Fingerprinting of *Marsdenia tenacissima* by Capillary Electrophoresis Compared to HPLC

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Abstract

Capillary electrophoresis (CE) is employed for the first time in a fingerprint analysis of *Marsdenia tenacissima*. Because the CE method is a new approach to fingerprinting, it is necessary to compare it to the conventional one: high-performance liquid chromatography (HPLC). In HPLC separation, 15 components are separated in 55 min, while in CE separation, 10 stable components are separated by 200mM boric acid (pH 8.0) containing 10% methanol within 12 min. CE shows better performance in the analysis of *Marsdenia tenacissima*, which makes its fingerprint in a much lower analysis time than with HPLC. It is further proven that CE can be a feasible and cost-effective method for the development of the fingerprint of *Marsdenia tenacissima*.

Introduction

Marsdenia tenacissima (Chinese name Tongguangteng), the stem of *Marsdenia tenacissima* (Roxb.) Wight et Arn. (family Asclepiadaceae), grown widely in southern and southwestern China, is a traditional herbal medicine (1) that has long been used for treating asthma, cancer, trachitis, tonsillitis, pharyngitis, cystitis, pneumonia, etc (2). There are two kinds of major active constituents in *M. tenacissima*, phenolic acid and C_{21} steroidal glycosides (3). Because it is impossible to separate these two kinds of compounds using the same conditions, only phenolic acid was involved in this study. Chlorogenic acid is the major active component, with antimicrobial and hemostasis effects. The active components of *M. tenacissima* are influenced by such factors as geographic origin, climate condition, and soil. In order to develop a comprehensive method to evaluate the quality of *M. tenacissima*, a chromatographic fingerprint technique was applied, which will play an important role in the efficacy, safety, and therapeutic reproducibility of *M. tenacissima* (4).

Chromatography, including thin-layer chromatography, gas chromatography, and high-performance liquid chromatography (HPLC), is introduced for the fingerprint analysis (5–7). Among them, HPLC, because of its high precision and popularization, has been regarded as the conventional way of performing a fingerprint analysis of *M. tenacissima* in the laboratory. Recently, capillary electrophoresis (CE) has been attracting attention as a more meaningful analysis technique for controlling the quality of herbal samples, because it is suitable for a wider range of complex analytical problems (8). CE is an automated analytical technique that separates species by applying voltage across buffer-filled capillaries. It is generally used for the separation of charged compounds that move at different speeds depending on their size and charge. CE has the advantages of short analysis time, high separation efficiency, and minimal consumption of the samples and solvents (9).

In the present study, CE was applied to develop a characteristic fingerprint analysis of *M. tenacissima* for the first time. Throughout the study, we focused on the feasibility of this method by using CE to develop fingerprinting of *M. tenacissima*, and it was compared it to HPLC as a means of validating the CE assay.

Experimental

Materials and reagents

The *M. tenacissima* samples were collected from Yunnan Province, China (provided by Nanjing Sanhome Pharmeceutical Co. Ltd.), and identified by Professor Song Xuehua of the China Pharmaceutical University. The voucher specimens were deposited at China Pharmaceutical University (Nanjing, China). Chlorogenic acid was provided by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

Borate, phosphate, boric acid, and sodium hydrate were purchased from Nanjing Chemical Reagent Factory (Nanjing, China). All were of analytical reagent grade. Solutions were prepared in deionized water. Methanol and acetonitrile, of chromatographic grade, were from Merck (Darmstadt, Germany).

Apparatus and operating conditions

HPCE

All of the experiments were carried out on a Hewlett-Packard G1600A (Hewlett-Packard, Palo Alto, CA) capillary electrophoresis instrument equipped with a diode array detector, temperature-controlled equipment, a HP chemical station, and an automatic injector. The applied voltage was held constant at 30 kV. The separation capillary was an untreated fused silica capillary with a total length of 50 cm and an effective length of 42 cm (50-µm i.d.). The UV detector was operated at 328 nm. The temperature of the capillary cartridge was maintained at 20°C. Before use, the buffer solutions were filtered through 0.22-µm films and degassed. Daily conditioning of the capillary was done by washing for 5 min with 0.1N NaOH, 5 min with water and 5 min with buffer. Between the runs, the capillary was purged for

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3 min with water and 3 min with buffer. After every 4 analyses, the buffer was replaced. Samples were introduced from its anodic end with gas pressure for 5 s.

HPLC

The HPLC system consisted of two Shimadzu LC-10AD delivery pumps, a Shimadzu LC-10AVP UV detector, and a model 7725i manual injector valve with a 20-µL sample loop (Shimadzu, Kyoto, Japan). The signals from the detector were connected and analyzed by a computer equipped with software of the N-2000 system (Zhejiang University).

