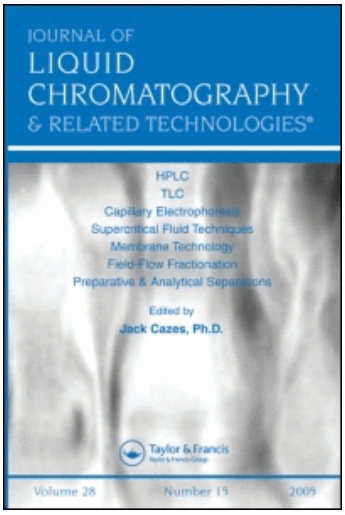

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Separation and Determination of Water Soluble Active Components in *Salvia miltiorrhiza* Bunge and Its Pharmaceutical Preparations by Capillary Zone Electrophoresis with Diode Array Detection

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Separation and Determination of Water Soluble Active Components in *Salvia miltiorrhiza* Bunge and Its Pharmaceutical Preparations by Capillary Zone Electrophoresis with Diode Array Detection

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Abstract: A novel, simple, and rapid method is developed for separating water soluble active components in *Salvia miltiorrhiza* Bunge and its pharmaceutical preparations by capillary zone electrophoresis with a diode array detector within 11 min under the optimal conditions. The repeatability (defined as relative standard deviation, RSD) was less than 4.45% with peak area evaluation and 2.13% with migration time evaluation. Regression equations revealed linear relationships (r: 0.9964–0.9987) between the peak area of each analyte and the concentration. The detection limits (S/N = 3) of Protocatechuic Aldehyde, Danshensu, and Protocatechuic Acid were 0.46, 0.62, and 0.28 μg/mL, respectively. The proposed method has been satisfactorily applied to the analysis of water soluble active components in *Salvia miltiorrhiza* Bunge and its pharmaceutical preparations with recoveries in the range of 94%–101%.

Keywords: Capillary zone electrophoresis, Danshensu, Separation, Pharmaceutical preparations

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INTRODUCTION

Traditional Chinese medicines have been extensively used to prevent and cure human diseases in Oriental countries for over a millennium. Because of their low toxicity and good therapeutical performance, traditional Chinese medicines have attracted considerable attention in many fields.[1] Also, their worldwide use has increased in the last decade.[2] *Salvia miltiorrhiza* Bunge, Danshen in Chinese, a popular traditional Chinese medicinal herb, has been applied in a wide range for the treatment of coronary heart disease, cerebrovascular disease, hepatitis, hepatocirrhosis, chronic renal failure, bone loss, arthritis, menstrual disorder, menostasis, dysmenorrhea, neuroasthenic insomnia, various neural diseases, osteoporosis, and diabetic complications.[3–10] Recent studies have confirmed many of its traditional properties and also uncovered some additional properties including anticoagulant, antibacterial,[11] antitumor,[12] antiatherosclerotic, antihypocholesterolemic, antihypertrophy,[13,14] and anti-inflammatory activity.[15] At the same time, there are many traditional Chinese medicine preparations (TCMPs) containing Danshen, such as Compound Danshen Tablets (CDT), Compound Danshen Dripping Pills (CDDP), Danshen Injection (DSI), Danhong Injection (DHI), Guanxinning Injection (GXNI), and Xiangdan Injection (XDI), among which Danshen is the major component. The TCMPs containing Danshen were mainly used to treat coronary heart disease, heart stroke, cerebrovascular diseases, and cardiovascular diseases.[16,17]

Compound Danshen Dripping Pill (Chinese name Fufang-Danshen-Diwan), for instance, an oral herbal medicine, has been well sold over a few years as a diet supplement or drug in a number of countries such as USA, Russia, Singaporea, and South Korea. *Salvia miltiorrhiza* Bunge contains two classes of major active compounds, namely tanshinones which are lipophilic and salvianolic acids which are hydrophilic.[18] Both of them are considered to be biologically active.[19,20] Before the 1970s, the studies were mainly focused on the lipophilic diterpenoid, which had been considered to be responsible for the clinical efficacy. However, due to the wide use of Danshen in clinical treatment, the water soluble constituents in *Salvia miltiorrhiza* Bunge were investigated.[21,22] Through pharmacological and clinical investigation, these phenolic acids were found to be the real active principles other than the lipophilic diterpenoids as reported previously.[23,24] In recent years, the water soluble components of *Salvia miltiorrhiza* Bunge has attracted increasing attention because of their effectiveness in improving the renal function of rats with adenine induced renal failure, their potential in treating Alzheimer's disease, and acting as an antioxidant for the removal of free radicals.[25–27]

