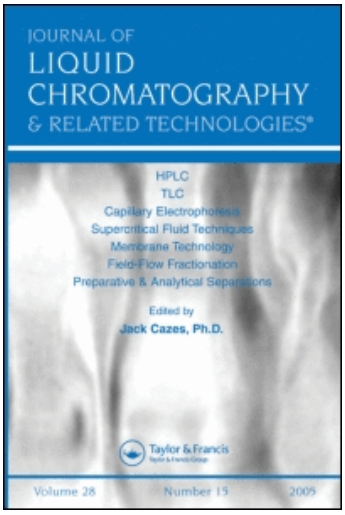

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Separation and Determination of Major Bioactive Components in *Radix Tinosporae* by Gradient Pressurized Capillary Electrochromatography

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Abstract: An approach of gradient pressurized capillary electrochromatography with UV detection was utilized for the separation and determination of major bioactive components in *Radix Tinosporae* extract, including two botanical steroids and three alkaloids. The assay was performed on a 30 cm ODS capillary column with acetonitrile-water gradient elution. Under the optimum conditions, five investigated compounds were eluted within 25 minutes, and were achieved with acceptable linearity, precision, repeatability, and accuracy. The method was successfully applied to analyze five major components in various *Radix Tinosporae* samples, and was sufficed to be utilized as a novel quality control approach for traditional Chinese medicine.

Keywords: Pressurized capillary electrochromatography, Alkaloid, *Radix Tinosporae*, Traditional Chinese medicine

INTRODUCTION

The ever increasing worldwide attention to the therapeutic or pharmaceutical use of traditional Chinese medicine (TCM) has made it absolutely essential to

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carry out stringent quality control measures. So far, it is widely accepted that multiple constituents are responsible for the therapeutic effects of TCM and in order to ensure its quality, it is necessary to quantitatively determine the multi-bioactive components of TCM. Over the past decades, several methods, such as gas chromatography (GC) and high-performance liquid chromatography (HPLC) have been employed in the multi-components determination of TCM,[1–2] among which HPLC is the most widely applied due to its precision and versatile selectivity. The microcolumn separation techniques, such as capillary electrophoresis (CE), capillary HPLC (cHPLC), and capillary electrochromatography (CEC)[2–3] were also developed for the analysis of TCM. These methods, undoubtedly, have the advantages of ultra-low sample and solvent consumption with high resolution and column efficiency, while CE has poor reproducibility, and CEC has problems in practice associated with bubble formation and column dry-out.[4–5]

Pressurized capillary electrochromatography (pCEC), which combine the advantages of CE and HPLC, is a novel hybrid microcolumn electroseparation technique. In the pCEC system, the mobile phase is driven by both an electro-osmotic flow (EOF) and a pressurized flow. It provides the versatile selectivity, larger sample capacity, lower time consuming, solvent consuming, and higher separation efficiency with the introduction of high voltage power;[6–8] moreover, it has a coupled micro HPLC pump, which could minimize the bubble formation.[9–10] Therefore, it is a potential powerful separation tool for the analysis of complex mixtures, especially for TCM samples. It, thus, suffices to be a novel quality control approach for TCM.

To date, most of the analytical applications of pCEC focus on the analysis of pesticides residue,[11] estrogens,[12] peptides,[13] proteins,[14] and chemical medicines,[15] while it was seldom applied in the field of quality control of TCM. So far there were only two papers reporting the determination of constituents in TCM by pCEC,[16,17] in which, however, only isocratic elution was employed and structural related multi-components can be determined. To the best of our knowledge, very limited literatures reported the gradient pCEC methods,[18,19] and no method has been developed to quantity different structures of multi-components. It is well known that, in TCM, the bioactive constituents are very complicated and often have different structures. Therefore, it is meaningful to develop a gradient pCEC method to simultaneously determine different structural types of bioactive components.

