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Short communication

Determination of paeonol and paeoniflorin in Chinese medicine Cortex Moutan and 'Shuangdan' granule by micellar electrokinetic capillary chromatography

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Abstract

An easy, simple and rapid micellar electrokinetic capillary chromatography (MEKC) method was developed for the separation of two active components paeonol (PN) and paeoniflorin (PF) within 7 min. Capillary electrophoresis (CE) was performed using a 50.0 cm (42.0 cm to the detector window) ×75 μm i.d. fused-silica capillary. The optimal running buffer containing 10 mM borate and 25 mM SDS at pH 9.54 was employed. The applied voltage 15 kV and the temperature 25 °C was used in CE separation. The linearities between peak areas and the concentrations of the analytes were investigated, and they exhibited excellent linear behavior over the investigated concentration ranges (R^2: 0.9945 for PN and 0.9992 for PF). The method was successfully applied to the determination of these two components contained in Cortex Moutan and 'Shuangdan' granule. The average recoveries ranged between 97.6 and 105.3% for PN and 95.3 and 106.1% for PF, respectively.

Keywords: Micellar electrokinetic capillary chromatography; Paeonol; Paeoniflorin; Cortex Moutan; 'Shuangdan' granule

1. Introduction

Cortex Moutan (Chinese name Danpi) has been shown to clear heat, cool blood, promote blood flow, activate blood circulation, eliminate blood stasis, rid of clots, drain pus and reduce swelling due to the presence of many bioactive ingredients [1–3]. 'Shuangdan' granule, made from Salvia Miltiorrhiza and Cortex Moutan, is one well-sold traditional Chinese medicine to treat the cardiovascular diseases. Paeonol (PN) is a major phenolic component of Cortex Moutan, and it is known to have anti-aggregatory, anti-oxidant and anti-inflammatory activities. The mechanisms by which PN exerts its anti-inflammatory and analgesic effects had been investigated [4]. Even inhibitory effects of PN on angiogenesis and tumor metastasis were studied [5]. The effects of PN on anaphylactic reaction and its mode of action were also elucidated [6]. Paeoniflorin (PF), a naturally anti-hyperlipidemic agent [7], is another important component contained in Cortex Moutan. Its anti-thrombotic effect has been evaluated, and it can significantly prolong thromobosis time [8]. The chemical structures of PN and PF are shown in Fig. 1.

The main obstacle for the worldwide use of traditional Chinese medicines is the quality evaluation and control in productions, therefore, the quantitative determination of the active components is the crucial technique for the evaluation and popularization of traditional Chinese medicines [9,10]. Up to now, several methods such as thin-layer chromatography, high-performance liquid chromatography and gas chromatography have been used for the determination of PN and/or PF in Cortex Moutan or other natural products. However, the chromatographic methods above have limitations of lower efficiency, time-consuming, and requiring larger amounts of organic solvents [11,12]. Therefore, it is urgent need of a new and reliable method capable of rapidly and simultaneously separating and determining these two active components in Cortex Moutan.

Capillary electrophoresis (CE) has been successfully applied to the analysis of the complex matrices because of its superiorities such as high resolution, small sample and solvents consumption, and high separation efficiency [13–16]. Micellar electrokinetic capillary chromatography (MEKC) is one important separation mode of CE, and it can be employed for the

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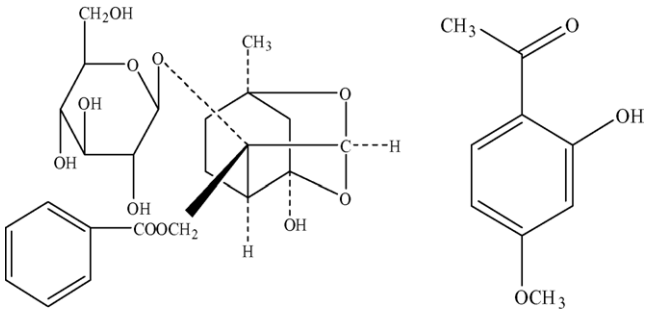