Pharmacological tests showed that the water soluble phenols, Protocatechuic aldehyde (3,4-dihydroxybenzaldehyde) (PAH), Danshensu (3,4-dihydroxyphenyllactic acid) (DS), and Protocatechuic acid (3,4-dihydroxybenzoic acid) (PA) are major active components of *Salivia miltorrhrza* Bunge.[28,29] Danshensu, the major active components of *Salivia miltorrhrza*

Bunge, can dilate isolated coronary artery, decrease the biosynthesis of cholesterol in cells, and inhibit lipoprotein oxidation.[30] Therefore, a simple and rapid method to simultaneous determine the three components is highly desired. So far, there were several literatures reported for determination of Protocatechuic aldehyde and Protocatechuic acid, including thin-layer chromatography (TLC),[31] reversed phase high performance liquid chromatography (RP–HPLC), and HPLC–MS.[32–34] However, these methods were limited, owing to complex preparation procedures, high expenditures, or low sensitivity. Compared with the methods mentioned above, capillary electrophoresis (CE) can surpass high performance liquid chromatography (HPLC) in terms of simplicity, resolution, and economy.[35]

Capillary electrophoresis (CE) is a widely applied technique in separation science on account of its highly efficient peak separation, short analysis time, rapid rate of separation, lower cost, and it offers satisfactory results in some pharmaceutical analyses.[36] Capillary electrophoresis (CE) has become a popular tool for the determination of a variety of compounds in the last decade. Until now, only a few capillary electophoresis methods have reported for the determination of Protocatechuic aldehyde and Protocatechuic acid. Amperometric detection[37] and chemiluminescence determination[38] were used in these methods. Compared with UV detection, these two detectors are not very common. Further, Danshensu, the key factors of quality control for *saliva milto*rrhrza in Chinese Pharmacopoeia, has not been mentioned in all capillary electophoresis methods. In our results, Danshensu are the major components in all samples including *salvia miltiorrhiza* Bunge and its pharmaceutical preparations.

In order to ensure the clinical effects, it is important to develop a simple and rapid method for simultaneously monitoring the quality of Protocatechuic aldehyde, Danshensu, and Protocatechuic acid of preparations of *Salvia miltiorrhiza* Bunge.

In this study, a capillary zone electrophoresis (CZE) with diode array detection (DAD) method was developed for simultaneous determination of Protocatechuic aldehyde, Danshensu, and Protocatechuic acid. The determination of Danshensu using capillary zone electrophoresis was first reported. The method was simple, inexpensive, and precise, and it was also applied to the analysis of Protocatechuic aldehyde, Danshensu, and Protocatechuic acid in *Salvia miltiorrhiza* Bunge and its pharmaceutical preparations in order to control the quality of this important Chinese herb.

EXPERIMENTAL

Instruments

A HP-3D capillary electrophoresis system (Agilent Technologies, Waldbronn, Germany) equipped with a diode array detector was used. Instrumental control

and data acquisition were carried out with CE ChemStation software (Agilent Technologies). Capillary electrophoresis was performed using a 48.5 cm (40 cm from the inlet to the detector) × 75 μm i.d. uncoated fused silica capillary (Yongnian Photoconductive Fiber Factory, Hebei, China).

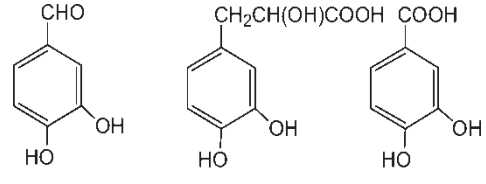
Materials

Procatechuic aldehyde (No.110810–200506), Danshensu (No.110855–200506), and Procatechuic acid (No.110809–200503) were purchased from the National Institute for Control of Pharmaceutical and Biological Products of China (Beijing, China). Their structures were shown in Figure 1.