Radix Tinosporae is the dried root of *Tinospora capillipes Gagnep*, and has been used as a natural remedy since ancient times. According to the theory of TCM, *Radix Tinosporae* has the effects of heat clearing and detoxicating and is often used as an antipyretic in clinics.[20] It possesses well documented properties of anti-inflammatory and antiviral effects.[21–22] As reported previously,[23] columbamine, jatrorrhizine, 20-hydroxyecdysone (HES), palmatine, and 2-deoxyecdysone (DES) are five major bioactive components in *Radix Tinosporae* (see chemical structures in Figure 1), among which, palmatine, jatrorrhizine, and columbamine are isoquinoline

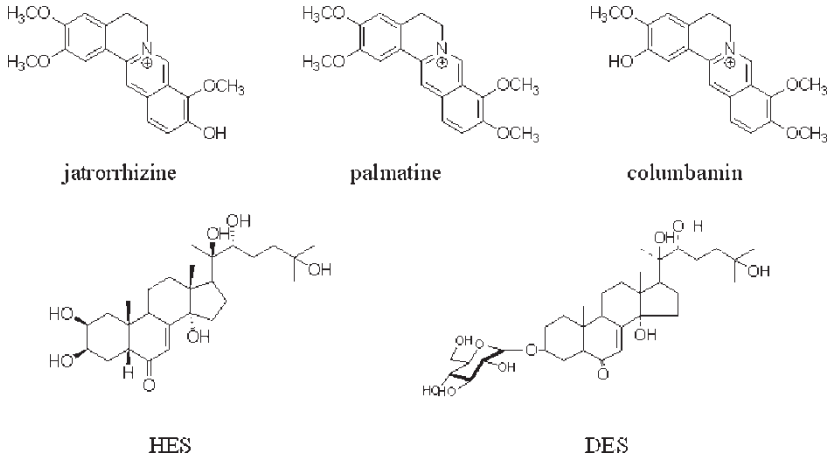

Figure 1. Chemical structures of five major bioactive components in *Radix Tinosporae*.

alkaloids, and HES and DES are botanic steroids. In this study, a novel approach of gradient pCEC has been developed, and the investigated compounds were separated and simultaneously determined successfully and rapidly. The proposed method can be readily utilized as a novel quality control approach for TCM.

EXPERIMENTAL

Instrumentation

The assay was carried out on a Trisep^{TM} 2100GV CEC system (Unimicro Technologies, Pleasanton, CA, USA) equipped with a solvent gradient delivery module, a high voltage power supply (+30 and −30 kV), a UV detector, a micro fluid manipulation module (including a six-port injector) and a data acquisition module. Two high pressure syringe pumps were used to provide supplementary flow to the CEC column. The mobile phase is driven by EOF, as well as pressurized flow, and enters into a six-port injection valve.

Materials and Reagents

The capillary column (100 μm ID × 375 μm OD, total length 55 cm, 30 cm packed with 5 μm ODS particles) was purchased from Unimicro Technologies Co. HPLC grade acetonitrile, methanol, ammonium acetate, and formic

acid were purchased from Merck Company Inc. (Merck, Darmstadt, Germany). Other reagents were of analytical grade. The reference standards of columbamine, jatrorrhizine, palmatine, HES, and DES were prepared in our laboratory (over 98% purity) and their chemical structures were identified by spectral analysis (to be reported elsewhere). Seven *Radix Tinosporae* samples, marked as samples 1∼7, were collected in various provinces in China. Sample 4 and Sample 5 were from Guangxi, and Samples 1, 2, 3, 6, 7, respectively, were from Hunan, Hubei, Sichuan, Jiangxi, and Yunnan.

Preparation of Standard and Sample Solutions

Five references were accurately weighted, dissolved in methanol, and diluted to the appropriate concentration. Stock solutions of the mixture of these references, containing columbamine (23.6 μg/mL), jatrorrhizine (19.4 μg/mL), palmatine (22.0 μg/mL), HES (33.0 μg/mL), and DES (20.4 μg/mL), were prepared in methanol. The stock solutions were further diluted to make working solutions.

