Separation and Determination of Three Phenylpropanoids in the Traditional Chinese Medicine and Its Preparations by Capillary Electrophoresis

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Abstract

Three phenylpropanoids (ferulic acid, chlorogenic acid, and caffeic acid) are simultaneously separated and determined within 13 min by a new capillary electrophoresis method using 15 mmol/L sodium borate (pH 8.71) as run buffer. The optimum conditions for the separation as well as the analytical characteristics, such as the calibration graph and limit of detection (LOD) for the determination of these three compounds, are studied. The linear range for the determination of ferulic, chlorogenic, and caffeic acid is 5.0~70.0, 8.0~112.0, and 9.0~64.0 µg/mL, with the LOD as 1.5, 2.25, and 6.0 µg/mL, respectively. The method, which is very simple, rapid, and of requisite sensitivity and reproduction, is satisfactorily used for the separation and determination not only of ferulic, chlorogenic, and caffeic acid in *Cimicifuga foelida Li* and its preparation (Yin-huang-han-pian), but also of ferulic acid and chlorogenic acid in *Ligusticum chuanxiong hort.* and *Angelica sinensis (Oliv.) Diels.*

Introduction

Ferulic, chlorogenic, and caffeic acid (structures shown in Figure 1) are widely existent or coexistent in many traditional Chinese medicines and Chinese preparations, such as Yin-huang-han-pian, as major bioactive components for the treatment of many diseases (1). It is reported that ferulic acid can inhibit thrombosis and blood platelet gather, chlorogenic acid is provided with antimicrobial and blood-stopping effects, and caffeic acid can be used as antimicrobial, antiphlogistic, and immunization accelerant. Therefore, the analysis of these phenylpropanoids is of pharmacological importance.

Several analytical methods have been developed for the determination of these compounds (shown in Figure 1), such as thin-layer chromatography (TLC) (2,3), high-performance liquid chromatography (HPLC) (4–8), reversed-phase (RP) HPLC (9–12), and HPLC–mass spectrometry (MS) (13). Moreover, some capillary electrophoresis (CE) methods, including the micellar electrokinetic chromatography (MEKC) (14) and capillary zone electrophoresis (CZE) (15–19), have also been reported. In these CZ methods, the literature (15,16,19) reports the quantitative determination for only ferulic acid or for only caffeic acid (18). The method proposed in the literature (17) could be used for the separation of six phenolic acids, including ferulic and chlorogenic acid, but only for the quantitative determination of chlorogenic acid. In fact, none of these methods reported in the literature (2–19) could be applied for the separation and quantitative determination of the three target analytes simultaneously, and only one method in reference (13) could be used for the simultaneous identification of all of these phenylpropanoids in the crude extract of yacon *(Smallanthus sonchifolius*) leaves.

In this study, a new CE method for the separation and quantitative determination of the three selected phenylpropanoids in either the crude or Chinese preparations has been established for the first time. Compared with some of

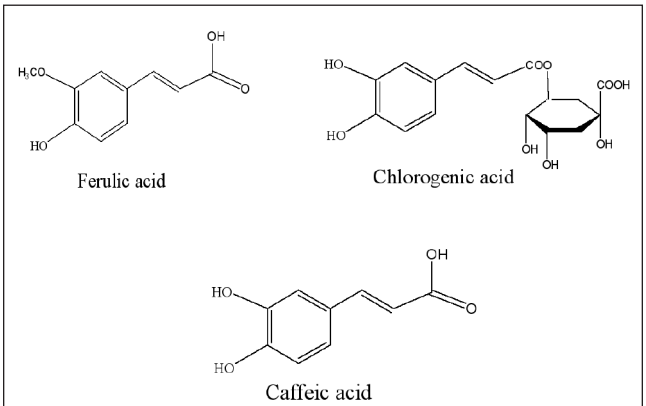

Figure 1. Structures of analytes.

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the reported CE methods (15–19) (the results are summarized in Table I), the method presented in this paper is more simple, rapid, and possesses higher sensitivity and efficiency.

Experimental

Regents and materials

Authentic ferulic, chlorogenic, and caffeic acid were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). *Cimicifuga foelida Li, Ligusticum chuanxiong hort., Angelica sinensis (Oliv.) Diels*, and its preparation (yin-huang-han-pian) were obtained from the pharmaceutical store of Kunming (Yunnan, China).

Ferulic (0.7 mg/mL), chlorogenic acid (0.4 mg/mL), and caffeic acid (0.32 mg/mL) were prepared by diluting in methanol (stored at 4°C). All other chemicals were of analytical grade, and the redistilled water was used for the preparation of buffer and related aqueous solutions.