HPLC grade methanol was purchased from Yucheng Chemical Reagent Co. (Shandong Province, China). Deionized water was purified by Milli-Q system (Millipore, Bedford, MA, USA). Phosphate buffer was prepared from 0.5 M phosphate acid solution adjusted to pH 7.2 with 2 M sodium hydroxide solution prepared from sodium hydroxide pellets. Phosphoric acid was of analytical grade from Beijing Beihua Fine Chemicals Co. Ltd. (Beijing, China). All other chemicals were of analytical reagent grade. *Salvia miltiorrhiza* Bunge, Compound Danshen tablets (CDT), Compound Danshen Dripping Pills (CDDP), Danshen Injection(DSI), Danhong Injection(DHI), Guanxinning Injection(GXNI), and Xiangdan Injection (XDI) was purchased from Lanzhou Pharmaceutical Company in Lanzhou, Gansu Province, China.

Preparation of Standard Solution

A concentrated stock solution of Procatechuic aldehyde, Danshensu, and Procatechuic acid was prepared in 10 mL solution, from which analytical solutions were prepared by appropriate dilution with deionized water to a final concentration of 2.2, 2.0, and 2.7 mg/mL. All of the stock solutions were stored at 4°C in a refrigerator and were diluted to required concentration with running buffer.



(1) (2) (3)

Figure 1. Structures of Protocatechuic aldehyde (1), Danshensu (2) and Protocatechuic acid (3).

Sample Preparation

Salvia miltiorrhiza Bunge, Compound Danshen Dripping Pills (CDDP) were finely powdered and ground, the sugar coatings of Compound Danshen tablets (CDT) were washed off with water, and the tablets were dried followed by being grinded into fine powder, and then 1.00 g, 1.00 g, 2.0 g powder were respectively weighed and extracted with 15 mL methanol in an ultrasonic bath for 1 h (*Salvia miltiorrhiza* Bunge) and 30 min (Compound Danshen tablets and Compound Danshen Dripping Pills). The final volume was made up to 15 mL after the solution was cooled down, and then the extract was centrifuged for 10 min with a rotation speed set as 1000 rpm. After being centrifuged, the extracts were concentrated to 10 mL, respectively. The solutions were passed through 0.45 μm membrane filters, and were injected into the capillary electrophoresis system. Danshen Injection (DSI), Danhong Injection (DHI), Guanxinning Injection (GXNI), and Xiangdan Injection (XDI) was diluted with deionized water at the ratio of 2 to 8 and filtrated through 0.45 μm filters for capillary electrophoresis analysis.

Capillary Electrophoresis Procedure

A new capillary column was activated by washing consecutively with each of 0.1 M hydrochloric acid (20 min), 0.1 M sodium hydroxide (30 min), and deionized water (30 min). At the beginning of each working day, the capillary was prewashed with 0.1 M HCl for 10 min, 0.1 M NaOH for 10 min, and running buffer for 20 min, respectively. Before each analysis, the capillary was consecutively rinsed with 0.1 M NaOH (2 min), water (2 min), and running buffer (5 min). The sample was loaded onto the column by pressure injection for 5s at 50 mbar. The running buffer consisted of 50 mM phosphate buffer (pH 7.2). Column temperature was set at 25°C. A constant voltage of 15 KV was applied during analysis. Diode array detection was set at 210 nm.

RESULTS AND DISCUSSION

Effect of Buffer Concentration

The buffer concentration plays a prominent role in electrophoretic separation. Consequently, more close examination of the effects of buffer concentration on the separation of the three analytes is highly desired. The running buffers consisting of phosphate at different concentration (30, 40, 50, 60, 70 mM) at pH 7.0 were investigated. The result is shown in Figure 2. The migration time and resolution of the three analytes increased with an increase of the concentration of running buffer. This is as a result of the decreased EOF, since this effect is directly related to the decrease of the

