Rapid Determination of Paeoniflorin and Three Sugars in Radix Paeoniae Alba by Capillary Electrophoresis

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A method based on capillary electrophoresis with electrochemical detection has been developed for the separation and determination of paeoniflorin, sucrose, glucose, and fructose in traditional Chinese medicine, Radix Paeoniae Alba. The effects of several important factors, such as the concentration of NaOH, the separation voltage, the injection time, and the detection potential, were investigated to determine the optimum conditions. The detection electrode was a 300- μ m diameter copper disc electrode at a working potential of +0.60 V (*versus* SCE). The four analytes can be well separated within 8 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 the peak current and the analyte concentration was linear over about 3 orders of magnitude with detection limits ($S/N = 3$) ranging from 1 to 2 μ M for all analytes. The proposed method has been successfully applied for the determination of the paeoniflorin and sugars in real plant samples with satisfactory assay results.

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Introduction

As a commonly used traditional Chinese medicine (TCM), Radix Paeoniae Alba is the dried root of *Paeonia lactiflora* Pall., family Ranunculaceae. It can nourish and stabilize the blood, pacify the liver, alleviate pain, reduce and smooth irritability, edginess, and mood swings.¹ Radix Paeoniae Alba has been frequently used as an important ingredient in many traditional prescriptions. Paeoniflorin is the major active constituent of Radix Paeoniae Alba. It has broad pharmacological effects, such as analgesic, anti-diuretic, anti-inflammatory, anti-convulsant, vasodilatic, *etc.*^{1,2} The paeoniflorin content is an important parameter for evaluating the quality of Radix Paeoniae Alba. The Pharmacopoeia of China requires the content of paeoniflorin in Radix Paeoniae Alba to be no less than 0.8%.¹ As the primary metabolites, sucrose, glucose, and fructose are found to be widely presented in plants. Han *et al.* have found that higher contents of sugars can indicate a better quality of some herbal drugs.³ Owing to the differences in the species, cultivation, harvesting and processing, the quality of Radix Paeoniae Alba as a commercial medicinal herb varies to a considerable extent. Hence, it is interesting to establish some rapid, simple, and accurate approaches for the determination of paeoniflorin, sucrose, glucose, and fructose in Radix Paeoniae Alba.

Liquid chromatography (LC) has been widely used for the determination of paeoniflorin in Radix Paeoniae Alba,⁴⁻⁶ composite herbal preparations with Radix Paeoniae Alba as their ingredient,⁷⁻⁹ and biological samples.¹⁰⁻¹² In addition, a quantitative enzyme-linked immunosorbent assay (ELISA) has been used to survey paeoniflorin in crude drugs and multi-component traditional Chinese herbal medicines.¹³ Capillary

electrophoresis (CE) has also been employed for the determination of paeoniflorin and other constituents in Radix Paeoniae Alba.^{14,15} However, the sensitivity and selectivity of the ultraviolet (UV) detector used is relatively low.

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.^{16,17} Electrochemical detection (ECD) typically operated in the amperometric mode can be coupled with CE to provide high sensitivity and selectivity for the determination of electroactive substances.¹⁸⁻²⁰ CE has been widely used in the analysis of sugars and their derivatives.²¹⁻²³ The advances in the separation and detection approaches of derivatized and underivatized carbohydrates are reviewed by El Rassi.²⁴ Direct electrooxidation at platinum or gold electrodes has gained some popularity for the detection of underivatized carbohydrates.^{25,26} To reduce the fouling of platinum and gold electrodes, the applied potential for these electrodes must be continuously pulsed based on a complicated procedure. Alternatively, the work on copper electrodes by Baldwin and co-workers is of particular interest for the direct detection of carbohydrates in a strongly alkaline medium, where signal responses are achieved at a constant potential without any electrode fouling.^{22,27} It can be employed to detect not only carbohydrates, themselves, but also their derivatives.²²

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. As a glucoside, paeoniflorin (its molecular structure is shown in Fig. 1) is electroactive at a copper electrode in an alkaline solution because of its glucose moiety. In this study, CE-ECD was employed for the determination of paeoniflorin, sucrose, glucose, and fructose in

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Fig. 1 Molecular structure of paeoniflorin.

