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Determination of glycosides and sugars in Moutan Cortex by capillary electrophoresis with electrochemical detection

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Abstract

A method based on capillary electrophoresis with electrochemical detection has been developed for the separation and determination of paeoniflorin, sucrose, paeonoside, glucose, and fructose in Moutan Cortex for the first time. Effects of several important factors such as the concentration of NaOH, separation voltage, injection time, and detection potential were investigated to acquire the optimum conditions. The detection electrode was a 300 μm diameter copper disc electrode at a working potential of +0.60 V (versus saturated calomel electrode (SCE)). The five analytes can be well-separated within 12 min in a 40 cm length fused silica capillary at a separation voltage of 12 kV in a 75 mM NaOH aqueous solution. The relation between peak current and analyte concentration was linear over about 3 orders of magnitude with detection limits (S/N = 3) ranging from 0.9 to 1.3 μM for all analytes. The proposed method has been successfully applied to monitor glycoside and sugar contents in the real plant samples with satisfactory assay results.

Keywords: Moutan Cortex; Glycoside; Sugar; Capillary electrophoresis; Electrochemical detection

1. Introduction

As a commonly used traditional Chinese medicine (TCM), Moutan Cortex is the dried root cortex of *Paeonia suffruticosa* Andrews, which belongs to the paeoniaceae family. It can cool blood, promote blood circulation, and remove blood stasis without inducing bleeding [1]. Moutan Cortex has been frequently used as an important ingredient in many traditional prescriptions. Recent investigation demonstrated its effects such as antiaggregatory [2], radical scavenging [3], and inhibition of phenylhydroquinone-induced oxidative DNA cleavage [4], etc. A variety of physiologically active compounds (such as paeonoside, paeonolide, apiopaeonoside, paeoniflorin, oxypaeoniflorin, benzoyloxypaeoniflorin, benzoylpaeoniflorin, paeonol, etc.) have been found presented in Moutan Cortex. They have close relation with the quality of the herbal drug because different functions of the herbal drug come from different active constituents [5]. As the primary metabolites, sucrose, glucose, and fructose are found widely presented in plants. It has been demonstrated that higher contents of sugars can indicate the better quality of some herbal drugs [6]. Hence, it is interesting to establish some rapid, simple, and accurate approaches for the determination of the bioactive substances and some co-existent constituents in Moutan Cortex.

Liquid chromatography (LC) was the most commonly used method for the determination of bioactive constituents in Moutan Cortex [7], composite herbal preparations with Moutan Cortex as their ingredient [8], and biological samples [9,10]. In addition, micellar capillary electrophoresis [11] and gas chromatography [12] have also been employed for the quantitative determination of paeonoside and some co-existent bioactive constituents in Moutan Cortex and multi-component traditional Chinese herbal medicines. Separation and determination of various constituents in plant drugs is always a complicated and challenging task. Nowadays, the application of CE for the separation of various active constituents in medicinal plants has become increasingly widespread because of its minimal sample volume requirement, short analysis time and high separation efficiency [13,14]. Electrochemical detection (ED) typically operated in the amperometric mode can be coupled with CE to provide high

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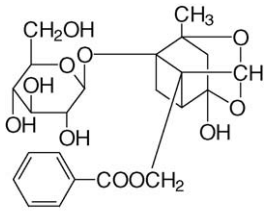
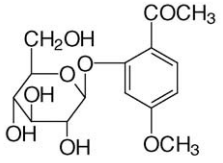


Paeoniflorin



Paeonoside

Fig. 1. Molecular structure of paeoniflorin and paeonoside.

sensitivity and selectivity for the determination of electro-active substances [15,16]. Nowadays, it is of high importance to control the quality of herbal drugs based on their active constituents and some co-existent substances. In 2000, the U.S. Food and Drug Administration (FDA) published a draft of Guidance for Industry Botanical Drug Products. Before a plant drug can become legally marketed, its spectroscopic or chromatographic fingerprints and chemical assay of the characteristic markers are required. CE should find more applications in this area.