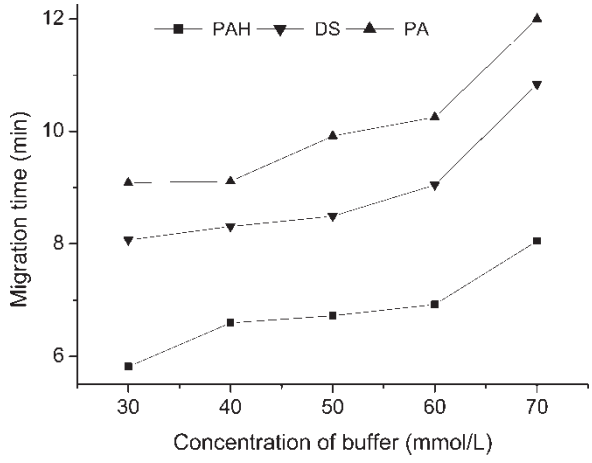


Figure 2. Effect of phosphate buffer on migration time. Analysis conditions: buffer, 30–70 mM phosphate (pH 7.0); applied voltage, 15 kV; temperature, 25°C; detection wavelength, 210 nm; uncoated fused-silica capillary, total length 48.5 cm (40 cm effective length) 75 μm i.d.

zeta potential at the capillary wall solution interface. The best resolution was achieved at 50 mM phosphate buffer.

Effect of Buffer pH

In electrophoretic separations of ionizable compounds, pH plays an important role as it determines the extent of ionization of each individual analyte, so it was a key step to manipulate the pH of the buffer in optimizing the conditions of the separation. In this study, the effect of pH on the separation was investigated in the range of 6.0 to 8.0 using 50 mM phosphate buffer solutions and 15 kV applied voltage, 25°C. Figure 3 showed the effect of buffer pH on the migration time. It can clearly be seen from Figure 3, that the migration time increased with the increasing the buffer pH. At pH < 6.50, PAH, and PA could not be separated. Separation of the analytes can be achieved from pH 6.5 to 8.0. When pH is 7.2, the best separation was obtained. Moreover, higher pH value results in longer analysis time. With concurrent consideration of peak area, resolution, and migration time, pH 7.2 was therefore preferred for further studies.

Effect of Organic Modifier

Generally speaking, the addition of organic modifier often plays an important role in the improvement of viscosity of medium, dielectric constant, and

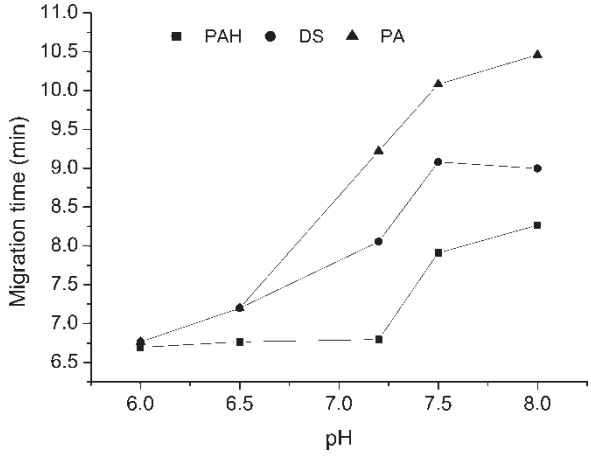


Figure 3. Effect of pH on migration time. Analysis conditions: buffer, 50 mM phosphate; pH, 6.0–8.0; applied voltage, 15 kV; temperature, 25°C; detection wavelength, 210 nm; uncoated fused-silica capillary, total length 48.5 cm (40 cm effective length) 75 μm i.d.

structure of the electric double layer of the capillary wall. In this study, various additives such as isopropanol, acetonitrile, and methanol were examined. After a series of designed experiments, we have found that the addition of organic modifier did not signally improve the resolution of analytes, so the organic modifier was not used in this experiment.

Effects of Temperature and Separation Voltage

The effect of capillary temperature on the resolution of Procatechuic aldehyde, Danshensu, and Procatechuic acid was investigated in the range of 15–35°C (Figure 4). The results indicated that resolution was not remarkably improved with increasing temperature, however, a slightly decrease in the migration time was observed. This is probably due to the fact that increased working temperature decreases run medium viscosity, resulting in a slight decrease in migration time; 25°C was selected for shorter analysis time and eclectic resolutions.

The influence of the separation voltage on the migration time and the separation of the analytes were also studied in this experiment. The higher voltage was necessary for rapid analysis, which could reduce molecular diffusion in the mobile phase and band spread. However, the resolutions of Procatechuic aldehyde, Danshensu, and Procatechuic acid also decreased with increasing working voltage from 15 to 35 KV (Figure 5). With concurrent considerations in the migration time and resolution, the separation voltage finally chosen was 15 kV.