Samples of *Radix Tinosporae* were dried at 50°C until constant weight. Each dried material was pulverized to 40 meshes. Pulverized powder, 10 g, was accurately weighted and then extracted with 100 mL 95% ethanol by a Universal Extraction System B-811 (BUCHI Labortechnik AG, Flawil, Switzerland) in solvent warm mode. The dry material was extracted for 3 h and rinsed for 0.5 h with the lower and upper heating temperatures set at 60°C and 100°C, respectively. The solution was evaporated under vacuum till dryness. The residue was dissolved accurately into 250 mL methanol. All the obtained solutions were filtered through syringe filters (0.22 μm) and aliquots (12.5 nL) were subjected to pCEC analysis.

pCEC Analysis Conditions

The mobile phases were composed of A (water containing 0.04% formic acid, 5 mM ammonium acetate) and B (acetonitrile/water = 60/40, v/v). The linear gradient was as follows, 0 min, 15% B; 25 min, 60% B; and 30 min, 100% B. The electrochromatogram for the sample and mixture of five standards is shown in Figure 2. Mobile phases were filtered through 0.45 μm membrane filters and degassed by ultrasonication for 5 min. A negative voltage was added on the column outlet and the column inlet was grounded with the applied voltage of −8 kV. Pressure was applied to the column inlet during the separation. The flow rate of the pump was set at 0.05 mL/min. The wavelength of the UV/Vis detector was set at 247 nm and the backpressure regulator was set at 13.8 MPa.

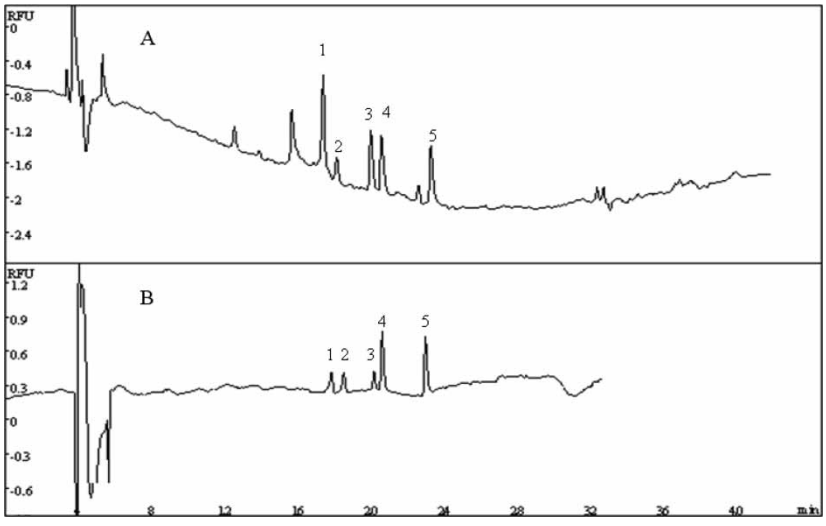

Figure 2. Representative electrochromatograms of sample (A) and references (B). 1-HES, 2-DES, 3-columbamine, 4-jatrorrhizine, 5-palmatine.

RESULTS AND DISCUSSION

Optimization of pCEC Conditions

The analytical method was selected on the basis of the classic HPLC method.[24] However, for pCEC analysis, several factors, including the ionic strength, pH of mobile phase, and gradient program, etc., can affect the separation by different mechanisms from HPLC with the application of the voltage, which needs to be considered to chose a most optimal condition to maximize column efficiency, separation ability, and detector response.

The acetonitrile-water system had a similar separation effect with the methanol-acetonitrile-water system, which was chosen as the HPLC condition,[24] and is easy to apply. Therefore, in our study, the acetonitrile-water system was employed for separation. The concentration of ammonium acetate was an important parameter of EOF, and accordingly affects the migration velocity and resolution. The increase of the buffer concentration will lead to the decrease of the thickness of the electric double layer at the liquid solid interface and the decrease of the ξ potential.[25] As the initial concentration range of 4 to 15 mM, the average currents ranging from 5.8 μA to 8.4 μA were detected. This indicates that a higher concentration of ammonium acetate leads to a higher ionic strength and higher current which permits faster separation, while peak resolution will decline to a degree. To achieve optimal ionic strength, the proper concentration of salt was often required to add into the mobile phase. Combining the

effect of EOF, ionic strength, and chromatographic behavior, 5 mM ammonium acetate was used and thus good separation and shorter run time were achieved.

