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Original article

Simultaneous Determination of Five Hydrophilic and Lipophilic Components from roots of *Salvia miltiorrhiza* by HPLC

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ABSTRACT

Objective A reversed-phase HPLC method was established for the simultaneous determination of five hydrophilic and lipophilic components in the roots of *Salvia miltiorrhiza*. **Methods** Hydrophilic components including danshensu, protocatechuic aldehyde, and salvianolic acid B, and lipophilic components such as cryptotanshinone and tanshinone IIA, were successfully separated on a Waters Symmetry C_{18} reverse-phase column (250 mm × 4.6 mm, 5 μm), with acetonitrile-0.5% phosphoric acid (gradient elution) as mobile phase, the detection wavelength was set at 281 nm with flow rate of 1.0 mL/min, and the column temperature was maintained at 30 ℃. **Results** The recovery of the method was in the range of 95.1%–102.5% and the precision was less than 3% for all five analytes. All the compounds showed good linearity ($R^2 > 0.9990$) in a relatively wide concentration range. Therefore, this HPLC method demonstrated good reproducibility, stability, and accuracy in validation studies. **Conclusion** Simultaneous quantification of the multiple components by HPLC would be a better strategy for the quality evaluation on the roots of *S. miltiorrhiza*.

Key words

cryptotanshinone; danshensu; HPLC determination; protocatechuic aldehyde; *Salvia miltiorrhiza*; salvianolic acid B; tanshinone IIA

1. Introduction

Salviae Miltiorrhizae Radix (roots of *Salvia miltiorrhiza* Bunge from Labiatae) is a commonly used herbal medicine in China. Due to its better performance and fewer side effects as confirmed in the long-time clinical use, the roots of *S. miltiorrhiza* are widely adopted in the preparations of traditional Chinese medicine (TCM) to treat cardiovascular disease, cerebrovascular disease, coronary heart disease, chronic renal failure, atherosclerosis, myocardial infarction, angina pectoris,

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myocardial ischemia, dysmenorrhea, neurasthenic insomnia, and liver fibrosis and cirrhosis (Wasser et al, 1998; Liu et al, 2000; Chae et al, 2004; Ling et al, 2005; Wang et al,2013). The active compounds in the roots of *S. miltiorrhiza* were divided into two groups by chemical and pharmacological investigations, phenolic acids such as protocatechuic aldehyde (PTAL), salvianolic acid B (SAB), and danshensu (DSS), which are hydrophilic, and tanshinones including tanshinones IIA (TSIIA), I, IIB, and cryptotanshinone (CTS), which are lipophilic (Lu and Foo, 2002). TSIIA and SAB are both considered by *Chinese Pharmacopoeia 2010* to be marker compounds for the quality control of the roots of *S. miltiorrhiza*. Furthermore, DSS and PTAL have been identified as the main water-soluble constituents (Luo et al, 2008; Li et al, 2008). Multi-component quantification would be much more comprehensive. Therefore, the development of multi-component analysis method to improve the quality evaluation criteria for the roots of *S. miltiorrhiza* would be of great practical significance.

Up to now, the quantitative determination of the active constituents in the roots of *S. miltiorrhiza* has been only focused on hydrophilic or lipophilic compounds, and one or two compounds, which could not reflect the overall quality for the roots of *S. miltiorrhiza* (Liu et al, 2007), and the reports on simultaneous quantification of them were scarce. There are some reports concerning the determination of active components in the roots of *S. miltiorrhiza* by HPLC. Simultaneous determination of either water-soluble (Ma et al, 2006) or lipid-soluble (Shi et al, 2005) compounds in the roots of *S. miltiorrhiza* has been carried out. Zhou et al (2006) have established the HPLC methods for the simultaneous determination of several active compounds including water-soluble and lipid-soluble components in the roots of *S. miltiorrhiza*. Nevertheless, all the methods above had some problems such as complex experimental procedures, complex extraction method, and incomplete simultaneous extraction of water-soluble and lipid-soluble components. Therefore, a simple method to determine hydrophilic and lipophilic compounds simultaneously is highly desired for controlling the drugs quantity validly. In the present paper, based on the previous methods (Ma et al, 2007; Zeng et al, 2008), a simple and reliable method by HPLC was developed for the quality evaluation on the roots of *S. miltiorrhiza* through simultaneous determination of both hydrophilic and lipophilic components including three major active hydrophilic constituents, namely DSS (**1**), PTAL (**2**), and SAB (**3**), and lipophilic constituents, namely CTS (**4**) and TSIIA (**5**), though it was still time-consuming. Their structures are shown in Figure 1.

