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Determination of puerarin, daidzein, paeoniflorin, cinnamic acid, glycyrrhizin, ephedrine, and [6]-gingerol in Ge-gen-tang by micellar electrokinetic chromatography

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Abstract

This paper presents a micellar electrokinetic chromatography (MEKC) method with diode-array detection to analyze for seven major components in the traditional Chinese medicinal preparation of Ge-gen-tang. Those analytes were successfully separated within 9 min using a pH 9.0 borate buffer containing 20 mM Sodium dodecyl sulfate at 30°C. Experimental results indicated that the relative standard deviations of the analytes' migration times were <0.43% and the correlation coefficients of the analytes' linear calibration graphs exceeded 0.996. The quantities of the seven components in four different Ge-gen-tang samples were determined by the MEKC method coupled with a relatively simple extraction method. © 1997 Elsevier Science B.V.

1. Introduction

Ge-gen-tang (Pueraria Combination) is a traditional Chinese medicinal preparation of *Puerariae radix* (named as 'Ge-gen' in Chinese) combined with other crude herbs. Among those herbs include *Paeoniae radix, Cinnamomi ramulus, Glycyrrhizae radix, Ephedrae herba, Zingiberis rhizoma,* and *Zizyphi fructus* [1]. The medicine is frequently used to treat diseases such as the common cold, influenza, neuralgia, and shoulder stiffness. Traditional Chinese medicine has become increasingly popular in recent years owing to the advantages of low toxicity and rare complications. Moreover, the extracted medicine powder from crude herb preparations makes Chinese medicine taking much more convenient than with the traditional time-consuming preparation process of Chinese medicine. However, knowledge regarding the composition of bioactive ingredients in Chinese medicinal preparations is limited. Therefore, establishing an appropriate analytical method is necessary to investigate the active ingredients and quality of complicated Chinese medicinal preparations.

Seven major components of those crude herbs constituting Ge-gen-tang were selected as the target analytes. Puerarin and daidzein are isoflavonoid compounds of *Puerariae radix*. Their pharmaceutical effects on biological action have been examined [2,3]. Paeoniflorin, cinnamic acid, glycyrrhizin, ephedrine, and [6]-gingerol are the major components in *Paeoniae radix, Cinnamomi ramulus, Glycyrrhizae*

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radix, Ephedrae herba, and *Zingiberis rhizoma*, respectively [2]. Those compounds have been studied for their potential pharmaceutical activities. Previously, liquid chromatography (LC) was employed to determine puerarin and daidzein in various samples containing *Puerariae radix* [3–6]. The separation usually lasted more than 30 min.

Micellar electrokinetic chromatography (MEKC), a modified capillary electrophoresis (CE) technique, has been extensively applied to separate various compounds including neutral and hydrophobic compounds [7–10]. Among the advantages of MEKC technique are high resolution, high separation efficiency, rapid analysis, and a minute sample requirement. A few Chinese crude drug and medicinal preparations have been analyzed by MEKC methods [11–14]. Buffers employed in those studies contained either sodium dodecyl sulfate (SDS) or bile salts to form micelles. Cyclodextrins or organic solvents were found to effectively improve the separation efficiency for these relatively hydrophobic Chinese medicine [13,14]. However, those studies only focused on analyzing for compounds from one or two crude herbs [11–14]. Because Chinese medicinal preparations are frequently composed of several crude herbs, a comprehensive analysis of marker analytes for each crude herb is necessary to study complicated Chinese medicinal preparations.

In this study, we employed a MEKC method to simultaneously determine seven analytes in the Chinese medicinal preparation of Ge-gen-tang. The effects of buffer pH values and SDS concentrations on the analytes' migration behavior were examined. Moreover, the extraction efficiencies of different extraction solutions were discussed. Four concentrated commercial Ge-gen-tang preparations were analyzed under the optimized MEKC conditions.