Sample preparation

Pulverized herbal drug (1.000 g) (drying at 60°C) or preparation were immersed with 10 mL methanol overnight (24 h) and then an ultrasonic extract for 30 min twice. The extract was filtered and washed several times with methanol. Then the filtrate was combined and dried by evaporation, and the residue was dissolved to a certain concentration with methanol. This solution was passed through a 0.45-µm membrane filter. The filtration was directly injected into the CE system.

Apparatus and CE conditions

Experiments were carried out on a Beckman P/ACE MDQ CE system (Beckman Coulter, Fullerton, CA) equipped with a photodiode array detector operating at 254 nm and a 57 cm (50 cm effective length) × 75 µm i.d. uncoated fused-silica capillary (Yongnian Optical Fiber Factory, Hebei, China). A pHS-2C pH meter with a combination electrode (Rex Instrument Factory, Shanghai, China) was applied for pH measurements.

The sample was injected by pressure (0.5 p.s.i, 3447.38 Pa) for 5 s at the cathode of the capillary. All separations were achieved at 25°C by 20 kV and computer-controlled using 32 Karat software. The electrolyte, which was a buffer solution of 15mM sodium borate (pH 8.71), was filtered through a 0.45-µm membrane filter before use. The capillary was conditioned with 0.1M sodium hydroxide (3 min), distilled water (2 min), and separation buffer (4 min) previous to start-up. The data used to construct figures were the average results of three measurements of each point.

Theory

In conventional CE, μ_{eff}, the effective mobility of the analyte M with negative charge, is easily determined by the classical relationship:

$$\mu_{eff} = \frac{l \cdot L}{V}\left(\frac{1}{t_M} - \frac{1}{t_{eof}}\right) \quad \text{Eq. 1}$$

where, t_M and t_{eof} are the migration time(s) of the analyte and a neutral marker [methanol, the negative peak produced by methanol at the detection wavelength of 254 nm was used to measure the electroosmotic flow (EOF)], respectively. L, l, and V are the total length of capillary (cm), the capillary length to the detector (cm), and the applied voltage (V), respectively.

Results and Discussion

The molecular structures of these three target analytes

Table I. Contrast the CE Methods for the Determination of Ferulic, Chlorogenic, and Caffeic Acid

Method	Constituents	Migration time	Linear range (µg/mL)	Buffer solution	Reference
1	Ferulic acid	16.13 min	10–100	10mM borate	15
2	Ferulic acid	10 min	4–400	60mM borate (pH 9.45)	16
3*	Chlorogenic acid	17 min	27.7–665	10mM phosphate + 20mM boric acid + 5% ethanol (pH 7.00)17	17
4	Caffeic acid	8 min	10–100	20mM (pH 9.18)	18
5†	Ferulic acid	14 min	28.80–144	50mM borate (pH 9.00)	19†
6	Ferulic acid Chlorogenic acid Caffeic acid	8.38 min 8.95 min 12.25 min	5.0–70.0 8.0–112.0 9.0–64.0	15mM borate (pH 8.71)	This work

* Six phenolic acid including ferulic acid and chlorogenic acid was separated, but only chlorogenic acid was quantitatively determined.
† Ligustrazin was also Determined in this method.

(shown in Figure 1) pointed out that they could be easily converted into an anion in an alkaline medium and yield a complex with borate anions through the phenolic group, according to the complexation mechanism postulated by Lorand and Edwards (20). In fact, some application of this theory for CE of phenylpropanoids has been previously reported (15, 16, 18, and 19). Therefore, a CE method using borate as the background electrolyte was considered to be used for separation in this work.

Effect of pH

To verify the effect of buffer pH on migration behavior and determination, the preliminary experiments were performed with an electrolyte system only containing 15 mmol/L $Na_2B_4O_7$ at different pHs ranging from 6.93 to 9.95 (adjusted with 0.1 mol/L NaOH and 0.1 mol/L HCl). The results indicated that the pH value played an important role in changing not only the migration time of the three compounds, but also the sensitivity of detection. Figure 2 shows the effects of buffer pH on the relative migration time. It could be observed that the complete separation for these three analytes were obtained in the whole range of buffer pH tested except pH 9.0 or so. However, the peaks of the analytes, especially for caffeic acid, broadened seriously when buffer pH was below 8.0, which resulted in a difficulty in separating the target analytes from the coexisting constituents in the real samples. The same question resulted from the split of the peak of caffeic acid existing in the separation of the three compounds when pH of run buffer was more than 9.18. Because the degrees of dissociation of the analytes were different at varying pH levels, the optimum peak shape as well as the shortest migration times for all the target analytes were obtained in the pH range of 8.10~9.18. The sensitivity (peak area) for the determination of the analytes also varied with buffer pH. The results shown in Figure 3 indicate that the peak area of caffeic acid, which is of the lowest sensitivity in the three analytes, is low at a pH less than 8.0, and then increases with the increasing of pH. Although the optimum sensitivity for the determination of each compound could be obtained at a pH greater than 9.18, pH 8.71 was finally chosen for the separation in this paper, considering the sensitivity together with the analysis time and resolution.