Radix Paeoniae Alba without derivatization. This method is simple, sensitive, reliable, and efficient, providing not only a way for evaluating the quality of Radix Paeoniae Alba and plant medicines made from Radix Paeoniae Alba in marketplaces, but also an alternative approach for quality control in medicinal factories and constituent investigations of other plants. To our best knowledge, there are no earlier reports published on the determination of paeoniflorin and sugars in Radix Paeoniae Alba by CE-ECD. The optimization, detailed characterization, and advantages of the CE-ECD approach are reported in the following sections in connection to the measurement of paeoniflorin and three sugars.

Experimental

Reagents and solutions

Paeoniflorin was supplied by National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Sucrose, glucose, and fructose were all purchased from Sigma (St. Louis, MO, USA), and were used as received without further purification. All aqueous solutions were made up in doubly distilled water. Other chemicals were of analytical grade.

Stock solutions of paeoniflorin, sucrose, glucose, and fructose (10 mM) were prepared in doubly distilled water, and were kept in a 4°C refrigerator. They were stable for at least 1 month. The electrophoretic separation medium was a 75 mM NaOH aqueous solution, unless mentioned otherwise. The stock solutions were diluted to the desired concentration with the separation medium just prior to use.

Apparatus

The CE-ECD system used has been described previously.^{20,28} 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 the ground potential. The separations were proceeded in a 40 cm length of 25 μ m i.d. and 360 μ m o.d. fused silica capillary (Polymicro Technologies, Phoenix, AZ, USA).

A three-electrode electrochemical cell consisting of a laboratory-made 300 μ m 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 to the outlet of the capillary

with the aid of a three-dimension 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 approximately 50 μ m using a micrometer caliper of a micromanipulator. The electropherograms were recorded using a LKB $\times$ REC 1 chart record (Pharmacia, Sweden). An YS73-4A-3KVA alternate constant-voltage power supply (Shanghai Keyi Instrumental Factory, Shanghai, China) was employed to suppress the voltage fluctuation of the power line. The whole system was assembled in a 10 m² Faraday room that was air-conditioned at 25°C to minimize the effects of external noise sources.

Sample preparation

Three samples of Radix Paeoniae Alba were obtained from Sun-Tian-Tang Traditional Chinese Medicine Store (Shanghai, China). They were all dried at 60°C for 2 h, and then pulverized. About 2.0 g of the powder was accurately weighed 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 a 75 mM NaOH aqueous solution at a ratio of 5 (1 to 5) just prior to CE analysis.

Procedures

Before use, the copper disc electrode was successively polished with emery paper and alumina powder, and sonicated in doubly distilled water. 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 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, several nanoliters of the sample solution were 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 separation medium were all filtered through a syringe cellulose acetate filter (0.22 μ m) prior to their use. Peak identification was performed by the standard-addition method.

Results and Discussion

Hydrodynamic voltammograms (HDVs)

Carbohydrates and their derivatives are not normally electroactive at carbon electrodes, the most commonly used working electrode in ECD. 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.^{22-26,29,30} 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.^{22,27} 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

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

with C-C bond cleavage involved.^{31,32} As a glucoside, paeoniflorin contains a 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, 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 (HDVs) for the analytes to obtain the optimum potential. Figure 2 depicts the HDVs for the catalytic oxidation of paeoniflorin, sucrose, 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 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.