2. Experimental

2.1. Apparatus

All experiments were performed on a Beckman P/ACE 5500 capillary electrophoresis system (Fullerton, CA). The diode-array detector can scan wavelengths from 190 to 600 nm. In addition, the detection wavelength can be programmed to alter during the separation process. Separation was performed in a 47 cm (40 cm to detector) × 50 μm i.d. fused silica capillary tube (Polymicro Technologies, Phoenix, AZ). The capillary column was assembled in a cartridge format and the temperature was maintained by a thermostated system. Samples were pressure injected at 0.5 p.s.i. (0.034 bar). The voltage of the electrophoresis separation was 20 kV. A personal computer was used to control the P/ACE instrument and to perform data analysis using System Gold software (Beckman).

2.2. Chemicals

Sodium dodecyl sulfate (SDS), borax (sodium borate), boric acid, and (−)-ephedrine hydrochloride were purchased from Sigma (St. Louis, MO). Cinnamic acid and glycyrrhizin were bought from Nacalai Tesque (Kyoto, Japan). Puerarin, [6]-gingerol, and paeoniflorin were obtained from Yoneyama (Osaka, Japan). Daidzein was purchased from Extrasynthese (Genay, France). Methanol was bought from Merck (Darmstadt, Germany). Four concentrated Ge-gen-tang samples manufactured by different GMP medicinal companies were purchased from local drug stores in Taiwan. All other chemicals were of analytical grade and were purchased from Merck. All of the chemicals were used as received. Water was purified by a Milli-Q water system (Millipore, Bedford, MA) and filtered through a 0.22 μm filter.

2.3. Procedure

Standard solutions (5 mg ml^{-1}) of seven analytes were prepared in methanol. Sample solutions with various concentrations were prepared by diluting the standard solution with 40% (v/v) methanol aqueous solution. Electrophoresis borate buffers were prepared by mixing appropriate amounts of 0.1 M borax with 0.1 M sodium hydroxide or with 0.1 M boric acid in deionized water. A 2.0 g sample of each concentrated commercial medicinal preparations was accurately weighed. The concentrated samples were mixed and extracted with 20 ml of solution (50%, 70% or 100% methanol aqueous solution) for 15 min in an ultrasonic bath. The sample was then filtered through a filter paper. The extracting and filtering procedure was repeated three times. A total of 60 ml extracted sample solution was evaporated to dryness. Then a methanol

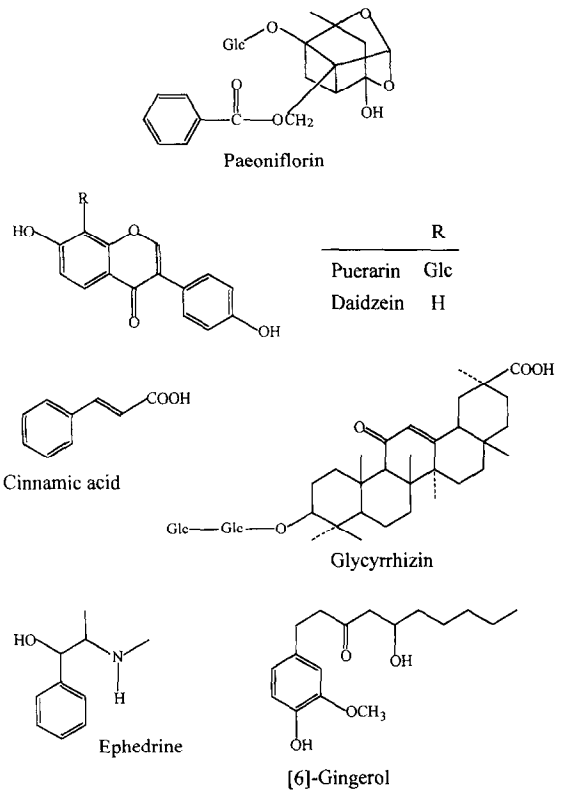

Fig. 1. Molecular structures of the seven analytes.