In this study, CE-ED was employed for the determination of paeoniflorin, sucrose, paeonoside, glucose, and fructose (the molecular structures of paeoniflorin and paeonoside are shown in Fig. 1) in Moutan Cortex without derivatization for the first time. Because all the five constituents in the crude drugs contain nearby hydroxyl groups that are electro-active at modest oxidation potential on copper electrode in alkaline medium, ED was employed for their sensitive and selective detection in this work. This method is simple, sensitive, selective, and efficient, providing not only a way for evaluating the quality of Moutan Cortex and plant medicines made from Moutan Cortex in marketplaces, but also an alternative approach for quality control in medicinal factories and constituent investigations of other related plants. In a previous report, CE-ED has been employed for the determination of paeoniflorin in Radix Paeoniae Alba, the dried root of *Paeonia lactiflora* Pall [17]. To our best knowledge, there are no earlier reports published on the determination active constituents in Moutan Cortex by CE-ED. The optimization, detailed characterization, and advantages of the CE-ED approach are reported in the following sections in connection to the measurement of the five active constituents in the crude drugs.

2. Experimental

2.1. Reagent and solutions

Paeoniflorin and paeonoside were supplied by National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) while sucrose, glucose, and fructose were all purchased from Sigma (St. Louis, MO, USA). All aqueous solutions were made up in doubly distilled water. Other chemicals were of analytical grade.

Stock solutions of paeoniflorin, sucrose, paeonoside, glucose, and fructose (20 mM) were all prepared in doubly distilled water and were kept in a 4 °C refrigerator. They were stable for at least 1 month. The *background electrolyte* was 75 mM NaOH aqueous solution unless mentioned otherwise. The stock solutions were diluted to the desired concentration with the background electrolyte just prior to use.

2.2. Apparatus

The CE-ED system used has been described previously [17,18]. A ±30 kV high-voltage dc power supply (Shanghai Institute of Nuclear Research, China) provided a separation voltage between the ends of the capillary. The inlet of the capillary was held at a positive potential and the outlet of capillary was maintained at ground. The separations were carried out in a 40 cm length of 25 μm i.d. and 360 μm o.d. fused silica capillary (Polymicro Technologies, Phoenix, AZ, USA).

A three-electrode electrochemical cell consisting of a laboratory-made 300 μm diameter copper disc working electrode, a platinum auxiliary electrode, and a saturated calomel electrode (SCE) as the reference electrode, was used in combination with a BAS LC-4C amperometric detector (Bioanalytical Systems Inc., West Lafayette, IN, USA). The filter of the detector was set at 0.1 Hz. The working electrode was positioned carefully opposite the outlet of the capillary with the aid of a micromanipulator (CORRECT, Tokyo, Japan) and arranged in a wall-jet configuration. The distance between the tip of the working electrode and the capillary outlet was adjusted to ~25 μm by comparison with the bore (25 μm) in the capillary while being viewed under a microscope. The electropherograms were recorded using a LKB·REC 1 chart record (Pharmacia, Sweden). A YS 38–1000, 220 V alternate constant-voltage power supply (Shanghai Instrumental Transformer Factory, Shanghai, China) was employed to suppress the voltage fluctuation of the power line. The whole system was assembled in a *laboratory* that was air-conditioned at 25 °C to minimize the *temperature fluctuation*.

2.3. Sample preparation

Two samples of Moutan Cortex were obtained from Sun-Tian-Tang Traditional Chinese Medicine Store (Shanghai, China). They were all dried at 60 °C for 2 h and then were pulverized. About 2.0 g of the powder was weighed accurately and dispersed in 100 ml doubly distilled water. The mixture was kept in a 90 °C water bath for 2 h. After cooling, it was sonicated for 30 min and filtered through a filter paper. The extract was diluted using 75 mM NaOH aqueous solution at a ratio of 5 (1–5) just prior to CE analysis.