The column was an Alltech ODS column (4.6 × 250 mm, 5 µm) (Lexington, Kentucky). The mobile phase was solvent A (CH_3CN) and solvent B (0.1% acetic acid–water). The gradient mode was as follows: from 0 to 30 min, 90–78% B; from 30 to 40 min, 78–74% B; and from 40 to 55 min, 74–67% B. The flow rate was 1.0 mL/min. The effluent was monitored at 328 nm.

Sample preparation

M. tenacissima was ground into fine powder, 4 g of which was weighed accurately and immersed with 50 mL of 50% methanol for 30 min in a conical flask. After ultrasonication for 30 min, it was cooled to room temperature and filtered though filter paper. This extraction was repeated twice. The extracts were combined and concentrated by evaporation at 40°C. The concentrated solution was diluted to 10 mL with the buffer (200 mmol/L boric acid containing 10% methanol, pH = 8.0) and filtered with 0.22-µm film as sample solution for future CE analysis.

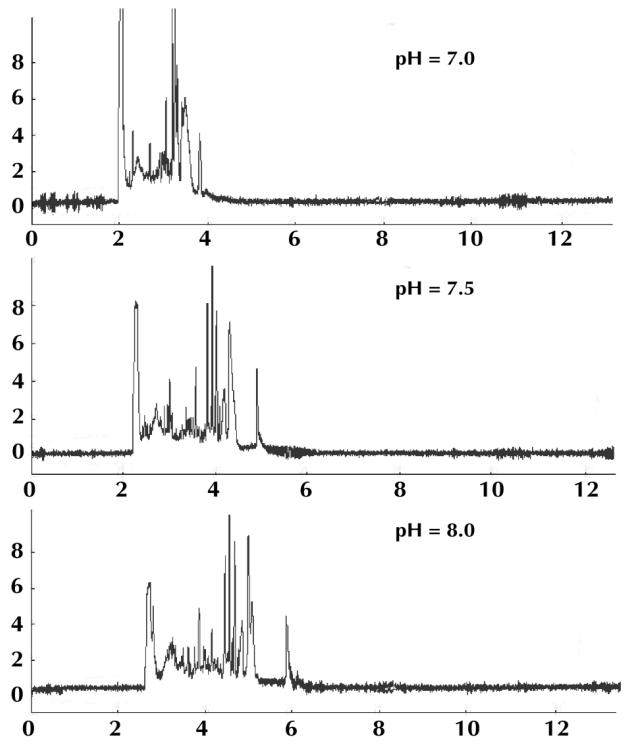

Figure 1. Chromatograms of the sample by CE analysis. Method: buffer, 100 mM boric acid (pH 7.0, 7.5, and 8.0); applied voltage, 30 kV; injection time, 5 s; temperature, 20°C; UV wavelength, 328 nm; capillary, 50 cm (effective length, 42 cm) × 50-µm i.d.

The concentrated solution was diluted to 10 mL with 50% methanol and filtered with 0.45-µm films as stock solution. Twenty microliters of the sample solution was used for HPLC analysis.

Results and Discussion

Optimization of separation conditions

In order to propose a specific and accurate way of developing a fingerprint of *M. tenacissima* by capillary CE, it is essential to find the best experimental conditions in which the analytes can be separated from each other. The following parameters were optimized: pH, composition and concentration of the buffer, organic modifier, and other electrophoretic parameters such as separation voltage, temperature, injection mode, etc.

Effect of pH

The pH of the buffer plays an important role in improving selectivity in CE, especially for closely related compounds because it affects both the overall charges of the solute and the electroosmotic flow (EOF). In the separation of *M. tenacissima*, the influence of pH was studied using seven pH values: 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, and 10.0. Figure 1 shows the influence of pH on the separation of this group of compounds. It is evident that when the pH increased, resolution and time analysis also increased. However, it was found that at a pH higher than 8.5, chlorogenic acid is labile due to the presence of two ortho-phenolic groups in its molecule. In this experiment, the chlorogenic acid displayed two peaks at a pH higher than 8.5 (Figure 2). Thus, a pH of 8.0 was selected as optimum in order to maintain good resolution and keep the chlorogenic acid stable.

Effects of buffer composition and concentration

Phosphate, borax, and boric acid buffers of 20–300 mmol were tested for use as the running buffer in this separation. It was found that boric acid showed higher separation efficiency than borax and phosphate. Good resolution between peaks was obtained with buffers at high concentration (200 mmol/L). Adding modifiers such as sodium dodecyl sulfate and sodium deoxycholate deteriorated the column efficiency and gave no improvement to the separation.