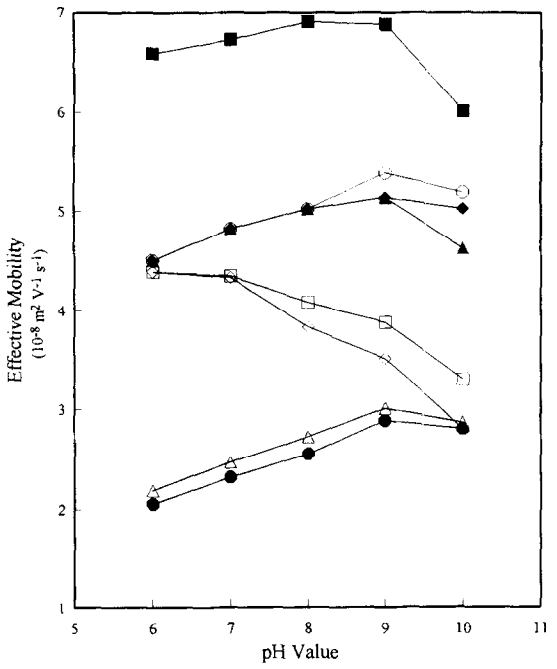

Fig. 2. Effect of buffer pH on effective mobility. Conditions: capillary, 47 cm (40 cm to detector)×50 μm i.d.; applied voltage, 20 kV; detecting wavelength, 200 nm; column temperature, 25°C. Key: ○, EOF; ◆, paeoniflorin; □, puerarin; ◇, daidzein; △, cinnamic acid; ●, glycyrrhizin; ■, ephedrine; ▲, [6]-gingerol.

aqueous solution was added to the dry sample to make it up to a final volume of 4.0 ml. The extracted sample was ready for analysis by MEKC.

The recovery study followed the same extraction procedure to prepare the sample for direct injection.

3. Results and discussion

Fig. 1 depicts the molecular structures of the seven target analytes in this study, i.e., paeoniflorin, puerarin, daidzein, cinnamic acid, glycyrrhizin, ephedrine, and [6]-gingerol. Their structures significantly differ except for puerarin and daidzein. Four analytes, i.e. puerarin, daidzein, cinnamic acid, and glycyrrhizin, had maximum absorbances around 270 nm, also had absorbances at 200 nm. The maximum absorbances of other three analytes were at 200 nm. Consequently, the detection wavelength was set at 200 nm to search for the optimum separation conditions. While quantitatively analyzing actual samples, the detecting wavelength was altered between 200 and 270 nm during the separation process to produce better signals for all analytes in one electropherogram.

Phosphate and borate buffers with various pH values ranging from 6.0 to 10.0 were initially used to separate the seven analytes. Variation of pH affected analytes' resolutions and migration times. Fig. 2 summarizes the effects of buffer pH on the separation. At pH 6.0, cinnamic acid and glycyrrhizin dissociated, thereby causing their effective mobilities to be slower than the electroosmotic flow (EOF). Ephedrine, carrying a positive charge, migrated faster than the EOF. The other four analytes had the same effective mobility as the EOF at pH 6.0. In pH 7.0 buffer, puerarin and daidzein migrated at an identical effective mobility which was slower than the neutral compounds (i.e., paeoniflorin and [6]-gingerol). The peaks of puerarin and daidzein could be adequately resolved at pH 8.0. The other analytes' migration order in pH

8.0 buffer was identical with those in previous buffers. The separation results in pH 9.0 buffer and in pH 8.0 buffer were similar except that paeoniflorin and [6]-gingerol separated from the EOF in pH 9.0 buffer. At pH 10.0, paeoniflorin and [6]-gingerol peaks were fully resolved. However, daidzein and glycyrrhizin migrated together. According to those results, the optimum separating resolutions for the analytes were achieved at pH 9.0. Nevertheless, further improvement was necessary to sufficiently separate all analytes.

3.1. Optimum MEKC conditions

Sodium dodecyl sulfate (SDS) surfactant was added to the pH 9.0 borate buffer to enhance the separation efficiency. Fig. 3 presents the effects of different SDS concentrations on the analytes' migration behaviors. As those results indicate, the effective mobilities of ephedrine and [6]-gingerol were significantly altered, implying that both analytes strongly interacted with SDS micelles. Although carrying one positive charge, ephedrine could interact with SDS through electrostatic and hydrophobic interactions. The effective mobilities of daidzein and paeoniflorin were influenced in the presence of SDS. More specifically, their mobilities were decreased with an increase in SDS concentration. Nevertheless, the other three analytes' effective mobilities were barely affected by adding SDS in the buffer because of their polar properties. Experimental results indicated that adding SDS in the borate buffer indeed improved the separation efficiency. The separation resolution was satisfactory in the buffer containing 20 or 40 mM SDS. Since the analytes' effective mobilities were greater in the 20 mM SDS buffer than in the 40 mM SDS buffer, the former was selected for subsequent use.

