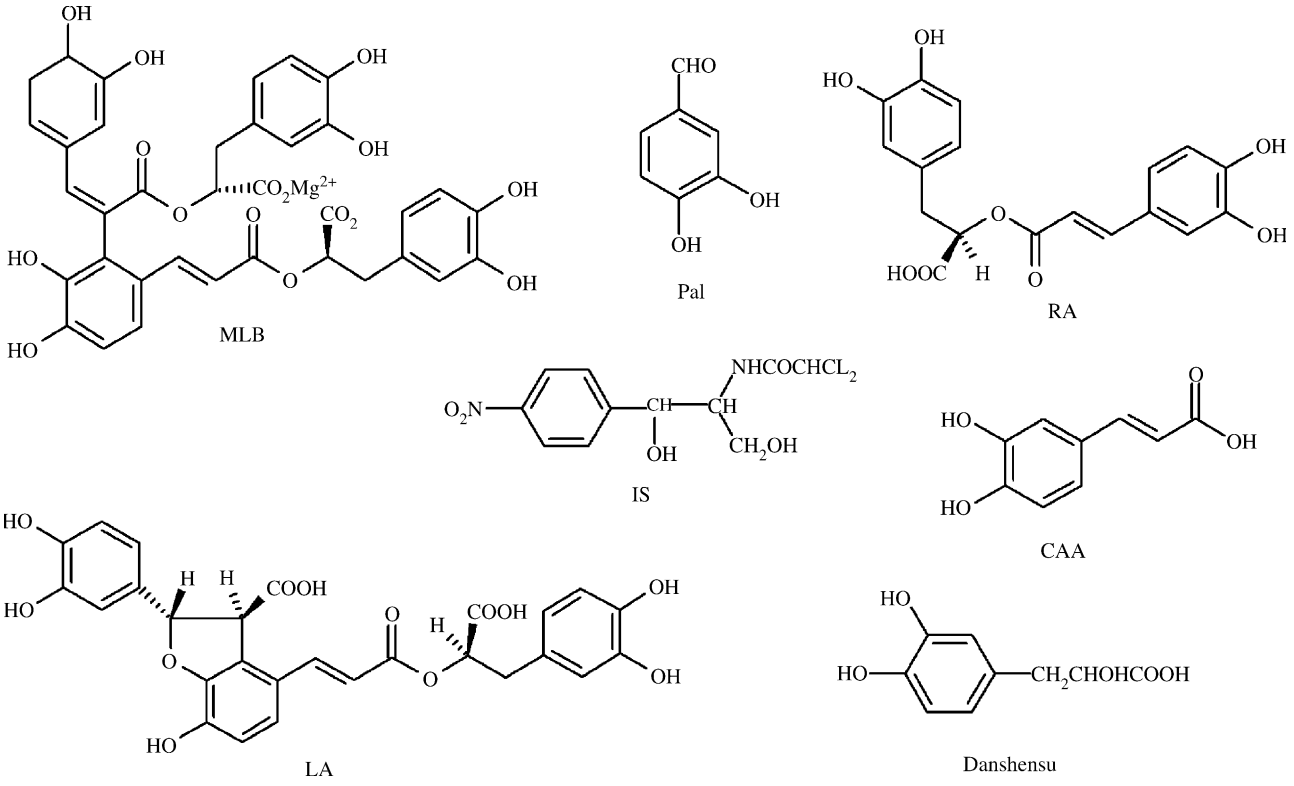

Fig. 1. Chemical structures of six phenolic constituents and IS (chloramphenicol).

Table 1
The compound-dependent instrumental parameters for six phenolic constituents and IS

Compounds	Q1 (*m*/*z*)	Q3 (*m*/*z*)	Time (ms)	Parameter[a]				
				DP (V)	FP (V)	EP (V)	CE (V)	CXP (V)
MLB	717.2	519.2	200	−36	−215	−9	27	−15
RA	359.1	160.6	200	−36	−280	−10	23	−12
LA	537.5	493.3	200	−30	−240	−9	12	−15
CAA	179.3	134.9	200	−45	−255	−14	21	−8
Pal	137.1	107.9	200	−40	−200	−10	32	−7
Danshensu	197.1	135.0	200	−36	−200	−9	27	−23
IS	321.3	151.9	200	−30	−170	−14	25	−10