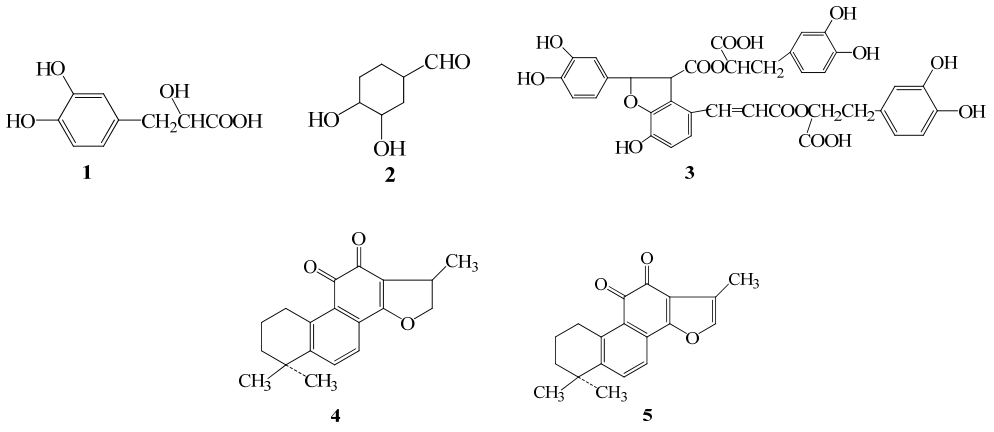

Figure 1 Chemical structures of five components in roots of *S. miltiorrhiza*

2. Materials and methods

2.1 Plant materials

Seven dried samples were collected from different habitats and identified by Prof. Jian-he Wei, Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College. The collection habitats and time of these samples are shown in Table 1. In this experiment, the seven samples were mainly collected from October, 2008 and 2009, dried in an oven at 60 ^{o}C until a constant weight, and comminuted for further extraction and separation. A voucher specimen (SM-10-09-26) has been deposited in the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences, Beijing, China.

2.2 Chemicals and reference sompound

The acetonitrile and methanol of HPLC grade were purchased from Fisher Scientific (USA). Deionized water was purified by the Milli-Q System (Millipore, USA). The phosphoric acid and ethanol of analytical grade were from Beijing Beihua Fine Chemicals Co., Ltd. (China). Authentic standards of danshensu (BN: A0062), proto- catechuic

aldehyde (BN: A0616), salvianolic acid B (BN: A0056), cryptotanshinone (BN: A0061), and tanshinone IIA (BN: 111605) were purchased from the National Institute for Control of Biological and Pharmaceutical Products (China). The purities of these reference compounds were determined to be above 95.0% by normalization of the peak areas detected by HPLC-DAD.

Table 1 *S. Miltiorrhizae* root samples collected from different habitats

Sample No.	Habitats	Collected time
DS002[1]	Shandong	October, 2008
DS003	Jiangsu	October, 2008
DS007	Henan	October, 2008
DS009A	Beijing	October, 2009
DS009B	Beijing	October, 2009
DS010	Beijing	October, 2009
DS012	Hubei	October, 2009

[1]danshen (DS) + gemplasm number (002) = DS002

2.3 Apparatus and analytical conditions

A Waters 1525 HPLC System (Waters Technologies, USA) comprising a binary solvent delivery system, an on-line degasser, a Waters 717 plus Autosampler, a column temperature controller, and a Waters 2457 Dual γ Absorbance Detector coupled with an analytical workstation were used. The column was a Waters Symmetry C_{18} reverse-phase column (250 mm × 4.6 mm, 5 μm). The sample injection volume was 10 μL.