2.4. Procedures

Before use, the copper disc electrode was successively polished with emery paper and alumina powder and sonicated in

doubly distilled water. The capillary used for the separation were treated before use by flushing with 0.1 M NaOH and doubly distilled water for 10 min each. Subsequently, the capillary was filled with the background electrolyte and was conditioned with the background electrolyte for at least 10 min at the voltage of 12 kV between the two ends of the capillary. CE was performed at a separation voltage of 12 kV, unless otherwise indicated. The potential applied to the working electrode was +0.60 V (versus SCE). Samples were injected electrokinetically into the capillary at 12 kV for 6 s. Before injection, both the anode end of the capillary and the platinum-wire anode were moved from the anode solution to the sample solution. After an injection voltage of 12 kV was applied between the two ends of the capillary for 6 s, the sample solution could be introduced into the capillary. The anode end of the capillary together with the anode was then quickly returned to the anode solution. A voltage of 12 kV was subsequently applied in the constant-voltage mode for CE separation. The amperometric detector was on during the injection procedures. Note that the cathode solution in the electrochemical detection cell, the anode solution, and the sample solution were all at the same level. Moreover, sample solutions, standard solutions, and the background electrolyte were all filtered through a polypropylene filter (0.22 μm, Shanghai Bandao Industry Co. Ltd., Shanghai, China) prior to their use. Peak identification was performed by the standard-addition method.

3. Results and discussion

3.1. Hydrodynamic voltammograms (HDVs)

Carbohydrates and their derivatives are not normally electroactive at carbon electrodes, the most commonly used working electrode in ED. Thus, a variety of metal electrodes made of copper, nickel, gold, and platinum have been employed for the electrochemical detection of carbohydrates and their derivatives [19–26]. Among them, copper and nickel are most widely utilized. Carbohydrates and their derivatives can be detected by copper electrodes at a constant applied potential in strongly alkaline media based on electrocatalytic oxidation [19,24]. It has been revealed that the minimum structural requirement for facile oxidation is the presence of at least two nearby hydroxyl groups in the compound to be oxidized. All of these oxidations were found to consist of many-electron processes with C–C bond cleavage involved [27,28]. As glucosides, both paeoniflorin and paeonoside contain glucose moiety. It is electro-active at a copper electrode in an alkaline media. In the present work, a copper disc electrode was employed for the electrochemical detection of paeoniflorin, sucrose, paeonoside, glucose, and fructose at constant applied potentials in a 75 mM NaOH aqueous solution.

The potential applied to the working electrode directly affects the sensitivity and the detection limits of this method, and it is necessary to determine the hydrodynamic voltammograms for the analytes to obtain the optimum potential. Fig. 2 depicts the HDVs for the catalytic oxidation of paeoniflorin, sucrose, paeonoside, glucose, and fructose using the copper disc-electrode detector. The curves were recorded pointwise from +0.4 to +0.7 V (versus SCE) in steps of 50 mV using a separation

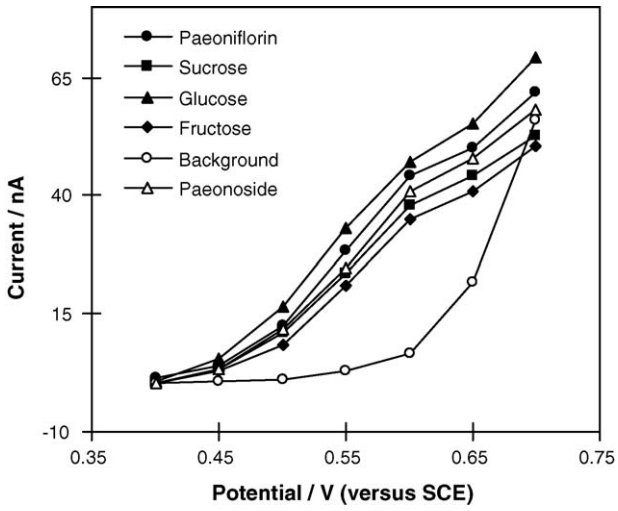


Fig. 2. Hydrodynamic voltammograms (HDVs) for 0.5 mM of paeoniflorin, sucrose, paeonoside, glucose, and fructose in CE. Fused-silica capillary, i.d. 25 μm × 40 cm length; *working electrode*, 300 μm diameter copper disc electrode; *background electrolyte*, 75 mM NaOH; separation and injection voltage, 12 kV; injection time, 6 s.

voltage of 12 kV. All of the analytes displayed similar profiles, with an increase of the response starting at +0.45 V (versus SCE). The current rose rapidly upon raising the potential above +0.50 V (versus SCE). Although an applied potential greater than +0.60 V (versus SCE) resulted in higher peak currents, both the baseline noise and the background current increase substantially. The high background current led to an unstable baseline, which is a disadvantage for the sensitive and stable detection. Considering the sensitivity and background current, subsequent amperometric detection work employed a detection potential of +0.60 V (versus SCE), which offered the most favorable signal-to-noise characteristics. The stability of the working electrode was good and the reproducibility was high at the optimum potential.