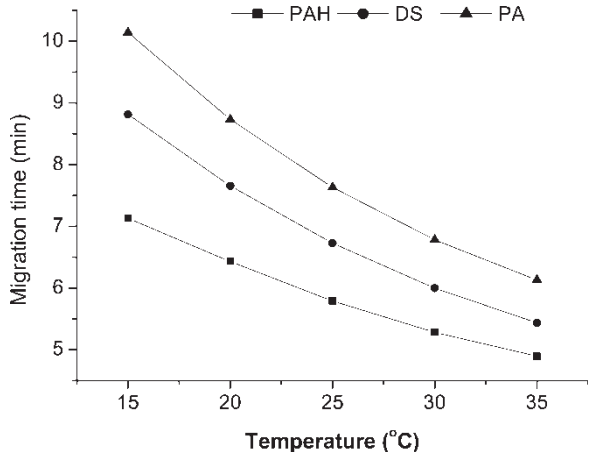


Figure 4. Effect of temperature on migration time. Analysis conditions: buffer, 50 mM phosphate; pH 7.2; temperature, 15–35°C; applied voltage, 15 kV; detection wavelength, 210 nm; uncoated fused-silica capillary, total length 48.5 cm (40 cm effective length) 75 μm i.d.

Final Optimization

Based on these results, the optimal conditions for the separation of Procatechuic aldehyde, Danshensu, and Procatechuic acid were set as follows: 50 mM phosphate buffer, pH 7.2, working voltage at 15 KV, temperature at

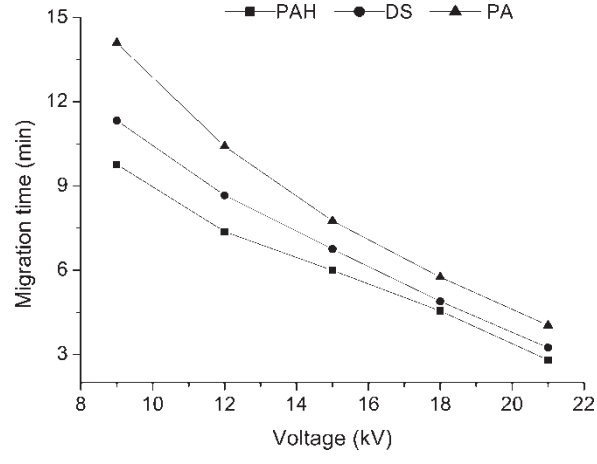


Figure 5. Effect of voltage on migration time. Analysis conditions: buffer, 50 mM phosphate; pH 7.2; applied voltage, 10–22 kV; temperature, 25°C; detection wavelength, 210 nm; uncoated fused-silica capillary, total length 48.5 cm (40 cm effective length) 75 μm i.d.

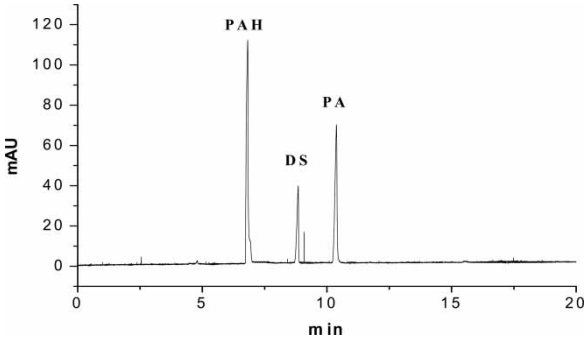


Figure 6. Electrophorogram of standard mixture of Protocatechuic aldehyde, Danshensu, and Protocatechuic acid. Analysis conditions: 50 mM phosphate; pH 7.2; applied voltage, 15 kV; temperature, 25°C; detection wavelength, 210 nm; uncoated fused-silica capillary, total length 48.5 cm (40 cm effective length) 75 μm i.d.

25°C. Figure 6 shows the separation of Procatechuic aldehyde, Danshensu, and Procatechuic acid in a capillary electrophoresis system under the proposed conditions.