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

SCIEX API-3000 triple-quadruple mass spectrometer (Sciex, Concord, ON, Canada) equipped with TurboIonSpray source, with Analyst^{TM} 1.3.1 controlling software. A negative TurboIonSpray mode was selected to get higher sensitivity than that in positive ion mode. The source conditions were typically as follows: nebulizer gas 12 L/min, curtain gas 10 L/min, collision gas 12 L/min, ionspray voltage −4500 V, and source temperature 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 the resolution of 0.7 Da peak width for Q1 and 1.0 Da peak width for Q3 in MRM mode.

LC separations were performed on a 5 μm CAPCELL PAK C18 column (100 mm × 2 mm i.d., Shiseido, Japan) with a mobile phase consisted of 56% water (containing 0.5% formic acid (v/v)) and 44% acetonitrile–methanol (1:1, v/v) at a flow of 0.20 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 a volume of 10 μL was injected into the HPLC system.

2.3. Extraction procedure

The serum sample (100 μL) and 10 μL of the IS solution (2.0 μg/mL of chloramphenicol) were added to a 2 mL polypropylene test tube, 200 μL formic acid–acetone–water (4:140:56, v/v/v) solution was added using an Eppendorf repeater pipette, followed by the addition of 1 mL 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 acetone–water (25:75, v/v). After centrifugation at 2500 × *g* for 5 min, a volume of 10 μL of the supernatant was introduced into the LC/MS/MS system.

2.4. Standard curves preparation

Standard stock solutions of six phenolic constituents were prepared in methanol at 1.0 mg/mL and serially diluted to working solution with water. All the stock and working solutions were stored at −20 °C. A 2 μg/mL chloramphenicol (internal standard) working solution was prepared in water. The serum concentrations of 2048, 1024, 512, 256, 64, 24, 8 ng/mL for six phenolic constituents were freshly prepared with blank serum and working solution. The serum samples for calibration were extracted and prepared under the same conditions as the test samples. Peak area ratios between compounds and IS were used for calculations and a weighted (1/concentration) regression analysis was used.

2.5. Validation procedures

A total of six replicates at each of three different concentrations (20, 800 and 1600 ng/mL) of six phenolic constituents validated the method presented here to determine the intra-day and inter-day accuracy and precision. Quantification was based on the ratio of the peak area of the analyte 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. The QC sample serum was freshly prepared with standard stock solution at 1.0 mg/mL and diluted with blank serum. Intra-day variability was tested on six different human serum QC samples using the same calibration curve and inter-day variability was tested on four different days using calibration curves obtained daily. The precision of the method at each QC concentration was expressed as the relative SD (RSD) by calculating the standard deviation as a percentage of the mean calculated concentration, while the accuracy of the assay was expressed as the relative error of the calculated concentrations.

The limit of detection was defined as the lowest concentration at which the analytical assay can reliably differentiate analyte LC peaks from background levels (signal-to-noise, 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 were calculated by comparing the peak areas obtained when QC samples were analyzed by adding the analytical reference standard and the internal standard in the extract of blank serum prior to and after

the extraction procedure. The recoveries were assessed at three different concentrations level (20, 800, 1600 ng/mL), using six replicates at each level. Samples spiked with 20, 800, 1600 ng/mL analytes were placed in the autosampler (4 °C) and analyzed at times 0, 2.5, 4, 8 h to test the stability.

2.6. Clinical studies

Two hundred fifty microliter of Danshen injection fluid was administrated to four human healthy volunteers through an i.v. drip infusion at a flow rate of 250 mL/h, and the LC/MS/MS method developed here was used to investigate the serum concentration–time profile of phenolic constituents. Blood (1 mL) was removed by venepuncture prior to dosage and at 0.25, 0.5, 0.75, 1, 1.083, 1.25, 1.5, 1.75, 2, 2.5, 3, 4, 6, 7, 9, and 13 h thereafter. After stored at 4 °C for 1 h, 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 of phenolic constituents were diluted five times with blank serum and then re-analyzed.