3.2. Effects of the NaOH concentration

Paeoniflorin, sucrose, paeonoside, glucose, and fructose are neutral compounds within the physiological pH range. In the present work, strongly alkaline background electrolyte (12.5–100 mM NaOH) was used to keep all the analytes in anionic form and the activity of the copper electrode for their electrocatalytic detection. The separation of these analytes by CE is based on their degrees of dissociation [19].

The NaOH concentration in the background electrolyte affect the zeta-potential (ζ) of the inner wall of the capillary, the viscosity coefficient of the solution, the electroosmotic flow (EOF) as well as the overall charge of the analytes, which determine the migration time, peak height, and the separation of the analytes [17]. The effect of NaOH concentration on the migration time of the analytes is illustrated in Fig. 3A. Upon raising the NaOH concentration from 12.5 to 100 mM, the migration time increases rapidly with the resolution improved for all analytes. When NaOH concentration is lower than 25 mM, the resolution of the five analytes is poor. At the concentration of 75 mM, the

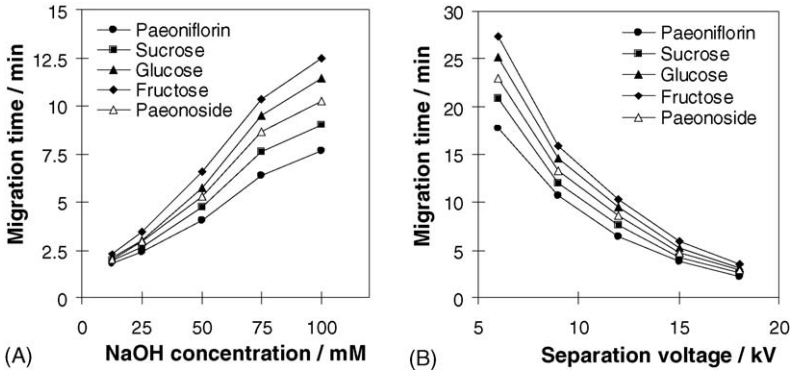


Fig. 3. Effect of: (A) NaOH concentration and (B) separation voltage on the migration time of the analytes. *Operation conditions*: separation voltage, 12 kV for (A) and 6–18 kV for (B); the concentration of NaOH used for separation, 12.5–100 mM for (A) and 75 mM for (B), working potential, +0.60 V (vs. SCE); other conditions as in Fig. 2.

five analytes can be well-separated within a relatively short time. Upon raising the NaOH concentration above 75 mM, the peak current was low and the peak shape became poor because the electric current in the capillary also increased resulting in Joule heating and peak broadening. In this experiment, 75 mM NaOH aqueous solution was chosen as the background electrolyte considering the peak current, resolution, and analytical time.

3.3. Effect of separation voltage and injection time

Fig. 3B illustrates the influence of separation voltage on the migration time of the analytes. As expected, increasing the separation voltage from 6 to 18 kV in steps of 3 kV dramatically decreases migration time for all the five multiple-hydroxyl analytes, from 17.7 to 2.2 min for paeoniflorin, from 20.9 to 2.6 min for sucrose, from 23.0 to 2.9 min for paeonoside, from 25.2 to 3.2 min for glucose, and from 27.4 to 3.5 min for fructose, respectively. The time for the analysis will be reduced markedly upon raising the separation voltage. It is found that higher separation voltages are not beneficial to the resolution of the five compounds. The peak of analytes will overlap when the separation voltage is above 15 kV. Moreover, higher separation voltage increases the baseline noise, resulting in poorer detection limits. However, too lower separation voltages will increase the analysis time considerably, which in turn cause peak broadening. Based on experiments, 12 kV was chosen as the optimum voltage to accomplish a good compromise.