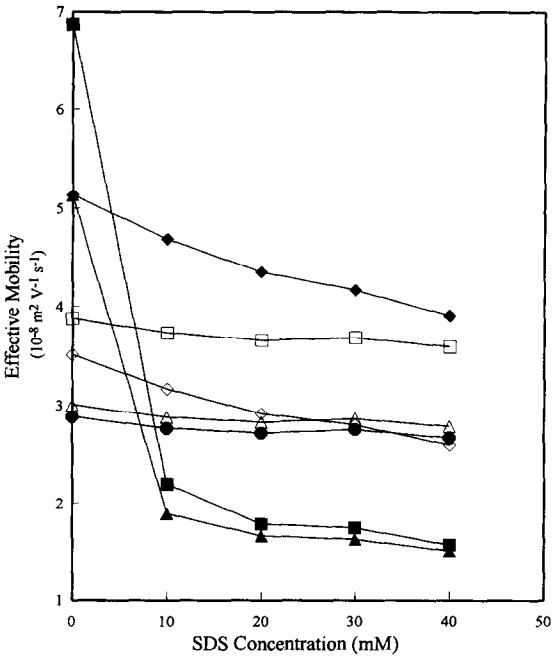

Fig. 3. Effects of SDS concentration on the effective mobility. Conditions: separation solution, SDS in 50 mM borate buffer (pH 9.0). Other conditions as in Fig. 2.

The optimum separation efficiency was achieved using pH 9.0 borate buffer containing 20 mM SDS. Under this circumstance, the seven analytes could be sufficiently separated within 10 min. However, when this buffer was applied to analyze concentrated Ge-gen-tang samples, ephedrine was interfered by an unknown compound despite the fact that all other analytes could be adequately resolved. To enhance the resolution of ephedrine, the temperature of the separation capillary was increased to 30°C. Fig. 4 depicts the electropherogram of seven analytes under these conditions. The satisfactory separation was completed within 9 min. Thus, increasing the temperature not only enhanced the exchange rate between SDS micelles and SDS monomer thereby yielding a better resolution, but also shortened the separation time.

Table 1 lists the average migration times, reproducibilities, correlation coefficients of calibration graphs, limits of detection, and separation efficiencies for those analytes. The relative standard deviations (RSDs) of the migration times were < 0.43%. The highest separation efficiency for the analytes was 4.67×10^5 for puerarin. The absorption wavelengths used for quantitative analysis were 200 nm for paeoniflorin, ephedrine, and [6]-gingerol, and 270 nm for puerarin, daidzein, cinnamic acid, and glycyrrhizin. The correlation coefficients of those linear calibration graphs exceeded 0.996 in the ranges of 60–480 $\mu g\ ml^{-1}$ for paeoniflorin, 26–210 $\mu g\ ml^{-1}$ for puerarin, 5–45 $\mu g\ ml^{-1}$ for daidzein, 15–120 $\mu g\ ml^{-1}$ for cinnamic acid, 112–900 $\mu g\ ml^{-1}$ for glycyrrhizin, 25–200 $\mu g\ ml^{-1}$ for ephedrine, and 13–110 $\mu g\ ml^{-1}$ for [6]-gingerol. For those analytes, the detection