The velocity of EOF is known to be dependent on the pH of the mobile phase due to its effect on the extent of dissociation of surface silanol groups. Generally, with constant ionic strength, the increasing of pH results in an increase of EOF and, therefore, the shorter migration time of the neutral analytes.[26] However, under high pH condition (pH $>$ 7), alkaloids have an ion exchange with the dissociated silanol groups, which would lead to peak tailing and decreasing of resolution. To investigate the effect, different pH's varying from 5 to 7 were used for pCEC analysis in mobile phase consisting of 25% ACN and 5 mM ammonium acetate as the initial condition. Considering the resolution and migration time, pH 5.0 was chosen for separation. During the applied gradient, the pH was at the range of pH 5.0$\sim$5.2.

The influence of applied voltage on the resolution and migration time was studied. Figure 3 shows the retention time of five components investigated at various applied voltages. The botanic steroids, HES and DES are neutral compounds, which are difficult to be ionized. With the same chromatographic condition, their movements were almost influenced by EOF. Therefore, with the increase of voltage, HES and DES, undoubtedly, have increased migration mobility, and will be eluted out earlier. Whereas, isoquinoline alkaloids compounds, including palmatine, jatrorrhizine, and columbamine are alkalescent, which are easy to be positively charged, behave with electrophoretic mobility opposite to the direction of EOF, and increased elute time. However, with the increase of voltage, the overall mobility of alkaloids is much more complex and the orders of all the investigated peaks appearance are disturbed. The peak orders were detected by the peak shape and peak height of the standard solution at the same elution condition. On the other hand, increasing of the applied voltage leads to the decreasing of resolution of peaks. In view of migration time and resolution, various voltages were

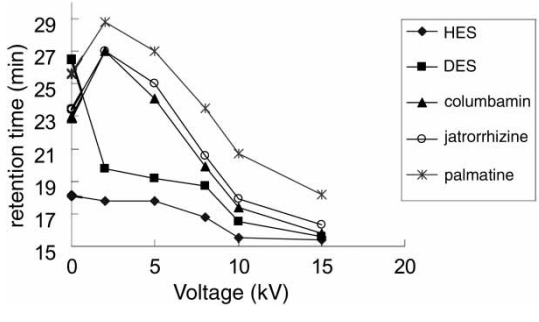

Figure 3. Retention time of the investigated components at various applied voltages.

investigated and the best separation and resolution was obtained with a voltage of −8 kV applied.

Maximally efficient detection can be obtained by selecting the wavelength where the component has the maximum absorption. However, there are two different kinds of components to be quantified, and it is often impossible for them to have the same maximum absorption. In this study, the maximum absorption wavelengths of alkaloids and steroids are 348 nm and 247 nm, respectively. At 348 nm, steroids almost have no responses; while at 247 nm, though it is not the maximum absorption wavelength, alkaloids have enough response strength and acceptable signal-to-noise ratio to be precisely qualified. Therefore, in this assay, 247 nm is selected as the monitoring wavelength.

Validation

The assay linearity was determined by triplicate analysis of five different concentrations of the standard solutions. The limit of detection (LOD) was determined as the concentration resulting in a peak height greater than three times the baseline noise level ($S/N > 3$). The intra-day and inter-day precision were determined by analyzing calibration samples during a single day and on four different days, respectively. The relative standard deviation (RSD) was taken as a measure. Table 1 shows the regression data and LODs of the components determined. Table 2 lists the results of intra-day and inter-day precision tests. It indicates that all the RSDs are less than 5%.

The accuracy tests were carried out by spiking known contents of standard samples into a sample and comparing the determined amount of these standards with the amount originally added. The obtained recovery rates are 105.4–118.1% for HES, 110.3–122.7% for DES, 92.2–110.1% for columbamin, 84.6–91.6% for jatrorrhizine, and 93.1–97.1% for palmatine, respectively.