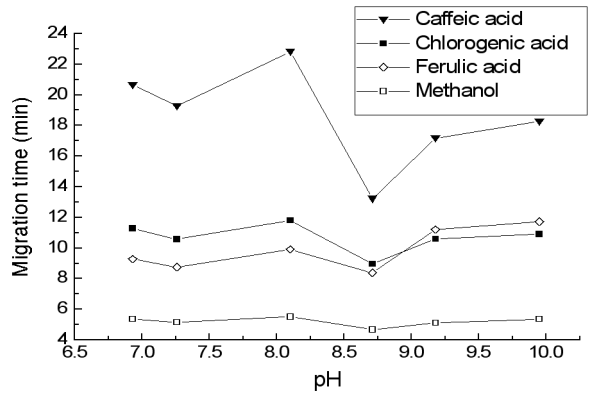

Figure 2. Effect of pH on the migration time. CE conditions were as follows: 15 mmol/L borate buffer, 75 i.d. μm × 57 cm (50.0 cm effective length) uncoated fused-silica capillary, applied voltage of 20 kV, capillary temperature of 25°C, injection at 0.5 p.s.i for 5 s, 254 nm.

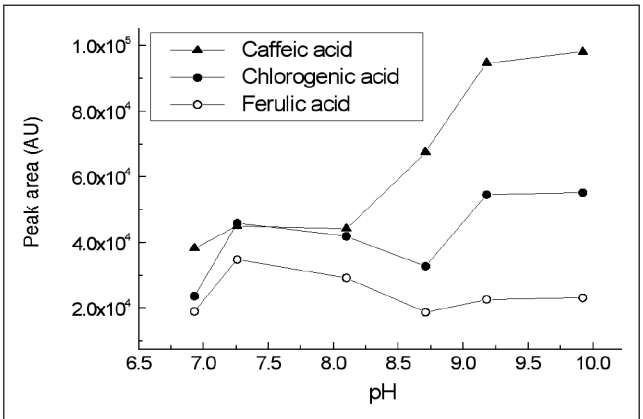

Figure 3. Effect of pH on the peak area. The concentration of ferulic, chlorogenic, and caffeic acid are 35, 40, and 64 μg/mL, respectively. The CE conditions are the same as those in Figure 2.

Effect of the sodium borate concentration

Electrolyte systems (pH 8.71) at different borate concentrations (varied from 10 mmol/L to 30 mmol/L) were used to investigate the dependence of the separation on electrolyte concentration. The results showed an obvious influence of the $Na_2B_4O_7$ concentration on the separation. As seen in Figure 4, the relative migration times of three compounds, which is defined as the migration time of the analyte relative to that of methanol, increased with increasing the concentration of borate when the $Na_2B_4O_7$ concentration was less than 20 mmol/L or greater than 25 mmol/L. These results indicated there should be a strong interaction between borate and the hydroxyl group in these compounds. The migration time of all three analytes was shortest at $Na_2B_4O_7$ concentration of 10 mmol/L, but the peaks of chlorogenic acid and caffeic acid broadened greatly at this condition. Figure 4 also points out

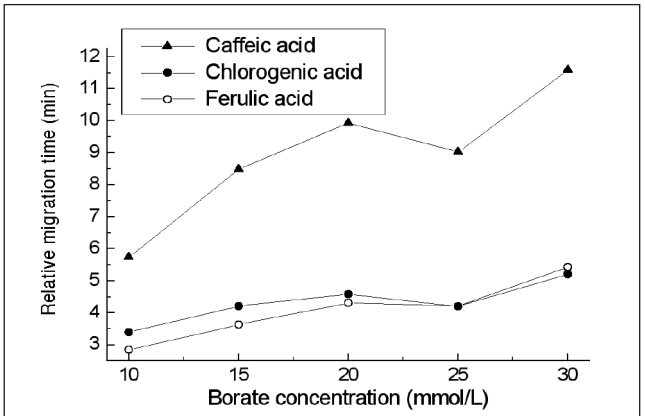

Figure 4. Effect of sodium borate concentration on relative migration time, which is defined as the migration time of the analyte relative to that of methanol. The carriers were buffer solutions (pH 8.71) at different borate concentrations varied from 10–30 mmol/L. The other separation conditions are the same as those in Figure 2.