Effects of the NaOH concentration

Paeoniflorin and the three sugars are neutral compounds within the physiological pH range. In the present work, a strongly alkaline separation medium (12.5 – 100 mM NaOH) was used to keep all the analytes in anionic form and the activity of the copper electrode for the electrocatalytic detection of paeoniflorin, sucrose, glucose, and fructose. The separation of these analytes by CE is based on their degrees of dissociation.²²

The NaOH concentration in the separation medium affects 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.²⁰ The effect of the NaOH concentration on the migration time of

Fig. 3 Effect of the NaOH concentration on the migration time of the analytes. Working potential, +0.60 V (*versus* SCE); other conditions, as in Fig. 2.

the analytes is illustrated in Fig. 3. Upon raising the NaOH concentration from 12.5 to 100 mM, the migration time increased rapidly with the resolution being improved for all analytes. When the NaOH concentration was lower than 25 mM, the resolution of the four analytes was poor. At a concentration of 75 mM, four analytes could 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, a 75 mM NaOH aqueous solution was chosen as the separation medium, considering the peak current, resolution, and analytical time.

Effect of the separation voltage and injection time

As expected, increasing the separation voltage from 6 to 18 kV in steps of 3 kV dramatically decreased migration time for all four multiple-hydroxyl analytes, from 11.0 to 2.3 min (paeoniflorin), from 13.1 to 2.6 min (sucrose), from 15.2 to 3.1 min (glucose), and from 17.8 to 3.5 min (fructose), respectively. The time for the analysis can be markedly reduced by raising the separation voltage. It was found that the higher separation voltages are not beneficial to the resolution of the four compounds. The peak of the analytes overlapped when the separation voltage was above 15 kV. Moreover, higher separation voltage increased the baseline noise, resulting in poorer detection limits. However, lowering the separation voltages increased the analysis time considerably, which in turn caused peak broadening. Based on our experiments, 12 kV was chosen as the optimum voltage to accomplish a good compromise.

In this study, samples were introduced into the capillary electrokinetically. The injection time directly affected the amount of sampling, which affected the peak height and the 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 increased with increasing the sampling time. When the injection time exceeded 6 s, the peak current leveled off and peak broadening became more severe. In this experiment, 6 s (at 12 kV) was selected as the optimum injection time, considering the separation and sensitivity.

Through the above-mentioned experiments, the optimum conditions for determining paeoniflorin, sucrose, glucose, and fructose were acquired. The typical electropherogram for a mixture containing 0.5 mM paeoniflorin, sucrose, glucose, and fructose is shown in Fig. 4A. Baseline separation for all four analytes could be achieved within 8 min.

Fig. 4 (A) Electropherogram for a mixture containing 0.5 mM of paeoniflorin (a), sucrose (b), glucose (c), and fructose (d). (B) Typical electropherogram for the diluted extracts from *Radix Paeoniae Alba* (Sample 1). Working potential, +0.60 V (*versus* SCE); other conditions, as in Fig. 2.

Table 1 Results of a 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
Paeoniflorin	$Y = -0.1664 + 56.017X$	0.9993	0.005 – 2.5	1.6
Sucrose	$Y = -0.0589 + 67.046X$	0.9989	0.005 – 2.5	1.3
Glucose	$Y = -0.2362 + 81.961X$	0.9996	0.005 – 2.5	1.0
Fructose	$Y = 0.0405 + 47.819X$	0.9991	0.005 – 2.5	1.8

a. Working potential is +0.60 V (*versus* SCE). Other conditions are the same as in Fig. 2.

b. Where 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.

Repeatability, linearity and detection limits

The repeatability of the peak current and migration time in this experiment was determined by repeated injection ($n = 7$) of a solution containing 0.5 mM paeoniflorin, sucrose, glucose, and fructose under the optimal conditions every 30 min. The relative standard derivations (RSDs) of the peak current and the migration time were 2.3% and 1.1% for paeoniflorin, 2.7% and 1.6% for sucrose, 2.9% and 1.7% for glucose, 3.2% and 2.0% for fructose, respectively. Such good repeatability reflects the reduced surface fouling of the copper electrode, and indicates that this approach is suitable for the analyses of real samples. The fused-silica capillary used in CE was usually rinsed with a 0.1 M NaOH aqueous solution to clean the inner wall. In this study, a 75 mM NaOH aqueous solution was used as the electrophoretic separation medium. Because it can minimize the fouling of the inner wall of the capillary and the surface of the working electrode during the CE analysis, the repeatability was improved. In addition, temperature control was applied in this work. In order to improve the repeatability of the peak current and migration time, the whole CE system was assembled in a small air-conditioned Faraday room that could reduce the temperature fluctuation.