Fig. 1. Structures of paeoniflorin and paeonol.

separation of not only charged but also neural compounds by means of its capacity to partition molecules between aqueous phase and the pseudo-stationary micellar phase. It has been proven that MEKC can compete with HPLC with regard to the efficiency and selectivity [17], and several MEKC methods have been developed for the analysis of natural products [18,19]. In this study, a simple and rapid MEKC method for the separation and determination of two active components PN and PF in Cortex Moutan and 'Shuangdan' granule was developed. The optimal separation was obtained based on the systematic investigation of the influences of the buffer pH, concentration of buffer and SDS, organic addictives, applied voltage, injection time and running temperature.

2. Experimental

2.1. Instruments

A HP^{3D}CE capillary electrophoresis system (Agilent Technologies, Waldbronn, Germany) equipped with a diode-array detector was used. Instrumental control and data acquisition was carried out with CE ChemStation software (Agilent Technologies). Capillary electrophoresis was performed using a 50.0 cm (42.0 cm from the inlet to the detector window) $\times$75 μm i.d. fused-silica capillary (Yongnian Photoconductive Fiber Factory, Hebei, China). Prior to the first use, the new capillary was conditioned with 1 M NaOH for 30 min and 0.1 M NaOH for 10 min, followed by demineralised water for 5 min and the running buffer for 5 min. Between two runs, capillary was washed with 0.1 M NaOH for 1 min, followed by demineralized water for 1 min, and then equilibrated with the running buffer for 2 min. The capillary was reconditioned with 1 M NaOH for 3 min and demineralized water for 2 min after every five runs. The running buffer was renewed after every three runs to ensure the good reproducibility. Direct UV detection was employed with a wavelength of 233 nm. The sample was introduced hydrodynamically at 30 mbar. A pHs-3C pH meter (Leici Instrumentation Factory, Shanghai, China) was used for pH measurements.

2.2. Chemicals and reagents

PN and PF were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Cortex Moutan was purchased from a Chinese medicine store in Hangzhou (Zhejiang, China). 'Shuangdan' granule was supplied by a Chinese medicine factory in Shangdong province of China. Sodium dodecyl sulphate (SDS) and borate were both obtained from Merck (Darmstadt, Germany). Other reagents were of analytical grade. Water used throughout the experiments was generated by a Milli-Q academic water purification system (Milford, MA, USA).

2.3. Buffer and standard solution preparation

Stock standard solutions of PN (1000 mg/L) and PF (1000 mg/L) were both prepared with methanol. Working standard solutions were prepared by the appropriate dilution from the stock solution. The running buffer, containing the borate and SDS with an appropriate pH value, was prepared daily in the water. The pH value of the buffer was adjusted with NaOH or HCl solution. All solutions for CE analysis were ultrasonically degassed and filtered through a 0.45 μm syringe filter before using.

2.4. Sample preparation

The powdered Cortex Moutan (2.0 g) was extracted with 16 mL of 70% (v/v) aqueous ethanol in an ultrasonic bath for 30 min and then centrifuged for 10 min. The extracts were filtered through a 0.45 μm membrane filter. The filtrate was diluted with 70% (v/v) aqueous ethanol to 25 mL, and 25-fold dilution was performed before injecting into the CE system. Sample preparation procedure of 'Shuangdan' granule was the same as that of Cortex Moutan, but only five-fold dilution was performed due to the lower concentration of PN and PF in the medicinal preparation.

3. Results and discussion

3.1. Selection of running buffer

Phosphate and borate are two common buffers used in CE separation. Though the separation of the standard solution by these two buffers was well accomplished, their performances in the real sample separation were quite different. Furthermore, the solvency of SDS in the phosphate buffer was much weaker than that in the borate buffer, and the stability of SDS solution in the phosphate buffer was much easier to be destroyed when the temperature and pH value slightly fluctuated. Thus, the borate buffer was used in this experiment.