3. Result and discussion

3.1. Chromatography and mass spectrometry

According to the direct full scan mass spectra in the positive and negative ionization modes, the signal intensities obtained with TurboIonSpray source were too low for quantitative measurement in the positive modes, while in the negative ion mode the sensibilities were high enough for the clinical detection for all the phenolic constituents, so the predominantly deprotonated molecules ($[M-H]^-$) were selected using the TurboIonSpray source. The product ions for phenolic constituents and IS were sensitive in the negative mode and stable fragments were found at *m/z* 519, 161, 493, 135, 108, 135, and 152, respectively (Fig. 2 displays the MS/MS spectra of six phenolic constituents and IS).

Acetonitrile, methanol, and formic acid were used in the mobile phase to bring high signal-to-noise ratios and shorten the peak tails and an isocratic elution at the flow rate of 0.2 mL/min was used. For our study, if a large binary dilution was used to elute these constituents with several kinds of HPLC columns, after injecting a sample of high concentration, large amount of residue for MLB, RA, and LA will be found in the next injecting blank sample.

The determination of six phenolic constituents and IS was finished in one chromatographic run, which was completed within 4 min, and very short retention times for six phenolic constituents and IS were found. Fig. 3 shows representative extracted ion chromatograms for blank and sample serum. There was very little background noise, and a stable baseline was maintained throughout.

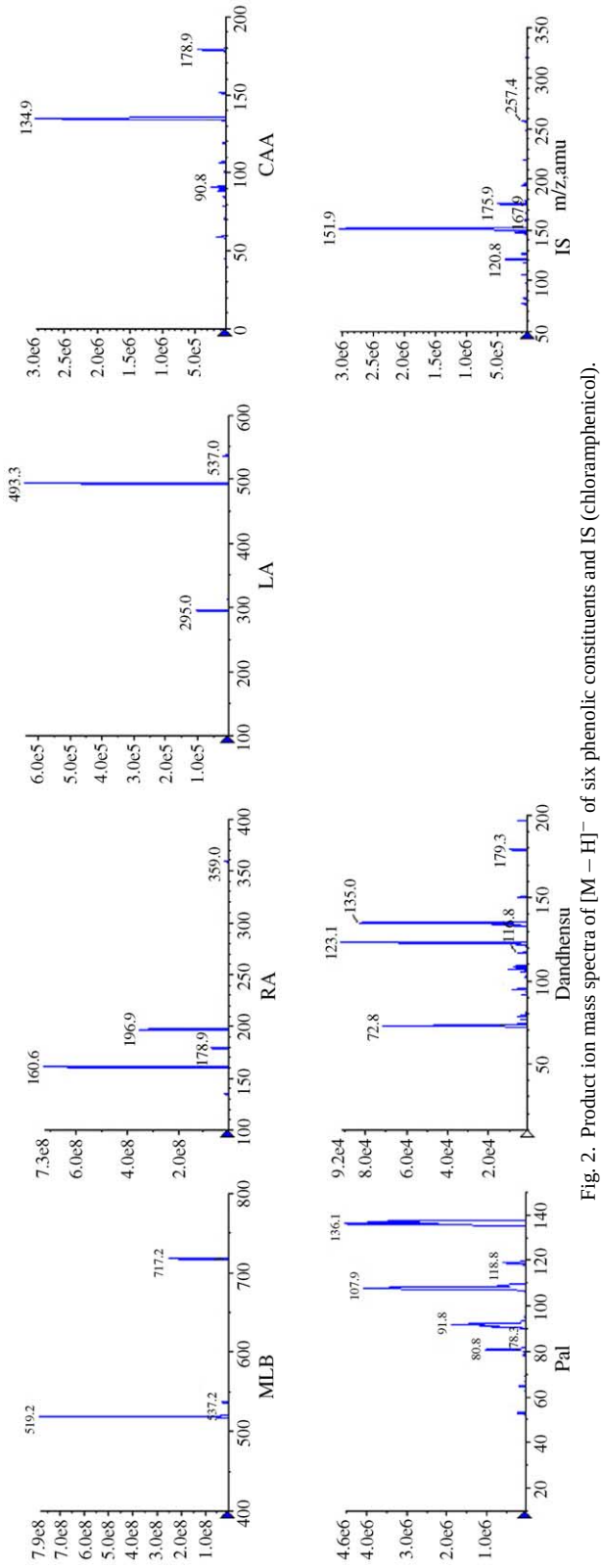

Fig. 2. Product ion mass spectra of $[M-H]^-$ of six phenolic constituents and IS (chloramphenicol).