The chromatographic conditions for hydrophilic and lipophilic components were as follows: the detection wavelength was set at 281 nm, the flow rate was 1.0 mL/min, and the column temperature was maintained at 30 ^{o}C. A gradient elution with acetonitrile (A) and 0.5% aqueous phosphoric acid (B) was used as follows: 0−10 min, 5%−14% A; 10−12 min, 14%−17% A; 12−16 min, 17%−19% A; 16−20 min, 19%−25% A; 20−35 min, 25% A; 35−45 min, 25%−75% A; 45−55 min, 75%−90% A; 55−60 min, 90%−5% A; 60−70 min, 5% A.

2.4 Preparation of sample solutions

The stock solutions of compounds **1**−**5** in 70% methanol were prepared at the concentration of 1 mg/mL, respectively. Then, the stock solutions were diluted to proper concentration ranges for the establishment of calibration curves.

Individual samples (0.5 g) were accurately weighed and suspended in 70% methanol, then extracted under reflux for 1 h. After cooling to room temperature, the methanol lost in refluxing was restored, the extracted solution was filtered through a membrane (0.45 μm), and then 10 μL was injected into the HPLC.

3. Results and discussion

3.1 Optimization of extraction procedure

Each extract was filtered and separated by the method described above. The chromatograms of five bioactive components in different solvent extracts with 50 mL of 30%, 50%, 70%, and 100% methanol, respectively. Figure 2 showed that the total content of the five reference substances in the extraction with 70% methanol was more than those with other solvent extracts. Therefore, 70% methanol was selected as extraction solvent. In order to investigate the volume of solvent, about 0.5 g dried sample DS003 was extracted with 30, 35, 40, 45, 50, and 60 mL 70% methanol, and the results showed that 50 mL 70% methanol had the highest extraction yield with almost equal extraction capacities. Therefore, 50 mL was selected as the solvent volume (Figure 3). The influence of extracting time on the efficiency of the extraction was also observed. The results showed that the highest amounts of the five bioactive components were obtained with the extracting time of 1 h.

3.2 Optimization of chromatographic conditions

In this study, the mobile phase, gradient mode, and operating conditions were optimized through several trials to achieve good resolution and symmetric peak shapes of the five reference compounds. Preliminary researches indicated that better separation and results were obtained using a mobile phase of water and acetonitrile rather than water and methanol. Therefore, in this work, water and acetonitrile were chosen as the mobile phase. Acid contained in the mobile phase, which could suppress the ionization of phenolic hydroxyl and carboxyl groups, was beneficial for good retention and separation of the five reference

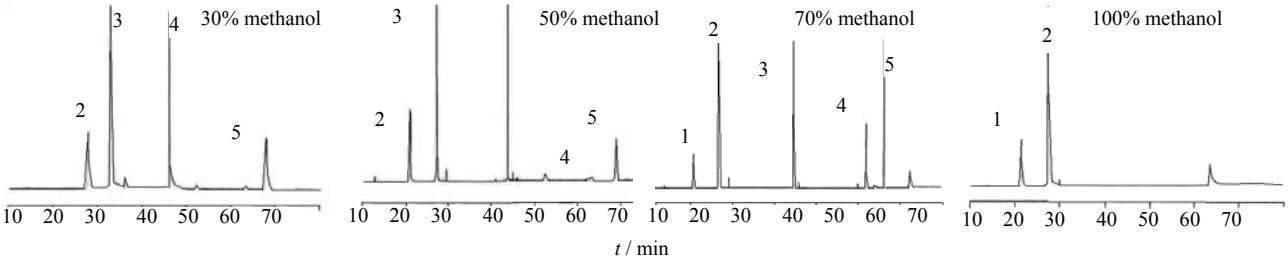

Figure 2 LC of five bioactive components from different extracts

1: danshensu; 2: protocatechuic aldehyde; 3: salvianolic acid B; 4: cryptotanshinone; 5: tanshinone IIA; same as below