In this study, samples were introduced into the capillary electrokinetically. Injection time directly affect the amount of sampling, which affects the peak height and peak shape. The effect of the injection time on CE separation was investigated by changing the sampling time from 2 to 10 s in increments of 2 s at an injection voltage of 12 kV. It was found that both the peak current and the peak width increase with increasing sampling time. When injection time exceeds 6 s, the peak current levels off and peak broadening becomes more severe. In this experiment, 6 s (at 12 kV) is selected as the optimum injection time considering the separation and sensitivity.

Through the experiments above, the optimum conditions for determining paeoniflorin, sucrose, paeonoside, glucose, and fructose were acquired. The typical electropherogram for a mixture containing 0.5 mM paeoniflorin, sucrose, paeonoside, glucose, and fructose is shown in Fig. 4A. Baseline separation for all the five analytes can be achieved within 12 min.

3.4. Reproducibility, linearity and detection limits

The *intra-day* precision was examined from a series of seven repetitive injections of a sample mixture containing 0.5 mM paeoniflorin, sucrose, paeonoside, glucose, and fructose under

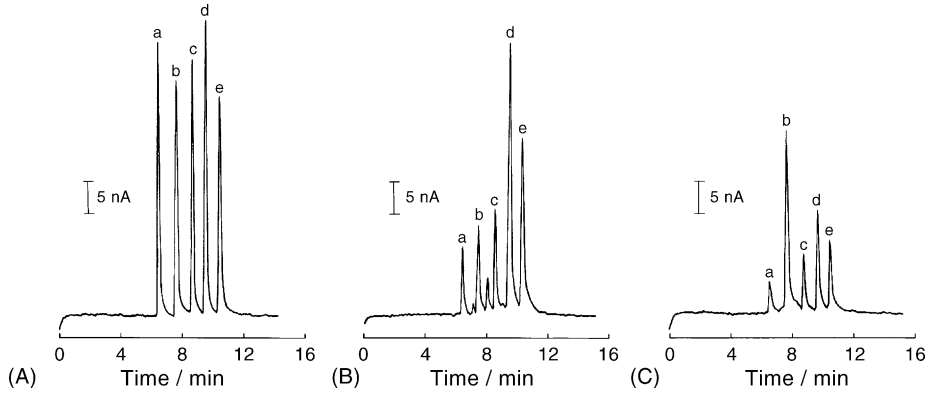


Fig. 4. (A) Electropherogram for a mixture containing 0.5 mM of: paeoniflorin (a), sucrose (b), paeonoside (c), glucose (d), and fructose (e). (B) Typical electropherograms for the diluted extracts from Moutan Cortex ((B) Sample 1; (C) Sample 2). Working potential, +0.60 V (vs. SCE), other conditions as in Fig. 2.

Table 1
The results of regression analysis on calibration curves and the detection limits[a]

Compound	Regression equation, $Y = a + bX$[b]		Correlation coefficient	Linear range (mM)	Detection limit (μM)[c]
	a	*b*			
Paeoniflorin	0.1452	88.234	0.9996	2.0–0.005	1.0
Sucrose	0.1741	76.050	0.9989	2.0–0.005	1.2
Paeonoside	0.1267	80.171	0.9997	2.0–0.005	1.1
Glucose	0.2135	94.179	0.9991	2.0–0.005	0.9
Fructose	0.0643	70.027	0.9993	2.0–0.005	1.3

[a] Working potential is +0.60 V (vs. SCE). Other conditions are the same as in Fig. 2.
[b] Here, the *Y* and *X* are the peak current (nA) and concentration of the analytes (mM), respectively.
[c] The detection limits correspond to concentrations giving a signal-to-noise ratio of 3.