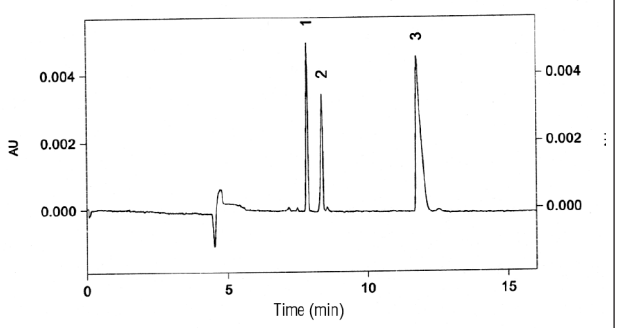

Figure 5. Electropherogram of the standard mixture of the three phenylpropanoids. Peak 1 to peak 3 are represented ferulic, chlorogenic, and caffeic acid. The buffer composition was 15 mmol/L $Na_2B_4O_7$ (pH 8.71). The apparatus conditions are the same as those in Figure 2.

Table II. Analytical Characteristics for the Determination of the Three Phenylpropanoids

Constituents	Linear range (µg/mL)	Regression equation*	*r*	LOD† (µg/mL)	RSD‡
Ferulic acid	5.0–70.0	A = 330.91C + 714.23	0.9997	1.50	1.64
Chlorogenic acid	8.0–112.0	A = 215.39C – 80.89	0.9994	2.25	1.64
Caffeic acid	9.0–64.0	A = 581.68C – 3469.96	0.9997	6.00	2.83

* A is peak-area and C is concentration expressed as µg/mL.
† LOD is the limit of detection at S/N = 3;
‡ RSD for the relative migration time (n = 7), which was defined as the migration time of analyte (M) relative to that of the negative peak given by methanol.

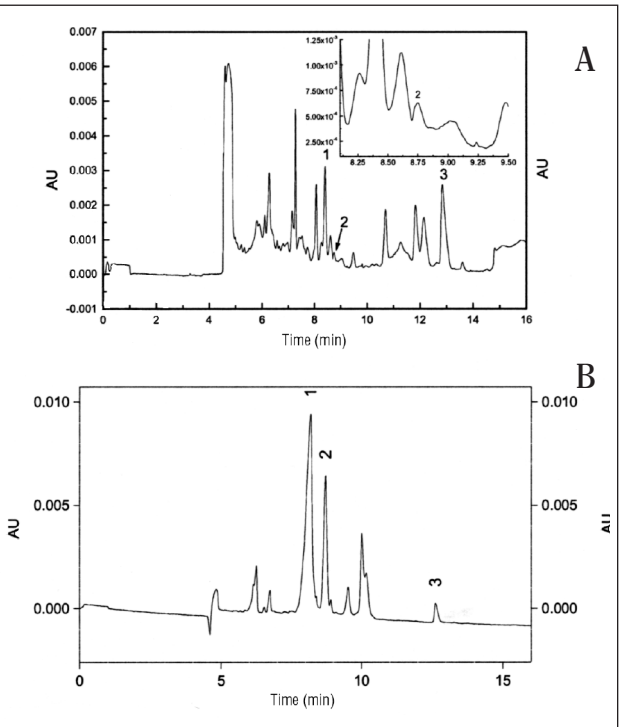

Figure 6. Electropherograms of the extract of *Cimicifuga foelida Li* (A) and Yin-huang-han-pian (B). The separation conditions and the peaks are the same as those in Figure 5.

that the resolution for ferulic and chlorogenic acid depended considerably on the borate concentration. With an increase of borate concentration, their resolution reduced at the beginning and then improved. When borate concentration reached 25 mmol/L, the peak of ferulic and chlorogenic acid were overlapped completely and, after that, their sequence reversed. Moreover, the noise and baseline aberration also manifest as the increasing of borate concentration and the baseline noise became serious when the concentration of $Na_2B_4O_7$ was greater than 20 mmol/L. To shorten the analysis time, lowering the baseline noise and obtaining a good peak shape 15 mmol/L borate (pH 8.71) was selected.