Method Validation

Appropriate method validation information concerning new analytical techniques for analyzing pharmaceuticals is required by regulatory authorities. Validation of such methods includes assessment of the stability of the solutions, linearity, reproducibility, and limits of detection and quantification.

Stability of the Solutions

The stability of standard and sample solutions was determined by monitoring the peak area and migration time of standard mixture solutions and sample solutions over a period of 1 week. The results showed that the migration time and peak area of each analyte remained almost unchanged and that no significant degradation is observed within the given period, indicating the solutions are stable for at least 1 week without the results being affected.

Linearity, Limit of Detection (LOD) and Limit of Quantification (LOQ)

The relationship between peak area and concentration was determined by preparing different concentration standard solutions for three analytes and

Table 1. Regression equation, linear ranges and detection limits of protocatechuic aldehyde Danshensu and protocatechuic acid

Analytes	Regression equation[a]	Correlation coefficient	Linear range (mg/mL)	Detection limit (μg/mL)
Protocatechuic aldehyde	$Y = 2332.08X - 49.67$	0.9982	0.01–0.73	0.46
Danshensu	$Y = 2178.38X + 72.14$	0.9964	0.01−1.33	0.62
Protocatechuic acid	$Y = 7683.42X + 184.46$	0.9987	0.0075–0.90	0.28

[a]In the regression equation, the X value is the concentration of analytes (mg/mL), the Y value is the peak area.

good linearity was obtained. The corresponding regression equations, as well as other characteristic parameters for the determination of Procatechuic aldehyde, Danshensu, and Procatechuic acid were listed in Table 1. The detection limit is evaluated on the basis of a signal-to–noise ratio of 3. The LOD of three analytes ranged from 0.28 to 0.62 μg/mL, and the LOQ is defined as the level at, or above, at which the measurement precision is satisfactory for quantitative analysis. In our case, LOQ was evaluated on the basis of a signal to noise ratio of 10. The LOQ were 1.31 μg/mL, 1.93 μg/mL, and 0.92 μg/mL for Procatechuic aldehyde, Danshensu, and Procatechuic acid, respectively.

Reproducibility and Accuracy

The relative standard deviations (RSD) of the run-to-run repeatability ($n = 6$) of migration times and peak areas of the method were, respectively, 0.56 and 2.67% for Protocatechuic Aldehyde, 1.33 and 2.23% for Danshensu, and 1.30 and 2.40% for Protocatechuic Acid. The RSD of day-to-day reproducibility ($n = 3$) of migration times and peak areas were, respectively, 0.99 and 4.34% for Protocatechuic Aldehyde, 2.01 and 4.36% for Danshensu, and 2.13 and 4.43% for Protocatechuic Acid. The accuracy of the method was determined by adding appropriate amounts of Procatechuic aldehyde, Danshensu, and Procatechuic acid to samples and calculating the recoveries. Mean recoveries (%, $n = 3$) for Procatechuic aldehyde, Danshensu, and Procatechuic acid were 96, 94 and 101%, respectively.

Applications

Quantitative analysis was performed under the optimum conditions obtained from the experiments described above. The method was applied to the

analysis of Procatechuic aldehyde, Danshensu, and Procatechuic acid in *Salvia miltiorrhiza* Bunge and its pharmaceutical preparations. The peaks were identified by comparing the migration times and the standard addition methods. Figure 7 showed the typical electropherogram and Table 2 lists the quantities of three analytes in *Salvia miltiorrhiza* Bunge and its pharmaceutical preparations and their relative standard deviations. The relatively