Table 2
Assay results (mg/g) of the analytes in the plant samples ($n = 3$)[a]

Sample	Paeoniflorin	Sucrose	Paeonoside	Glucose	Fructose
1	14.79 (3.3)[b]	15.67 (2.8)	16.48 (2.4)	20.45 (1.7)	17.80 (2.6)
2	7.125 (4.2)	32.00 (2.5)	9.143 (3.7)	7.745 (2.1)	7.218 (1.9)

[a] Working potential is +0.60 V (vs. SCE). Other conditions are the same as in Fig. 2.
[b] The data in the brackets are the R.S.D. (%).

the optimum conditions. The time for each run is about 30 min. Reproducible signals were obtained with R.S.D. of 3.6% (paeoniflorin), 2.8% (sucrose), 3.1% (paeonoside), 2.3% (glucose), and 3.7% (fructose) for the peak currents. Nine determinations of a mixture containing 0.5 mM paeoniflorin, sucrose, paeonoside, glucose, and fructose over 3 days (three times a day) resulted in the inter-day precisions of 4.8%, 4.6%, 5.1%, 4.3%, and 5.4%, respectively. Such good precision reflects the reduced surface fouling of the copper electrode and indicates this approach is suitable for the analyses of real samples. The fused silica capillary used in CE was usually rinsed with 0.1 M NaOH aqueous solution to clean the inner wall. In this study, 75 mM NaOH aqueous solution was used as the *background electrolyte*. It can minimize the fouling of the inner wall of the capillary and the surface of the working electrode during the CE analysis so that the repeatability was improved. In addition, temperature control has been applied in this work in order to improve the repeatability of the peak current and migration time.

A series of the standard mixture solutions of the five analytes with concentration ranging from 5.0 μM to 2.0 mM were tested to determine the linearity at the copper disc electrode in this method. The copper electrode detector offers a well-defined concentration dependence. The results of regression analysis on calibration curves and detection limits are presented in Table 1. The determination limits are evaluated on the basis of a single-to-noise ratio of 3. The calibration curves exhibit satisfactory linear behavior over the concentration range of about 3 orders of magnitude with the detection limits ranging from 0.9 to 1.3 μM for all analytes.

3.5. Sample analysis and recovery

Under the optimum conditions, CE-ED was applied for the determination of paeoniflorin, sucrose, paeonoside, glucose, and fructose in traditional Chinese medicine, Moutan Cortex. The typical electropherograms for the diluted extracts from two plant samples are shown in Fig. 4B and C. Peak identification was performed by the standard-addition method. The assay results are summarized in Table 2. By carefully comparing the electropherograms, it was found that both samples had similar constituents based on the migration times of the peaks. Because paeoniflorin, sucrose, paeonoside, glucose, and fructose were found presented in both samples, all five constituents can be defined as common peaks in the fingerprint of Moutan Cortex under the selected conditions. The paeoniflorin content in Moutan Cortex is well in agreement with a previous report [29]. In order to prevent the degradation of the analytes, the stock solution and the water extracts of the plant samples were diluted using a 75 mM NaOH aqueous solution just prior to CE analysis.

Recovery experiments were performed by adding accurate amounts of paeoniflorin, sucrose, paeonoside, glucose, and fructose to the diluted extract of Moutan Cortex in the separation medium. Subsequently, the standard-spiked sample solution was analyzed under the optimum conditions. The average recoveries and the corresponding R.S.D. were 96.7% and 3.5% for paeoniflorin, 98.1% and 2.7% for sucrose, 97.9% and 3.1% for paeonoside, 101.3% and 3.3% for glucose, and 97.7% and 2.7% for fructose, respectively ($n = 3$). The results demonstrated that this method had both high *recovery* and good precision for the analytes tested.

4. Conclusions

For the first time, CE-ED was employed for the determination of paeoniflorin, sucrose, paeonoside, glucose, and fructose in Moutan Cortex. It is characterized by its higher resolution and sensitivity, lower expense of operation, and less amount of sample. The main advantage of CE as an analytical technique for the analysis of plant samples is that capillary is much easier to wash. Because a paeoniflorin, sucrose, paeonoside, glucose,

and fructose are directly detected on copper electrode, samples do not need derivatization before determination. It is concluded that CE-ED is an efficient approach for the constituent and fingerprint study of plant drugs due to its special attributes.

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