3.2. Influence of buffer pH

It is considered that the buffer pH is one of the most important parameters in CE separation. Twenty millimoles of borate buffer at different pH (8.00, 8.50, 9.00 and 9.50) were used to investigate the effect of pH on the separation. Each buffer contained 20 mM SDS. It was found that the migration time of PN and PF was quite stable in the investigated pH range. The initial pH value of the borate solution is approximately 9.54, and it was

performed well in the real sample separation, so pH 9.54 was adopted in the separation.

3.3. Influence of buffer concentration

The influence of borate (pH 9.54) concentration on the migration time of analytes was examined from 10 to 40 mM. It was shown that the migration time of PN and PF both was increased with the scale of borate concentration. The effect may be attributed to the decreased electroosmotic flow (EOF). The running current also increased with the increasing buffer concentration for the increased ionic strength. To decrease the Joule heating and the analysis time, the buffer with lower concentration was much better than others to perform the separation, thus 10 mM was selected as the experimental buffer concentration, and it worked well in the real sample separation.

3.4. Influence of SDS concentration

The effect of SDS concentration on the separation was tested in the range of 10–30 mM. The result (Fig. 2) showed that the migration time of PN and PF was increased with the increasing concentration of SDS. The effect was due to the decreased EOF. In the real sample analysis, the separation resolution of PN/PF to other components was well improved when the concentration of SDS exceeded 20 mM. Thus, 25 mM SDS was chosen since the baseline separation was achieved in the real sample separation.

3.5. Influence of applied voltage

The influence of applied voltage on the separation was studied in the range of 10–20 kV at pH 9.54 with 10 mM borate and 25 mM SDS, and the result was shown in Fig. 3. Linear relationship between the applied voltage and running current indicated that there was not the effect of Joule heating in this range of voltage. With the increase of the voltage, the electroosmotic flow (EOF) also increased, which resulted in a shorter analysis time. However, the peak areas of the analytes were decreased dramatically once the applied voltage was larger than 15 kV, because the peak width became much narrow when the mobility of the analytes passed the detector window was accelerated. So,

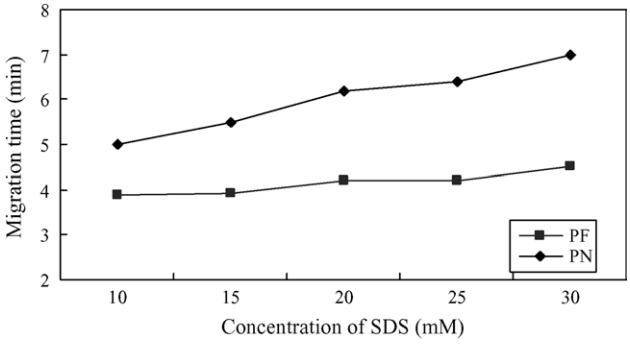

Fig. 2. Effect of SDS concentration on the migration time of the analytes. Buffer: 10 mM borate, pH 9.54: 50.0 cm (42.0 cm to the detector window) ×75 μm i.d.; applied voltage: 15 kV; temperature: 25 °C; detection: 233 nm; sample injection time: 3 s with a 30 mbar pressure.

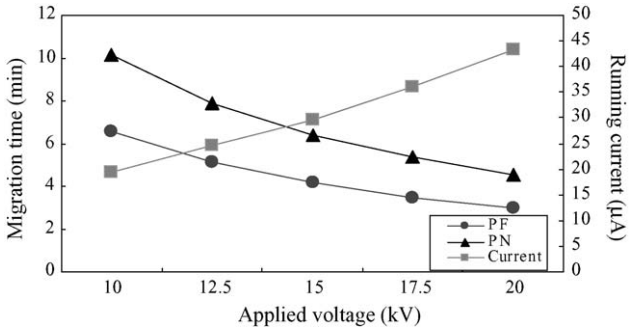

Fig. 3. Effect of applied voltage on the migration time and running current. Buffer: 10 mM borate, 25 mM SDS, pH 9.54; other condition as in Fig. 2.

the applied voltage 15 kV was applied, and it worked well in the real sample separation.