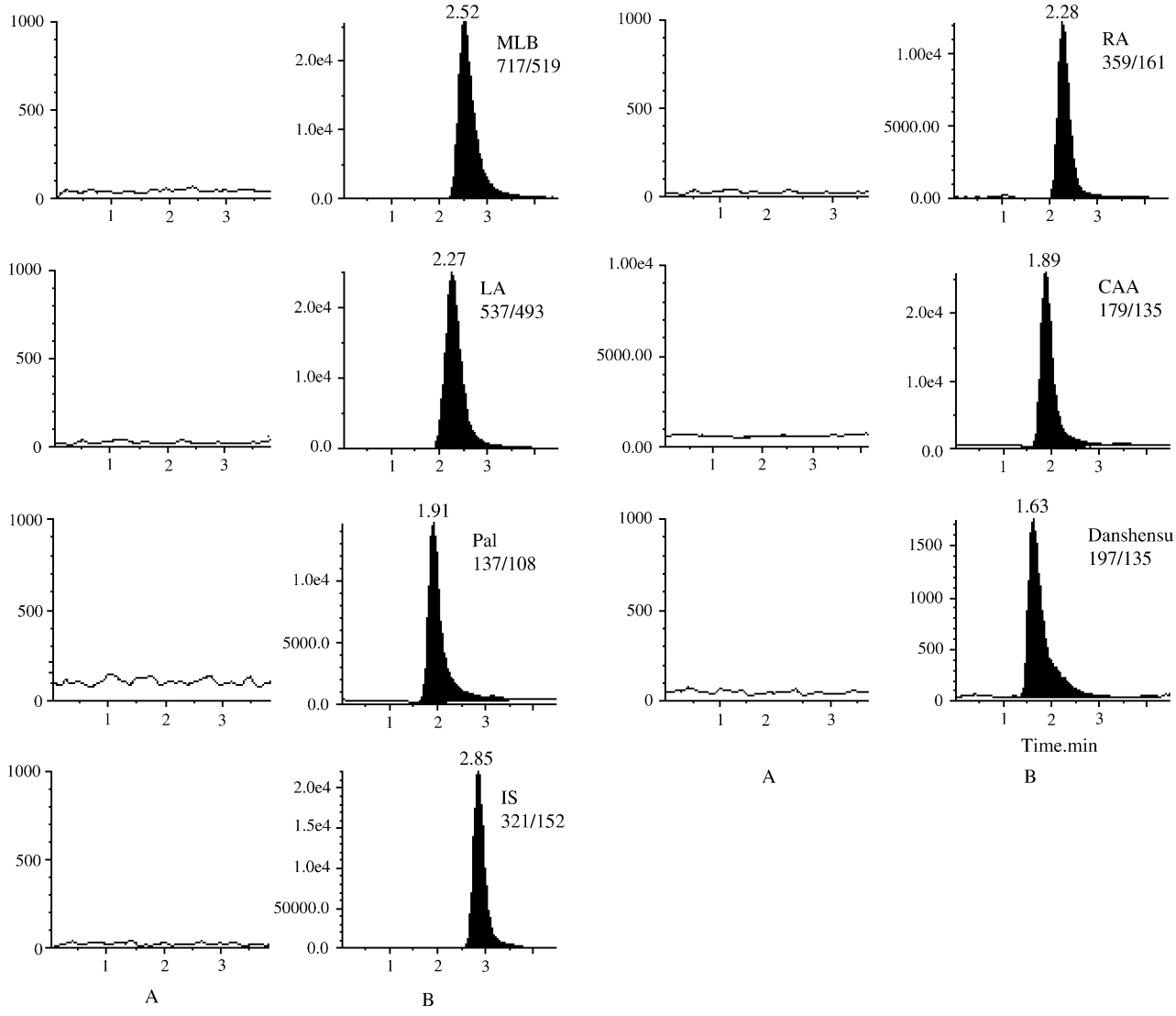

Fig. 3. Chromatograms of six phenolic constituents and IS in human serum. (A) Blank serum sample. (B) Serum sample spiked with phenolic constituents 128 ng/mL and IS. (intensity, cps.).