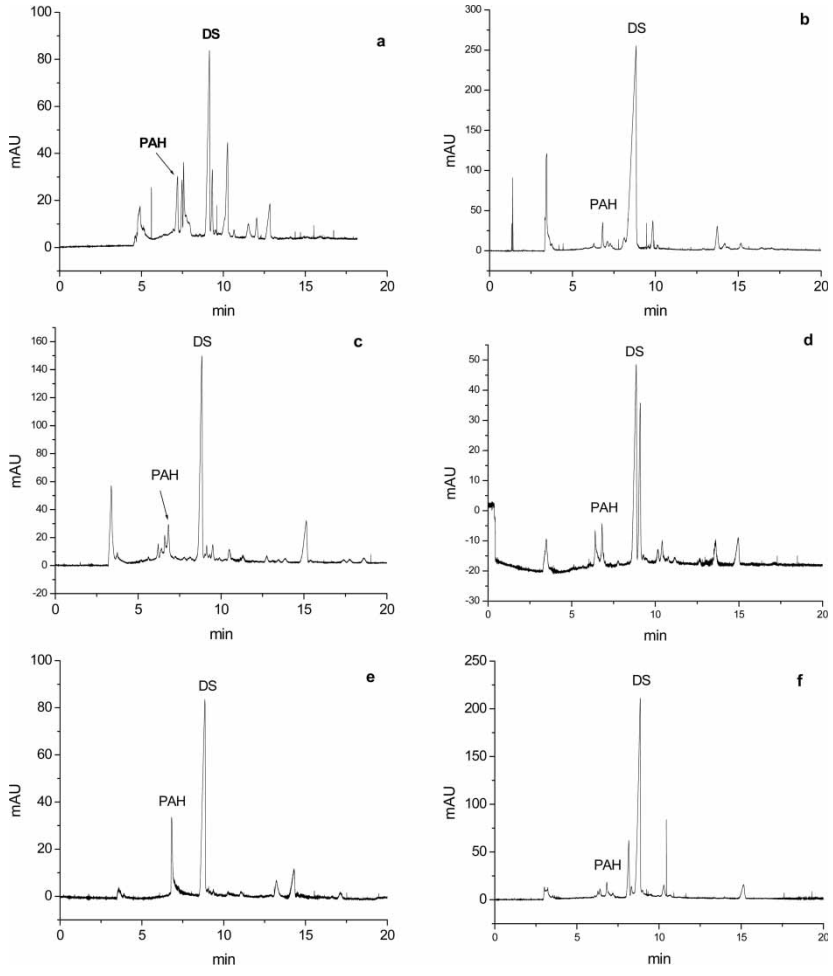


Figure 7. Electrophorograms in *Salvia miltiorrhiza* Bunge and its pharmaceutical preparations. a, *Salvia miltiorrhiza* Bunge; b, Compound Danshen Dripping Pills; c, Guanxinning Injection; d, Danhong Injection; e, Danshen Injection; f, Compound Danshen Tablets. Analysis conditions: 50 mM phosphate; pH 7.2; applied voltage, 15 kV; temperature, 25°C; detection wavelength, 210 nm; uncoated fused-silica capillary, total length 48.5 cm (40 cm effective length) 75 μm i.d.

Table 2. Results of sample analysis experiments ($n = 3$)

Sample	Component	Content	RSD (%)
Salvia miltiorrhiza bunge	PAH	2.20 mg/g	0.64
	DS	5.81 mg/g	0.53
Compound Danshen dripping pills	PAH	1.86 mg/g	1.38
	DS	9.89 mg/g	1.15
Guanxinning injection	PAH	0.44 mg/mL	0.97
	DS	1.50 mg/mL	1.02
Danhong injection	PAH	0.33 mg/mL	1.13
	DS	1.89 mg/mL	1.03
Danshen injection	PAH	0.18 mg/mL	0.86
	DS	0.76 mg/mL	0.95
Compound Danshen tablets	PAH	1.3 mg/g	1.12
	DS	15.7 mg/g	1.04

large R.S.D. of the real sample was probably due to the complexity of in *Salvia miltiorrhiza* Bunge and its pharmaceutical preparations and the heterogeneity of the concentrated powder. It was observed, that the present method was applicable in the analysis of herbal medicines and was easily used for the analysis of the herb. Especially, it is very simple and sensitive. The reproducibility and detection limits were better, and the analysis time was shorter.

CONCLUSIONS

A simple and fast method for the simultaneous determination of Procatechuic aldehyde, Danshensu, and Procatechuic acid in *Salvia miltiorrhiza* Bunge and its pharmaceutical preparations by capillary zone electrophoresis (CZE) with diode array detector (DAD) has been demonstrated for the first time. Under the optimized conditions, those bioactive compounds could be successfully determined within 11 minutes. The method can be used for both screening experiments and high throughput routine testing. The results in this report indicate that the capillary zone electrophoresis (CZE) with diode array detector method is very useful and reliable for the simultaneous identification and determination of Procatechuic aldehyde, Danshensu, and Procatechuic acid.

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