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Short communication

Determination of four water-soluble compounds in *Salvia miltiorrhiza Bunge* by high-performance liquid chromatography with a coulometric electrode array system

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Abstract

A method has been developed to determine the four water-soluble components—Danshensu (I), protocatechuic acid (II), protocatechuic aldehyde (III) and salvianolic acid B (IV) in Chinese medicine plant *Salvia miltiorrhiza Bunge* using high-performance liquid chromatography with a coulometric electrode array detection (HPLC–CEAD) system. Heat reflux extraction was used to pretreat the sample. This analysis was carried on a column of Hypersil C_{18} (250 mm × 4.6 mm, 5 μm) with a mobile phase of sodium acetate (pH 2.5, 50 mM) and acetonitrile in gradient mode. An ESA electrochemical detector monitored the four compounds. Potentials of four electrodes in series were set at 100, 150, 200 and 250 mV, respectively. Optimization of the pH of mobile phase and the proportion of acetonitrile were also performed. Calibration curve showed good linearity with correlation coefficients (r) more than 0.9937. Average recoveries of the four compounds were more than 92% and relative standard deviations were less than 6.6%. This method appeared to be stable, sensitive and reproducible for determination of the four water-soluble compounds in Chinese medicine plant *S. miltiorrhiza Bunge*.

Keywords: HPLC; Electrochemical detector; Chinese medicine plant; *Salvia miltiorrhiza Bunge*

1. Introduction

The root and rhizome of *Salvia miltiorrhiza Bunge* (Labiatae), Danshen in Chinese, has been commonly used in Chinese traditional medicine (CTM) in the treatment of coronary artery disease and cerebrovascular diseases including stroke [1]. The effective components of the roots could be classified as lipid-soluble and water-soluble. In recent years, the lipid-soluble components and their pharmacological functions have been largely researched. However, decoction with water is the conventional method of taking CTM. Danshensu (I), protocatechuic acid (II), protocatechuic aldehyde (III) and salvianolic acid B (IV) have been isolated and identified as the main constitutes in water-soluble compounds [2]. Pharmacological experiments have demonstrated that IV can effectively restrain lipid peroxidation of the brain, liver and kidney; I can protect cardiac muscle from lacking blood and oxygen; III can increase the flux of coronary [3]. Therefore, it is of great practical significance to establish an analytical method for determining them.

To detect water-soluble components in *S. miltiorrhiza Bunge*, colorimetric method [4], ultraviolet spectrophotometric method [5], high-performance capillary electrophoresis (HPCE) [6], thin layer chromatography (TLC) [7] and high-performance liquid chromatography (HPLC) [8–15] have been reported. Nevertheless, the first two methods are usually interfered by other compounds; the results of TLC have a bad repeatability and accuracy while the sensitivity of HPCE is restricted by low sample injection and short optical length. Among them, HPLC is most widely used.

The coulometric electrode array detection (CEAD) would be an ideal choice to separate the overlap peaks of the co-eluting analytes because of electroactive phenolic hydroxyl group in these four aqueous soluble compounds (Fig. 1) [16]. CEAD consists of 4–16 electrochemical cells arranged in series and the

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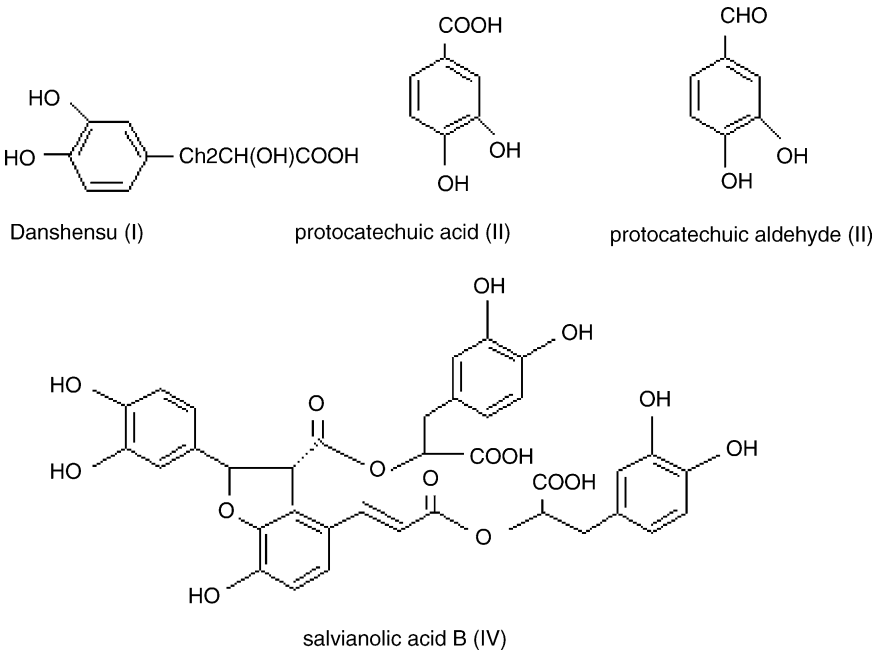