Table 2
Intra-day and inter-day validation of six phenolic constituents in human serum

Compounds	Low concentration (ng/mL)			Intermediate concentration (ng/mL)			High concentration (ng/mL)		
	Found (concentration)	RSD (%)	Accuracy (%)	Found (concentration)	RSD (%)	Accuracy (%)	Found (concentration)	RSD (%)	Accuracy (%)
Intra-day ($n = 6$)									
MLB	22.5 ± 2.5	11.3	112.3	846 ± 38	4.5	105.7	1672 ± 138	8.2	104.5
RA	20.3 ± 1.4	6.9	101.5	866 ± 57	6.6	108.3	1699 ± 121	7.1	106.2
LA	21.4 ± 1.8	8.7	106.8	831 ± 62	7.4	103.9	1613 ± 147	9.1	100.8
CAA	21.4 ± 1.3	5.9	107.0	836 ± 65	7.8	104.4	1612 ± 150	9.3	100.8
Pal	19.1 ± 0.9	4.8	95.4	767 ± 72	9.3	95.8	1450 ± 124	8.6	90.6
Danshensu	19.4 ± 1.7	8.7	97.0	758 ± 44	5.8	94.7	1532 ± 142	9.3	95.7
Inter-day ($n = 4$)									
MLB	23.2 ± 2.7	11.5	116.2	789 ± 79	10.0	98.6	1558 ± 170	10.9	97.4
RA	19.3 ± 2.4	12.3	96.5	820 ± 72	8.8	102.5	1650 ± 134	8.1	103.1
LA	20.8 ± 1.6	7.8	103.8	799 ± 62	7.8	99.9	1591 ± 139	8.7	99.4
CAA	21.1 ± 0.7	3.3	105.7	812 ± 96	11.8	101.5	1583 ± 162	10.2	98.9
Pal	17.6 ± 2.0	11.2	88.2	807 ± 33	4.1	100.8	1542 ± 77	5.0	96.4
Danshensu	19.1 ± 2.6	13.4	95.3	840 ± 67	8.0	105.0	1645 ± 95	5.8	102.8

Table 3
Six phenolic constituents in serum by LC/MS/MS: LOD, LOQ, and calibration results ($n = 4$)

Compounds	LOD (ng/mL)	LOQ (ng/mL)	Slope	Intercept	R^2
MLB	0.5	8	0.00652	−0.0171	0.997
RA	1.0	8	0.00207	−0.00038	0.998
LA	0.7	8	0.00607	−0.0116	0.998
CAA	1.5	8	0.00517	−0.00317	0.996
Pal	2	8	0.00229	0.0173	0.998
Danshensu	2.5	8	0.0004	−0.000529	0.998

3.2. Method validation

The precision of this analytical method was evaluated by calculating the RSD of four QC samples on the same day ($n = 6$) and on different days ($n = 4$), the resulting assay precision and accuracy data are presented in Table 2. The intra-day precision (CV) of the assay was less than 11.3% and the inter-day precision (CV) of the assay was less than 13.4% for each concentration on three QC samples, indicating good assay precision. Meanwhile, the accuracy ranged from 88 to 116%.

Sensitivity was investigated by determining the lower limit of detection based on the peak-to-peak noise of the baseline and on minimal value of signal-to-noise ratio of 3. The applied methodology provided LOD in the range of 0.5–2.5 ng/mL (Table 3). The lower limits of quantifications were 8 ng/mL, which were high enough to the detection of analytes in the clinical studies. The recoveries for six phenolic constituents at LLOQ were 94–113% with the CVs < 7.3%.

Standard curves exhibited good linearity in the range 8–2048 ng/mL, with all coefficients of correlation (r^2) ranging from 0.996 for CAA to 0.9998 for RA. Table 3 shows the slopes, intercepts, and correlation coefficients obtained for typical calibration curves. The CVs were determined to be <9.7% and the recoveries were 92–113%.