3.6. Influence of other factors

The influence of the organic additive was examined using the buffer containing 10 mM borate–25 mM SDS at pH 9.54, and 10% additive was used. The results of the real sample separation in the presence of acetonitrile, methanol and ethanol were shown in Fig. 4. The separation efficiency was improved when the organic additive was added. However, the detection sensitivity of PN was decreased by comparing Fig. 4D with Fig. 4A–C. Thus, the organic additive was not added to the running buffer.

Besides the factors mentioned above, other factors such as the injection time and the cassette temperature were also investigated. The separation was performed with the injection times ranged from 1 to 10 s at pressure 30 mbar. It was shown that the shorter was the injection time, the better was the separation efficiency. However, it was difficult to obtain the good injection repeatability once the injection time was too short. Therefore, the injection time was set at 3 s in the real sample separation. The separations at different cassette temperature (20, 25 and 30 °C) were also performed, and the influence of temperature on the separation was not notable. For the easy control, room temperature (25 °C) worked as the cassette temperature.

3.7. Optimized results

As the results mentioned above, the optimized separation condition was obtained with the running buffer containing 10 mM borate and 25 mM SDS at pH 9.54, 15 kV applied voltage, 25 °C cassette temperature, and 30 mbar × 3 s injection condition. The typical capillary electrophoregrams for the blank, standard, Cortex Moutan and ‘Shuangdan’ granule using the optimized condition were shown in Fig. 5. The separation was well achieved within 7 min.

3.8. Linearity, range and detection limits

Calibration curves for these two components were assessed at seven concentration levels, and triplicate injections were applied at each concentration. The linearity of each standard curve was

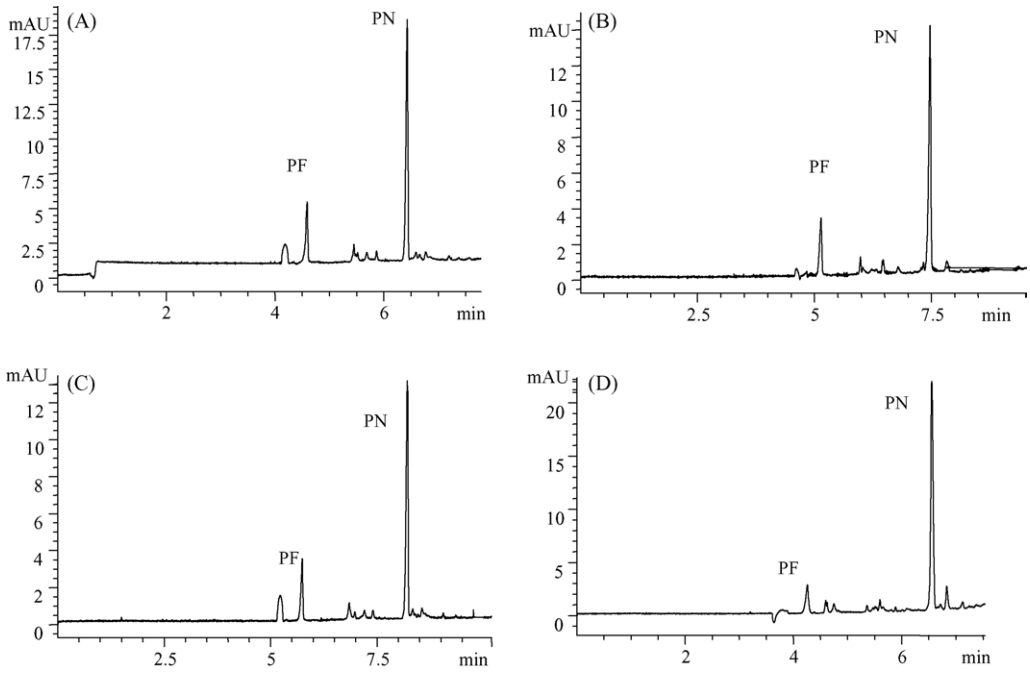

Fig. 4. Capillary electrophoregrams of Cortex Moutan sample solution with different additives (A, acetonitrile; B, methanol; C, ethanol; D, no additives). Buffer: 10 mM borate, 25 mM SDS, pH 9.54; capillary: 50.0 cm (42.0 cm to the detector window) ×75 μm i.d.; applied voltage: 15 kV; temperature: 25 °C; detection: 233 nm; sample injection time: 3 s with a 30 mbar pressure.