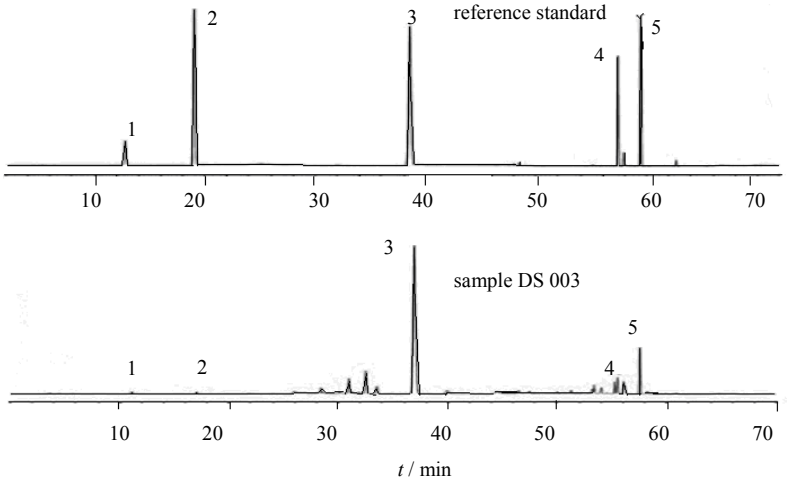

Figure 3 LC of five bioactive components

compounds, and so 0.5% phosphoric acid was selected for the aqueous phase. Thus, gradient elution program was carried out to separate these compounds in samples. The optimum eluting procedure was described above. The effect of temperature on the separation was investigated within the range of 25–40 °C and 30 °C was found to be the optimal temperature. The most suitable flow rate was 1.0 mL/min. On the basis of UV-vis spectra of the five compounds recorded by DAD detection in the range of 180–700 nm, experimental results indicated that the five compounds had the same UV absorption peaks at about 281 nm, so 281 nm was selected for monitoring. The chromatograms of the five compounds and sample DS003 are shown in Figure 3.

3.3 Calibration curves

A series of concentration levels were prepared and chromatography was carried out in six times for calibration and linear analysis of each standard compound. The calibration curves were constructed by peak-area of the analytes (*Y*) against the concentration of the calibration standards (*X*). The concentration of analytes in unknown samples was determined by interpolation from the calibration curve. Under the chromatographic conditions adopted in the study, all calibration curves exhibited good linearity ($r > 0.999\ 0$) in a relatively wide concentration range (Table 2).

3.4 Limits of detection and quantification

The stock solution of the five reference compounds was further diluted to a concentration with 70% methanol to test the limit of detection (LOD) and limit of quantification (LOQ). The LOD and LOQ values under the present chromatographic conditions were determined at signal-to-noise (S/N) ratio of 2 and 10, respectively. LOD and LOQ values for each reference compound are listed in detail in Table 2.

Table 2 Linear regression data for standard curves of reference compounds to be quantified

Compounds	Calibration curves	*r*	Linear range / (μg·mL^{-1})	LOD / (μg·mL^{-1})	LOD / (μg·mL^{-1})
1	$Y = 6.39 \times 10^5 X + 8730$	0.999 5	2.650 – 106.00	0.12	0.50
2	$Y = 4.84 \times 10^6 X + 58\ 600$	0.999 5	2.370 – 94.80	0.05	0.20
3	$Y = 9.63 \times 10^5 X - 991\ 000$	0.999 0	241.500 – 805.00	0.16	0.70
4	$Y = 2.00 \times 10^6 X + 20\ 400$	0.999 5	2.485 – 99.40	0.08	0.30
5	$Y = 3.39 \times 10^6 X - 7680$	0.999 8	2.540 – 30.48	0.06	0.24