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

Sample	Paeoniflorin	Sucrose	Glucose	Fructose
1	45.03 (4.8) ^b	77.94 (2.0)	19.64 (1.2)	8.540 (4.1)
2	33.59 (1.8)	71.48 (2.4)	16.99 (1.5)	9.544 (4.5)
3	37.17 (1.7)	83.73 (2.2)	21.69 (2.7)	12.56 (3.5)

a. Working potential is +0.60 V (*versus* SCE). Other conditions are the same as in Fig. 2.

b. The data in the brackets are the RSDs (%).

A series of the standard mixture solutions of the four analytes with concentrations ranging from 5.0 μM to 2.5 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 a regression analysis on the calibration curves and the detection limits are presented in Table 1. The determination limits were evaluated based on a single-to-noise ratio of 3. The calibration curves exhibit a satisfactory linear behavior over the concentration range of about 3 orders of magnitude with the detection limits ranging from 1 to 2 μM for all analytes.

Sample analysis and recovery

Under the optimum conditions, CE-ECD was applied for the determination of paeoniflorin, sucrose, glucose, and fructose in traditional Chinese medicine, *Radix Paeoniae Alba*. The typical electropherogram for the diluted extract from the plant sample is shown in Fig. 4B. 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 all samples had identical profiles based on the relative peak heights and the migration times. Because paeoniflorin, sucrose, glucose, and fructose were found present in all three samples of *Radix Paeoniae Alba*, all four constituents can be defined as common peaks in the fingerprint of *Radix Paeoniae Alba* under the selected conditions. The paeoniflorin content in *Radix Paeoniae Alba* is well in agreement with a previous report.¹⁵

As a monoterpene glucoside, paeoniflorin is also a benzoate. Hydrolysis of its ester bond in strongly alkaline solution is the major degradation mode, and has a close relation with its stability. In this work, no obvious decrease in the peak current of the paeoniflorin was observed in a repeatability test and in the analyses of real samples mentioned above based on the corresponding RSDs of 2.3% ($n = 7$) and 1.7 to 4.8% ($n = 3$), indicating the hydrolysis is slow enough during the CE analyses (typically 1 to 3 h). These results may be attributed to the low temperature (25°C) and the low NaOH concentration (75 mM) of the electrophoretic separation medium. 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. Honda *et al.* analyzed paeoniflorin in a 100 mM borate buffer (pH 10.5), an alkaline solution. The paeoniflorin during the analysis was quite stable because the reported RSDs for the response of paeoniflorin were in the range of 2.2 to 3.0% ($n = 8$).¹⁵

Recovery experiments were performed by adding accurate amounts of paeoniflorin, sucrose, glucose, and fructose to the diluted extract of *Radix Paeoniae Alba* in the running buffer. Subsequently, the standard-spiked sample solution was analyzed under the optimum conditions. The average recoveries and RSD were 99.6% and 2.5% for paeoniflorin,

97.2% and 1.9% for sucrose, 101.6% and 3.7% for glucose, and 98.6% and 2.1% for fructose, respectively ($n = 3$). The results demonstrated that this method had both high accuracy and good precision for the analytes tested.

Conclusions

For the first time, CE-ECD was employed for the determination of paeoniflorin, sucrose, glucose, and fructose in *Radix Paeoniae Alba*. It is characterized by its higher resolution and sensitivity, lower expense of operation, and smaller amount of sample. The main advantage of CE as an analytical technique for the analysis of plant samples is that the capillary is much easier to wash. Because all four analytes are directly detected on the copper electrode, samples do not need derivatization before a determination. It is concluded that CE-ECD is an efficient approach for constituent and fingerprint studies of plant drugs due to its special attributes.

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