confirmed by plotting the peak area (y) and the concentration (x, mg/L), and the linear regression equations were shown in Table 1. The calibration curves revealed good linear behavior over the investigated concentration range (R^2: 0.9945 for PN and 0.9992 for PF). Limit of detection (LOD) and quantitation (LOQ) were also given in Table 1.

3.9. Repeatability and precision

The repeatability of the injection and the analysis of the real sample solution were both shown in Table 2. R.S.D. value for the analysis repeatability was a little higher than that of the injection repeatability. The intra-assay precision was performed

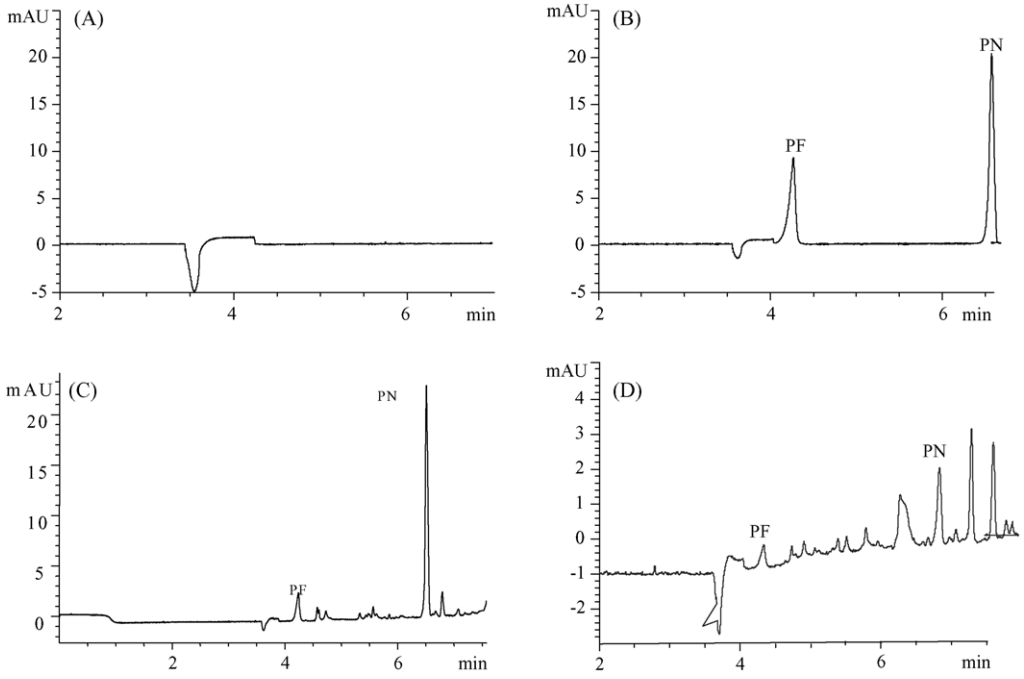

Fig. 5. Capillary electrophoregrams of the blank solution (A), spiked solution (B), Cortex Moutan (C) and 'Shuangdan' granule (D). Buffer: 10 mM borate, 25 mM SDS, pH 9.54; capillary: 50.0 cm (42.0 cm to the detector) × 75 μm i.d.; applied voltage: 15 kV; temperature: 25 °C; detection: 233 nm; sample injection time: 3 s with a 30 mbar pressure.