3.5 Precision and stability

The intra-day precision (presented as RSD) was determined for calibration sample by analyzing the six replicates on the same day, while the inter-day values were carried out over consecutive 6 d. The RSD was taken as a measure of precision. The sample stability test was performed with one sample at 0, 3, 6, 12, 24, and 48 h within 2 d. During this period, the solution was stored at room temperature. Table 3 shows the results of the tests of precision and stability of the five compounds. It indicated that the RSD values of the overall intra- and inter-day variations were less than 2.50% for all the five compounds. Further, the validation studies of this method proved that this assay had good reproducibility with RSD less than 2.27% for all the analyses and the sample solutions were stable during 48 h at room temperature with RSD less than 1.95%.

3.6 Accuracy

Each sample was analyzed in six replicates. The results showed that the developed analytical method had good accuracy with the overall recovery from 95.10% to 102.50% for the compounds concerned (Table 4). Therefore, the HPLC method was precise, accurate, and sensitive enough for simultaneously quantitative evaluation of the five compounds from the aerial part of *S. miltiorrhiza.*

3.7 Method performance of sample assays

Seven samples of *S. miltiorrhiza* roots from different habitats were extracted and separated as described above. Extraction solution (10 μL) was subjected to the determination under the selected chromatographic conditions. The results showed that compound **3** was the most abundant among five compounds in most samples, but with great variations (60.5−115.1 mg/g) (Table 5). The content of total bioactive compounds investigated in the roots of *S. miltiorrhiza* also showed high variations (61.58−118.82 mg/g). The higher degree of variability in the bioactive compounds contents (total and individuals) in samples from different geographical locations and TSIIA levels which did not meet the requirements of *Chinese Pharmacopoeia* 2010 (0.2%) could be due to various factors, such as climate, geographical source, harvest time, storage condition, and ages of the plant.

Table 3 Precision and stability of five compounds (*n* = 6)

Compounds	Precision / %	Stability / %	RSD / %	
			Intra-day	Inter-day
1	1.36	1.95	2.05	2.11
2	2.27	1.64	2.40	2.50
3	0.59	1.90	0.85	2.02
4	1.73	0.89	1.90	1.16
5	0.60	1.09	0.96	1.23

Table 4 Recoveries for five analytes in roots of *S. miltiorrhiza* (*n* = 6)

Compounds	Recoveries / %	RSD / %
1	102.50	2.22
2	95.10	2.21
3	97.20	1.73
4	99.01	2.81
5	99.50	2.92

Table 5 Determination of five compounds in roots of S. *miltiorrhiza* from five different samples（$\bar{x} \pm s$, n = 6）

	Contents of compounds / ($mg \cdot g^{-1}$)				
Sample No.	1	2	3	4	5
DS002	0.64 ± 0.02	0.01 ± 0.00	84.2 ± 2.53	0.18 ± 0.01	0.79 ± 0.02
DS003	0.95 ± 0.03	0.07 ± 0.00	115.1 ± 3.45	0.74 ± 0.02	0.73 ± 0.02
DS007	0.89 ± 0.03	0.02 ± 0.00	94.3 ± 2.83	0.82 ± 0.02	1.88 ± 0.06
DS009A	0.52 ± 0.02	0.01 ± 0.00	69.7 ± 2.09	0.69 ± 0.02	0.51 ± 0.02
DS009B	0.61 ± 0.02	0.01 ± 0.00	79.6 ± 2.39	0.25 ± 0.01	0.65 ± 0.02
DS010	0.44 ± 0.01	0.02 ± 0.00	60.5 ± 1.82	0.31 ± 0.01	1.20 ± 0.04
DS012	0.63 ± 0.02	0.01 ± 0.00	81.7 ± 2.45	0.12 ± 0.00	0.59 ± 0.02

4. Conclusion

A new analytical method for simultaneously quantitative evaluation of the five compounds from the roots of *S. miltiorrhiza.* is established to be precise and accurate.

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