Fig. 1. Structures of the four water-soluble compounds.

potential of each cell can be set independently. It can provide superior sensitivity over amperometric detectors and ultraviolet detectors commonly used together with HPLC. Yao and co-worker [17] determined multiple redox-active compounds using HPLC–CEAD. However, up to now, it has not been applied in determining the water-soluble compounds in *S. miltiorrhiza Bunge*.

This paper presented a sensitive and reproducible method for determining compounds I, II, III and IV in *S. miltiorrhiza Bunge* using HPLC–CEAD system. Influence of the pH of mobile phases and potentials of electrode were considered to optimize the analysis conditions. *S. miltiorrhiza Bunge* from Hebei province (China) was analyzed and the chromatograms were presented.

2. Experimental

2.1. Chemicals and reagents

Standards of Sodium Danshensu, II, III and IV with purity of 100% were bought from the Institute of Chinese Pharmaceutical and Biological Product Inspection (Beijing, China). Sodium acetate, glacial acetic acid and trifluoroacetic acid (TFA) of analytical-grade were from Sigma (St. Louis, MO, USA). Methanol and acetonitrile was HPLC purity reagent from Fisher Science (NJ, USA). The water used in all experiments had a resistivity of 18.2 MΩ cm.

2.2. Apparatus and chromatographic conditions

Analysis was performed using an ESA chromatographic system (ESA, Chemsford, MA, USA) equipped with two solvent pumps (ESA Model 582), a manual sample injector with a 20 μL loop (Rheodyne 7725i, CA, USA) and a 5600A 16 channels CoulArray detector. ESA software was used for data acquisition and processing. Sartorius BS 110S electronic balance (Beijing, China), WTW inolab level 1 pH meter (Weiheim, Germany), Dupont Sorvall RC 5C plus centrifuge (Newtown, CT, USA) and a Mill-Q water purification system (Millipore, Bedford, MA, USA) were used in our study.

The four compounds were analyzed by reversed-phase liquid chromatography using a ODS Hypersil (250 × 4.6 mm i.d. 5 μm, Hewlett-Packard, USA) column and a Hypersil guard column (Hypersil, 5 μm, Alltech Association, USA). The mobile phase was component A (50 mM sodium acetate–0.1% TFA, pH 2.5) and B (acetonitrile) in the gradient mode as follows: 0–15 min, 6%B; 15–45 min, 6–22%B; 0.8 mL/min. Electrode potentials were set at 100, 150, 200 and 250 mV. Injection volume was 20 μL and the analyses were carried out at room temperature.

2.3. Sample preparation

Fifteen grams dried powder (40 mesh) of *S. miltiorrhiza Bunge* and 500 mL water was placed in a flask (1000 mL) and the suspensions were made to boil with refluxing for 2 h. The mixture was centrifugated at 10,000 × *g* for 10 min. The supernatant was diluted by 10 and 800 times with mobile phase and filtered through 0.22 μm membrane before loading into the column.

3. Results and discussion

3.1. Optimization of the pH of mobile phase

Relationships between pH and retention time, pH and peak area were shown in Fig. 2a and b. From Fig. 2a, retention time of I was too short which made it difficult to separate I from the solvent at pH 2.2 and 4, while those of the other three compounds