Table 1
Results of regression analysis on calibration curves and detection limits

Components	Regression equation, $y = ax + b$[a]	R^2	Linear range (mg/L)	LOD (mg/L)[b]	LOQ (mg/L)[c]
PN[d]	$y = 1.0272x - 2.7059$	0.9945	25.5–204	0.55	1.83
PF	$y = 0.3029x - 0.4660$	0.9992	10.0–400	2.28	7.59

[a] y and x stand for the peak area and the concentration (mg/L) of the components, respectively.
[b] The limit of detection is defined as the concentration where the signal-to-noise ratio is 3.
[c] The limit of quantitation is defined as the concentration where the signal-to-noise ratio is 10.
[d] For abbreviations of the components, please see text.

Table 2
Repeatability of PN and PF in the real sample separation ($n = 5$)

Components	Migration time (mm)				Peak area (mAU s)			
	Injection repeatability		Analysis repeatability		Injection repeatability		Analysis repeatability	
	Average	R.S.D. (%)	Average	R.S.D. (%)	Average	R.S.D. (%)	Average	R.S.D. (%)
PN[a]	6.527	0.25	6.403	1.11	70.266	3.86	66.950	6.22
PF	4.247	0.21	4.215	1.06	10.821	4.46	11.295	6.46

[a] For abbreviations of the components, please see text.

Table 3
Intra-assay and inter-assay precision of PN and PF in the real sample separation ($n = 5$)

Components	Migration time (min)		Peak area (mAU s)	
	Average	R.S.D. (%)	Average	R.S.D. (%)
Intra-assay				
PN[a]	6.251	0.19	71.983	2.52
PF	4.245	0.17	11.073	3.96
Inter-assay				
PN	6.775	3.57	74.648	5.83
PF	4.307	1.67	10.914	4.36

[a] For abbreviations of the components, please see text.

by analyzing samples with the interval of 2 h in one day, and the inter-assay precision was performed over 5 days. R.S.D. values of inter-assay and intra-assay precision were both shown in Table 3.

3.10. Applications

The developed method was applied to the determination of PF and PN in Cortex Moutan and 'Shuangdan' granule. Each sample was quantitatively examined by analyzing five times with the developed method. The measurement results were given in Table 4. Because of the complexity of herbal matrix, there has neither a standard method for the determination of these two active components nor a standard reference. Therefore, recovery of the standard from samples is used to evaluate the accuracy of the developed CE method to determinate the components in herbs. The recovery of this method at three different levels was performed, and the results were shown in Table 5.

Table 4
Contents of PN and PF in Cortex Moutan and its related medicinal preparation ($n = 5$, mg/g)[a]

Samples	PN[b]	PF
Cortex Moutan	20.61 ± 0.986	11.44 ± 0.501
'Shuangdan' granule	1.96 ± 0.072	1.49 ± 0.065

[a] Each value represents the mean ± S.D. ($n = 5$).
[b] PN and PF are the abbreviations of two components, please see the text. Sample preparation procedure was given in Section 2.4.

Table 5
Recovery test of Cortex Moutan extracts and 'Shuangdan' granule ($n = 3$)[a]

Samples	Base value (mg/L)	Quantity added (mg/L)	Quantity found (mg/L)	Recovery (%)
Cortex Moutan				
PN[b]	73.86	183.60	265.60	104.0
		81.60	153.47	97.6
		68.00	148.08	103.5
PF	39.75	105.00	148.71	103.8
		43.20	80.92	95.3
		33.60	73.62	100.8
'Shuangdan' granule				
PN	22.39	81.00	101.68	97.9
		31.32	53.61	99.7
		24.84	48.54	105.3
PF	12.82	131.25	142.94	99.1
		25.20	38.56	102.1
		21.01	35.10	106.1

[a] Each value represents the mean. The experiments were performed in triplicate.
[b] PN and PF are the abbreviations of two components, please see the text. Sample preparation procedure was given in Section 2.4.

4. Conclusion

A MEKC method has been developed for the separation of PN, PF and other unknown compounds coexisting in Cortex Moutan and 'Shuangdan' granule within 7 min under the optimal conditions. The results showed that the method is simple and rapid for the determination of PN and PF. The proposed method is applicable for the quality control of Chinese herbal materials Cortex Moutan and its medicinal preparations.

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