3.3. Extraction recovery and analyte stability

The extraction recoveries of six phenolic constituents 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 the six phenolic constituents were in the range of 61–92% at the concentrations of 20, 800 and 1600 ng/mL, respectively. The extraction recovery of the IS was 93.8% (Table 4). The stabilities of the analytes in serum at autosampler (4 °C) were good and the recoveries were 91–106%. (Table 5).

Table 4
Recovery of six phenolic constituents in serum by the liquid–liquid extraction

Compounds	Serum concentration (ng/mL)	Recovery (%)	CV (%)
MLB	20	77.8	17.1
	800	74.5	4.1
	1600	80.3	4.9
RA	20	85	11.3
	800	85.6	5.8
	1600	89.2	6.8
LA	20	84.4	10.1
	800	79.1	11.3
	1600	88.4	5.9
CAA	20	73.9	10.6
	800	79.6	10.3
	1600	83.9	7.6
Pal	20	91.8	12.3
	800	73.4	9.9
	1600	79.9	7.0
Danshensu	20	61.9	15.3
	800	62.3	3.5
	1600	60.8	8.0
IS[a]	200	93.8	9.6

[a] $n = 18$.

3.4. Clinical studies

After an i.v. drip infusion of 250 mL Danshen injection fluid to four human healthy volunteers, the serum concentrations of six phenolic constituents were determined by the described LC/MS/MS method. Fig. 4 shows the mean serum concentration–time curves of the phenolic constituents after an i.v. drip infusion ($n = 4$). Although the high concentration of Pal was determined in the Danshen injection fluid, it was not observed in the serum.

Table 5
The stability of six phenolic constituents in serum in serum at autosampler (8 h at 4 °C) ($n = 4$)

Compounds	20 (ng/mL)			800 (ng/mL)			1600 (ng/mL)		
	Mean (concentration)	RSD (%)	Accuracy (%)	Mean (concentration)	RSD (%)	Accuracy (%)	Mean (concentration)	RSD (%)	Accuracy (%)
MLB	21.3	10.3	106.4	771	8.2	96.4	1518	8.7	94.8
RA	18.3	14.3	91.4	786	9.5	98.3	1588	10.0	99.2
LA	19.7	12.1	98.4	763	10.2	95.3	1520	8.6	95.0
CAA	20.7	5.2	103.3	798	9.7	99.8	1515	9.6	94.7
Pal	18.6	13.1	93.0	789	8.3	98.6	1528	6.7	95.5
Danshensu	18.5	14.5	92.5	796	9.6	99.5	1615	9.2	100.9

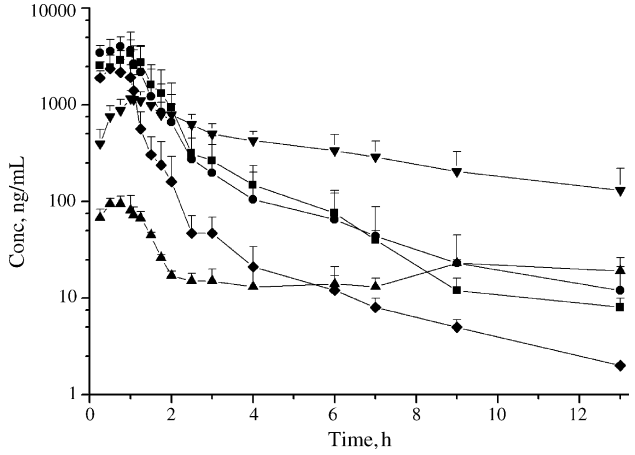

Fig. 4. Profiles of mean serum concentration of phenolic constituents versus time after i.v. drip infusion of 250 mL Danshen injection fluid to human healthy volunteers ($n = 4$). ((●) MLB; (♦) RA; (▼) LA; (▲) CAA; (■) Danshensu).

4. Conclusions

A new LC–MS/MS-based methodology for the simultaneous determination of a broad range of six phenolic constituents is described with good sensitivity and selectivity. The optimized method was validated to guarantee a reliable quantification of six phenolic constituents in human serum. It was successfully applied to a pharmacokinetic study of the phenolic constituents after i.v. drip infusion of 250 mL Danshen injection fluid to human healthy volunteers.

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