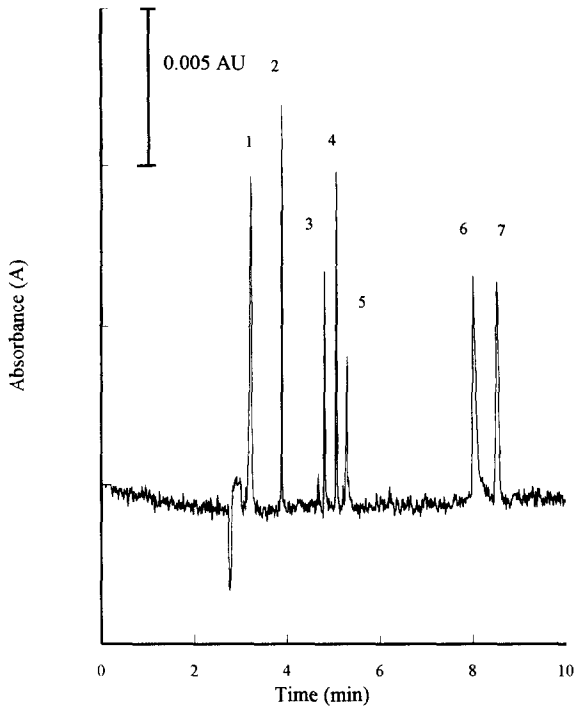

Fig. 4. Separation of the seven analytes by MEKC. Conditions: separation solution, 50 mM borate buffer containing 20 mM SDS (pH 9.0); column temperature, 30°C. Other conditions as in Fig. 2. Peaks: 1, paeoniflorin; 2, puerarin; 3, daidzein; 4, cinnamic acid; 5, glycyrrhizin; 6, ephedrine; 7, [6]-gingerol. An= absorbance.

limits ranged from 1.3 μg ml^{-1} for cinnamic acid to 57 μg ml^{-1} for glycyrrhizin. Under the optimum conditions, the separation efficiencies and peak resolutions were satisfactory for the standard analytes and Ge-gen-tang samples. Therefore, the optimized conditions were adopted herein to determine the seven markers in actual samples.

3.2. The extraction and analysis of real samples

The extraction efficiencies for the seven compounds were examined with three extraction solutions (50%, 70%, and 100% methanol). 50% methanol aqueous solution led to the lowest extraction efficiencies for all of the analytes. The highest extraction efficiencies for ephedrine, puerarin, and daidzein were achieved by pure methanol. However, glycyrrhizin could not be adequately extracted into pure methanol. Thus, 70% methanol aqueous solution was selected to extract Ge-gen-tang samples.

The extraction recoveries for the seven analytes in real samples were examined by spiking the standards into concentrated medicinal preparations. The recoveries for triplet extractions were 85.1% (RSD=4.11%) for paeoniflorin, 94.8% (RSD=2.37%) for cinnamic acid, 80.2% (RSD=3.77%) for puerarin, 89.2% (RSD=3.08%) for glycyrrhizin, 84.6% (RSD=0.66%) for daidzein, 81.2% (RSD=2.53%) for ephedrine, and 99.1% (RSD=3.56%) for [6]-gingerol.

The concentrated Ge-gen-tang samples were extracted according to the experimental procedure and were analyzed by MEKC. Fig. 5 depicts the separation of seven marker analytes in a Ge-gen-tang sample. The detection wavelength was changed from

Table 1
Average migration times, relative standard deviations, correlation coefficients of calibration graphs, limits of detection, and peak efficiencies for the analytes

	Migration time (min)[a,b]	RSD (%)[a,b]	Correlation coefficient of calibration graph[c]	Limit of detection (μg ml^{-1})[d]	Peak efficiency (N)
Paeoniflorin	3.00±0.010	0.34	0.998	19.5	19 000
Puerarin	3.89±0.012	0.30	0.999	3.2	467 000
Daidzein	4.81±0.019	0.39	0.998	1.4	456 000
Cinnamic acid	5.07±0.015	0.30	0.998	1.3	365 000
Glycyrrhizin	5.29±0.018	0.34	0.997	57.3	344 000
Ephedrine	8.01±0.034	0.43	0.996	9.3	132 000
[6]-Gingerol	8.52±0.026	0.30	0.999	2.8	191 000

[a] $n=12$.
[b] The concentrations of the analytes were 300 μm ml^{-1} for paeoniflorin, 60 μm ml^{-1} for puerarin, 15 μg ml^{-1} for diadzein, 20 μg ml^{-1} for cimmanic acid, 500 μg ml^{-1} for glycrrhizic acid, 100 μg ml^{-1} for ephedrine, and 30 μg ml^{-1} for [6]-gingerol.
[c] Triplicate results for each of five different analyte concentrations.
[d] S/N=3.