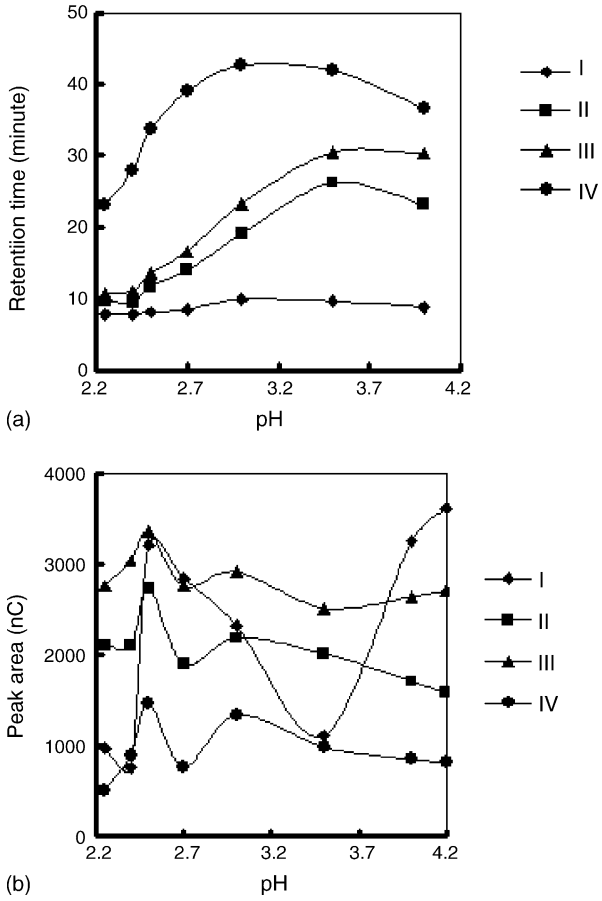

Fig. 2. Effect of pH on: (a) retention time of the four water-soluble compounds (b) peak area of the four water-soluble compounds (I 0.2 μg/mL; II, III and IV 0.1 μg/mL).

were too long from pH 2.5 to 3.5. In addition, from Fig. 2b, peak areas of the four compounds maximized at pH 2.5. Therefore, pH 2.5, at which all compounds could be separated well with short retention times, was chosen.

3.2. Voltammetric behavior of the four water-soluble compounds

Relationship between peak area (quantity of electricity) and potential (voltage) was shown in Fig. 3. It showed that I and IV could be oxidized at 50 mV, while II and III at 100 mV. Peak areas of I, II, III and IV could reach a plateau at 200, 250, 250 and 250 mV, respectively, where no increase in peak area occurred when the potential was increased. Based on the curve, the ratio between signals and noise was higher at 100, 150, 200 and 250 mV, which were selected finally for electrodes as the working potentials.

3.3. Calibration, linearity and limit of detection

Under the optimized experimental conditions, calibration curves of I, II and III showed good linearity in 0.03125–0.5 μg/mL, and IV showed good linearity in 0.075–1.0 μg/mL. Correlation coefficients (r) of these calibration curves were between 0.9937 and 0.9996. The detection limits were all no less than 12.5 ng/mL.

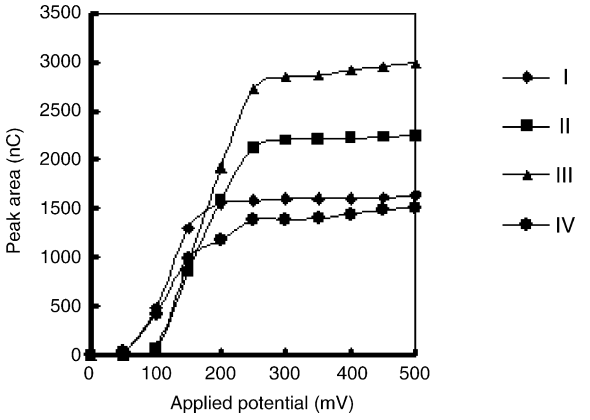

Fig. 3. Peak area–V curve for the oxidation of I, II, III and IV (I 0.2 μg/mL; II, III and IV 0.1 μg/mL).