Effect of apparatus conditions

The apparatus conditions such as: applied voltage, separation temperature, and injection time, which are the important parameters to control migration time and resolution, were studied using 15 mmol/L sodium borate (pH 8.71) as the run buffer. The results from these experiments indicated that increasing the applied voltage from 15 to 30 kV produced a decreased migration time and reduced separation efficiency because the excessive Joule-Heat caused by too much higher applied voltage. Thus, 20 kV was used as the applied voltage. The separation temperature influenced the viscosity of the solution, which in turn caused the change of EOF, and thus affected the separation. The variation of the separation temperature from 15°C to 30°C resulted in shortening the migration times of all compounds and lowering the selectivity of separation. When the temperature of the cartridge was greater than 30°C, the baseline separation for ferulic and chlorogenic acid could not be obtained. Therefore, 25°C was selected as the cartridge temperature. The effects of the injection time on separation pointed out that the sensitivity for the determination increased with the increase of the injection time, and the shape of the peaks broadened because of the fact that the injected volume occupied too large of a part of the capillary. Taking these two factors into account, a 5 s injection time was chosen as the optimum loading time.

Characteristic of quantitative analysis

Under the selected conditions [15 mmol/L borate buffer with pH of 8.71 as run buffer, 75-µm i.d. × 57-cm (50.0-cm effective length) uncoated fused-silica capillary, applied voltage of 20 kV, capillary temperature of 25°C, injection at 0.5 p.s.i for 5 s, 254 nm as the determination wavelength] for the separation and detection previously described, the typical electropherogram was obtained as shown in Figure 5, and calibration graphs [peak-area (A) vs concentration (C) in µg/mL] for the three constituents were constructed by analyzing a series of mixture solutions of the three analytes in the concentration range of 1.0–125.0 µg/mL. The linear ranges, regression equations, and correlation coefficients of the standard curves, and the relative standard deviation (RSD) of the relative migration time are

Table III. Result for the Determination of the Three Phenylpropanoids in Sample Extracts (n = 5)

	Ferulic acid			Chlorogenic acid			Caffeic acid		
Sample*	Value found (mg/g)	RSD (%)	Recovery (%)	Value found (mg/g)	RSD (%)	Recovery (%)	Value found (mg/g)	RSD (%)	Recovery (%)
1	0.25	2.73	104.2	0.005	3.21	103.2	0.29	2.65	97.6
2	1.06	1.59	104.4	0.72	1.99	102.9	–†	–	–
3	0.87	0.76	108.0	0.22	1.05	104.7	–	–	–
4	13.20	2.66	94.0	7.31	4.32	99.7	0.27	0.54	102.5

* Names and compositions of the samples: *Cimicifuga foelida Li*, 1; *Ligusticum chuanxiong hort*, 2; *Angelica sinensis* (*Oliv.*) Diels, 3; Yin-huang-han-pian, and the main compositions for Yin-huang-han-pian are the extraction of honeysuckle and scutellaria baicalensis, 4.
† – means no find in the sample.

summarized in Table II. The results showed that the method was of wide linear range and good reproducibility, and the correlation coefficients of all the calibration graphs were greater than 0.9990.

Determination of the phenylpropanoids in herbal drug and preparation

Methanol solution of sample extracts was at first filtered through a 0.45-µm membrane, then injected and separated under the selected conditions. Figures 6A and 6B were the electropherograms for one herbal drug (*Cimicifuga foelida Li*) and one preparation (Yin-huang-han-pian), and Table III shows the results for the quantitative determination of the three phenylpropanoids in all the samples. Peaks of the three analytes in electropherograms were identified by addition of standard substances of ferulic, chlorogenic, and caffeic acid. It could be seen that the peaks of ferulic (peak 1) and chlorogenic acid (peak 2) in Figure 6B looks broader than in Figure 5. It may be because the content of ferulic acid and chlorogenic acid in the sample extracts in Figure 6B are much higher that that in Figure 5, and the composition of the sample extracts is more complex than the standard mixture.

The appropriate concentration of the standard sample was added into the real sample extracts for the determination of recovery, which was calculated as the percent of the concentration of the standard sample recovered to that really added in the sample extracts. It can be seen that the results for the determination of these three compounds in the four samples were satisfactory.

Conclusion

CE has been shown to provide selectivity and high efficiency in the separation and determination of ferulic, chlorogenic, and caffeic acid in herbal drug and preparation by optimizing the pH and borate concentration within 13 min. There was no interference with any peak of the extracts in various samples, and it could be used as an indexing standard for the estimation of the quality of its preparation. This method is simple, economic, stable, and rapid. It is especially suitable for analyzing those bulky samples and for quality control in pharmaceutical plants.

Acknowledgments

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