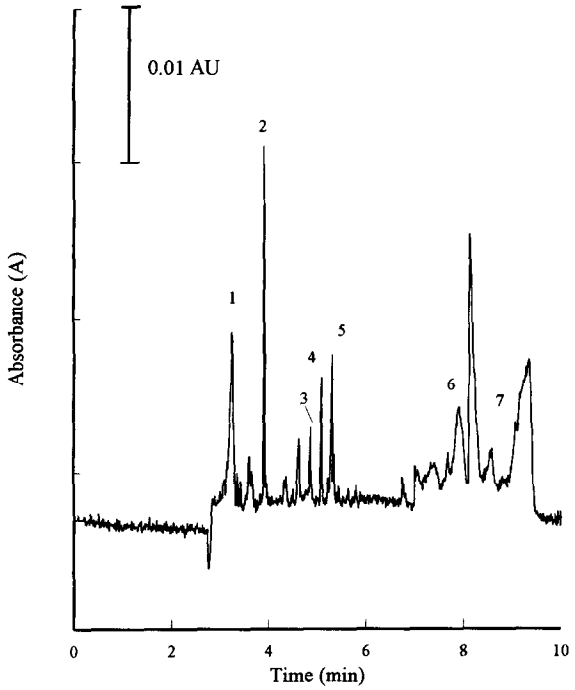

Fig. 5. Separation of Ge-gen-tang sample under the optimum MEKC conditions. Conditions: detecting wavelength, 200 nm (before 3.7 min and after 7.0 min), 270 nm (between 3.7 min and 7.0 min). Other conditions as in Fig. 4.

200 nm to 270 nm at 3.7 min, then was changed back to 200 nm at 7 min. The seven analytes were clearly identified by comparing each peak migration time and UV spectrum with those of the standard. Moreover, spiked standards in actual samples were employed to further confirm the identities of those analytes.

Table 2 lists the contents of Ge-gen-tang samples manufactured by four different companies, with correlation coefficients of UV spectra for the identification of analytes all above 0.97. As shown in Table 2, the contents of each analyte were markedly different in those samples, which is probably due to different sources of herbs, different manufacturing processes, or different composition formulas. Thus, quality control is critical for concentrated Chinese medicines. For the seven analytes, their RSD ranges for the samples varied from 0.63% (for glycyrrhizin, sample A) to 4.85% (for [6]-gingerol, sample D). Such variations in RSD can probably be attributed to the heterogeneity of the sample powder or different matrixes of those samples. Nevertheless, the electropherograms of those samples were similar to one another. Thus the results presented herein demonstrate that the MEKC method can serve as a quick and adequate method to analyze complicated Chinese medicinal preparations.

4. Conclusions

This study has successfully developed a high precision, high resolution, and rapid MEKC technique to analyze for seven marker components in Ge-gen-tang. Those analytes were completely separated within 9 min using a pH 9.0 borate buffer containing 20 mM SDS at 30°C. The sample extraction method for Ge-gen-tang was relatively simple and efficient. Therefore, the MEKC method is also a promising alternative to analyze other complicated Chinese medicinal preparations.

Table 2
Contents of paeoniflorin, puerarin, daidzein, cinnamic acid, glycyrrhizin, ephedrine, and [6]-gingerol in four different Ge-gen-tang samples[a]

Analyte	Sample A		Sample B		Sample C		Sample D	
	Mean (mg g^{-1})	RSD (%)	Mean (mg g^{-1})	RSD (%)	Mean (mg g^{-1})	RSD (%)	Mean (mg g^{-1})	RSD (%)
Paeoniflorin	9.80	1.44	11.76	0.96	15.13	2.22	4.02	3.68
Puerarin	2.10	1.69	2.23	0.93	1.47	1.42	1.38	1.27
Daidzein	0.16	1.66	0.09	0.82	0.07	2.71	0.07	3.93
Cinnamic acid	0.15	4.87	0.21	0.99	0.10	2.01	0.05	4.37
Glycyrrhizin	10.15	0.63	7.88	2.86	6.43	1.65	2.59	4.65
Ephedrine	2.47	4.68	2.99	1.84	2.23	1.90	1.32	1.61
[6]-Gingerol	0.26	3.12	0.85	3.79	0.59	2.95	0.57	4.85

[a] $n=3$.

Acknowledgements

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