Table 1. Regression data and LODs of the investigated components

Components	Regression equation $y = a + bx$	Correlation coefficient	Linear range (μg/mL)	LOD (μg/mL)
HES	$y = 112.05x - 64.08$	0.9982	11.0–66.0	4.13
DES	$y = 185.95x - 153.10$	0.9998	6.80–40.8	2.55
Columbamin	$y = 195.75x - 354.37$	0.9978	7.87–47.2	2.95
Jatrorrhizine	$y = 739.23x - 1138.20$	0.9958	6.47–38.8	1.22
Palmatine	$y = 728.41x - 1291.40$	0.9982	7.33–44.0	0.46

y and x denotes for the peak area and the concentration (μg/mL) of the analyte, respectively.

Table 2. Precision and accuracy data of the proposed pCEC method

	Precision					
	Intra-day (n = 5)		Inter-day (n = 4)		Accuracy (n = 5)	
Components	Mean (mg/g)	RSD (%)	Mean (mg/g)	RSD (%)	Mean (%)	RSD (%)
HES	3.10	2.25	3.08	1.05	110.7	4.49
DES	1.88	2.92	1.80	1.54	115.5	4.21
Columbamine	2.20	2.82	2.15	1.22	108.9	1.66
Jatrorrhizine	1.00	2.49	0.87	5.12	88.83	3.24
Palmatine	0.998	2.40	1.01	5.14	96.05	1.73

Conditions: Mobile phase: A (water consisting of 0.04% formic acid, 5 mM ammonium acetate) and B (acetonitrile: water = 60/40, v/v), gradient elution: 0 min, 85% A, 15% B; 25 min, 40% A, 60% B; and 30 min, 0% A, 100% B; flow rate: 0.05 mL/min, voltage: −8 kV, backpressure: 13.8 MPa, injection: 12.5 nL, detection: 247 nm, temperature: 20°C

Sample Analysis

The developed pCEC method was applied to the simultaneous determination of HES, DES, columbamin, jatrorrhizine, and palmatine in *Radix Tinosporae* from seven various provinces in China, and the results were presented in Table 3. It was shown that the contents of these components had a fluctuation with high RSD values over 10%, which would significantly influence the quality stability of this botanical medicine.

To ensure the quality of *Radix Tinosporae*, it is more important to keep the stability of the content of alkaloids components. Historically, *Radix Tinosporae* from Yunnan province (sample 7) is often considered as the most authentic one, which might mean *Radix Tinosporae* of Yunnan has the best

Table 3. Contents of the major bioactive components in *Radix Tinosporae* (mg/g)

Sample no.	HES	DES	Columbamin	Jatrorrhizine	Palmatine
1	2.25	1.40	0.74	0.40	0.61
2	2.66	1.64	0.65	0.43	0.63
3	3.12	1.94	0.93	0.62	0.69
4	2.21	1.41	1.10	1.31	1.29
5	0.61	0.35	2.28	1.83	1.81
6	3.57	2.20	0.55	0.39	2.29
7	3.09	1.94	2.29	1.01	1.09
Mean	2.50	1.55	1.22	0.86	1.20
RSD	0.387	0.391	0.614	0.647	0.539

therapeutic or pharmaceutical effects. From Table 3, we can also find that samples from Guangxi province (samples 4∼5) have similar alkaloids content to that of sample 7. The result suggests the samples of Guangxi and Yunnan province are very similar, which is to a degree, due to the contiguity of these two provinces.

CONCLUSIONS

In this study, an approach of gradient pressurized capillary electrochromatography has been established to separate and simultaneously quantify different structural types of components in *Radix Tinosporae*. Under the proposed optimal conditions, three alkaloids and two botanical steroids were base separated and achieved with acceptable linearity, precision, repeatability, and accuracy. The gradient pCEC method developed in this study has the potential advantages of separation and determination of multi-components in complex analytes, and, thus, suffice to be utilized as a novel quality control approach for TCM.

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