3.4. Sample analysis

Sample of *S. miltiorrhiza Bunge* from Hebei province (China) was investigated. Average contents of I, II, III and IV of three batch of samples were 9.54, 0.058, 0.42 and 14.13 mg/g. Their average relative standard deviations (R.S.D.) were all below 1.7%. Detection limits of samples were all no less than 20 ng/mL. Chromatograms were shown in Fig. 4a and b. The

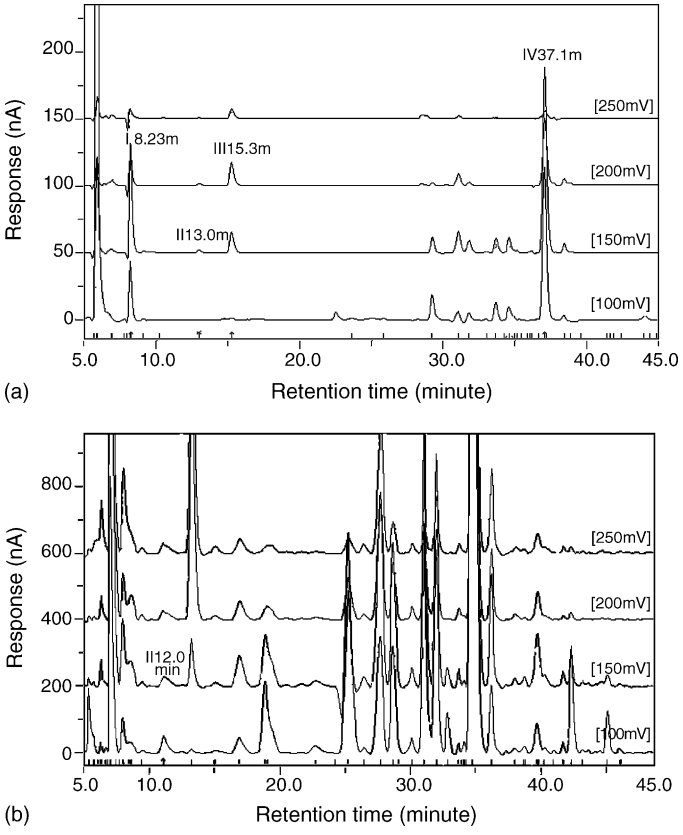

Fig. 4. HPLC chromatograms for: (a) sample diluted by 800 times; (b) sample diluted by 10 times.

Table 1
Recoveries of I, II, III and IV from *Salvia miltiorrhiza Bunge* samples ($n = 6$)[a]

Analytes	Spiked (μg/mL)	Inter-day			Intra-day		
		Concentration found		Recovery (%)	Concentration found		Recovery (%)
		Mean	R.S.D. (%)		Mean	R.S.D. (%)	
I	0.0625	0.0620	4.1	99.2	0.0618	4.2	98.9
	0.1250	0.1231	3.5	98.5	0.1229	3.6	98.3
	0.2500	0.2463	4.4	98.5	0.2424	4.8	97.0
II	0.0625	0.0621	6.5	99.4	0.0619	6.6	99.0
	0.1250	0.1240	3.5	99.2	0.1232	3.7	98.6
	0.2500	0.2450	2.7	98.0	0.2443	2.7	97.7
III	0.0625	0.0622	2.6	99.5	0.0623	2.7	99.7
	0.1250	0.1224	1.9	97.9	0.1224	2.0	97.9
	0.2500	0.2481	0.6	99.2	0.2487	1.0	99.5
IV	0.0625	0.0617	4.5	98.7	0.0617	4.5	98.7
	0.1250	0.1240	4.0	99.2	0.1236	4.1	98.9
	0.2500	0.2304	2.0	92.2	0.2364	2.4	94.6

[a] $n = 6$ means that the same sample has been pretreated by the same process on 6 consective days.

peak of I, III and IV was nice in Fig. 4a, but that of II was too small to be quantified. However, in Fig. 4b got from the sample diluted 10 times, the content of II could be gained.

3.5. Recovery test

Sample with known content was accurately weighed and added into 300 mL 0.0625, 0.125 and 0.25 μg/mL mixed standard solutions, respectively. They were pretreated and analyzed as Sections 2.3 and 3.4. Average recoveries of the four compounds were summarized in Table 1. From the table, it showed that the average recoveries were more than 92% and R.S.D. were all less than 6.6%. The results of intra- and inter-day were very similar which demonstrated good reproducibility and accuracy within the concentration range selected.

4. Conclusion

In summary, a method had been developed for determination of four water-soluble components in *S. miltiorrhiza Bunge*—Danshensu, salvianolic acid B, protocatechuic aldehyde and protocatechuic acid. The samples were pretreated with heat reflux extraction method. HPLC–CEAD system was chose to detect the target compounds. The whole method was stable, sensitive and reproducible. Therefore, this method also could be used to control the quality of *S. miltiorrhiza Bunge* from different places based on the accurate results.

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