Effects of organic modifier

Organic solvents can be added to the buffer solution in order to improve separation and resolution and to increase the elution window by reducing the EOF (10–12). The influence of organic modifiers was examined using a 200 mM boric acid buffer of pH 8.0. Figure 3 shows the separation of this group of compounds in the presence of methanol and acetonitrile. Although the addition of modifiers resulted in a decreasing of the EOF and, consequently, an increasing in time of analysis, an improvement in the separation efficiency was obtained. The best results were obtained when methanol was added to the buffer. The reason is that the addition of methanol enhanced the solubility of the analytes and improved the peak shapes. Keeping other parameters constant (200 mM boric acid, pH 8.0), the methanol concentration was varied from 10% to 20%. It was found that migration times as well as current increased when the concentration of

methanol increased. The resolution also increased for higher methanol concentrations, but no appreciable improvements were observed for methanol concentrations above 10%. Therefore, a 200 mM boric acid buffer (pH 8.0) containing 10% methanol was selected as optimal.

Effects of electrophoretic parameters

The applied voltage is another important factor affecting EOF in CE analysis, as well as separation efficiency and resolution. In this experiment, different voltages from 10–30 kV were applied to find an optimum value. It can be observed in the experiment that the analysis time decreased with the increase of voltage. 30 kV was chosen because of the shorter migration time and narrower peaks, and all analytes were baseline separated within 12 min. The temperature was fixed at 20°C.

Fingerprint of *M. tenacissima* by capillary zone electrophoresis

The optimized capillary zone electrophoresis (CZE) separation electrolyte was 200 mM boric acid containing 10% methanol (pH 8.0). Prior to each sample injection, the capillary was rinsed consecutively with 0.1 N–NaOH, water, and buffer for 5 min. Crude samples of *M. tenacissima* from the Yunnan Province, which is considered the original and genuine herbal medicine, were prepared in accordance with the method described in the Experimental section, sample preparation subsection.

The method validation of fingerprint analysis was assessed based on both the relative retention time (the ratio of peak retention time of sample constituents to the reference standard) and the relative peak area (the ratios of peak area of sample constituents to the reference standard). The average electropherogram from 10 batches was regarded as the standardized characteristic fingerprint of *M. tenacissima*. Peaks that existed in all 10 electropherograms were assigned as "common peaks" for *M. tenacissima* (Figure 4). There were 10 common peaks. Peak 4 was identified as chlorogenic acid based on its UV spectra, migration times, and standard addition due to its high and stable contents (Figure 5). The relative retention time and relative peak area of all common peaks are obtained on the basis of this substance. The precision of the proposed method, on the basis of analyzing five replicate samples, was below 3.0% for the relative standard deviations (RSD) of all peaks' relative retention times and relative peak areas, respectively. The reproducibility test was performed with 6 sample solutions extracted from one batch of the herb. The RSD of the relative retention times and the relative peak areas were both less than 5%. The stability test was performed with sample solutions for 24 h at room temperature. The RSD of the relative retention time was less than 5%, and the RSD of the relative peak area was less than 5%. The consistency in chromatograms of these representative samples reflects the similar chemical properties of *M. tenacissima*. RSD of relative migration times and the relative peak areas of the corresponding peaks in 10 batches were calculated. The average RSD were 3.5% and 4.1%, respectively. It was proven that CZE could be an effective method in fingerprinting. These results also provided useful information regarding the marked components for quality control of *M. tenacissima*. Based on this study, the indices for fingerprint could used to distinguish substitute and counterfeit *M. tenacissima*.

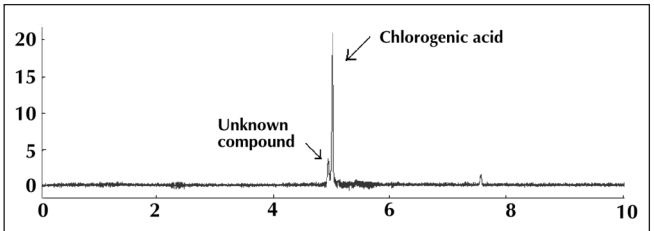

Figure 2. Chromatograms of chlorogenic acid standard sample by CE analysis. Method: buffer, 200 mM boric acid (pH 8.5); applied voltage, 30 kV; injection time, 5 s; temperature, 20°C; UV wavelength, 328 nm; capillary, 50 cm (effective length 42 cm) × 50-µm i.d.

Fingerprint of *M. tenacissima* by HPLC

In a comparison with CE, HPLC was used to develop the fingerprint of *M. tenacissima* in this study as described in Figure 6. In the 10 batches of samples, there are 15 common peaks eluted in 55 min by HPLC. Peak 2 was identified as chlorogenic acid based on its UV spectra, retention times, and standard addition. The average RSD values of the relative retention times and the relative peak area of the corresponding peaks in HPLC analysis were 2.1% and 3%.

Comparisons of CE and HPLC

Both CE and HPLC could display the distribution of the major active components of *M. tenacissima*, which is the most important characteristic of the fingerprint. CE is an automated analytical technique that separates species by applying voltage across buffer-filled capillaries. When voltage is applied, different migra-

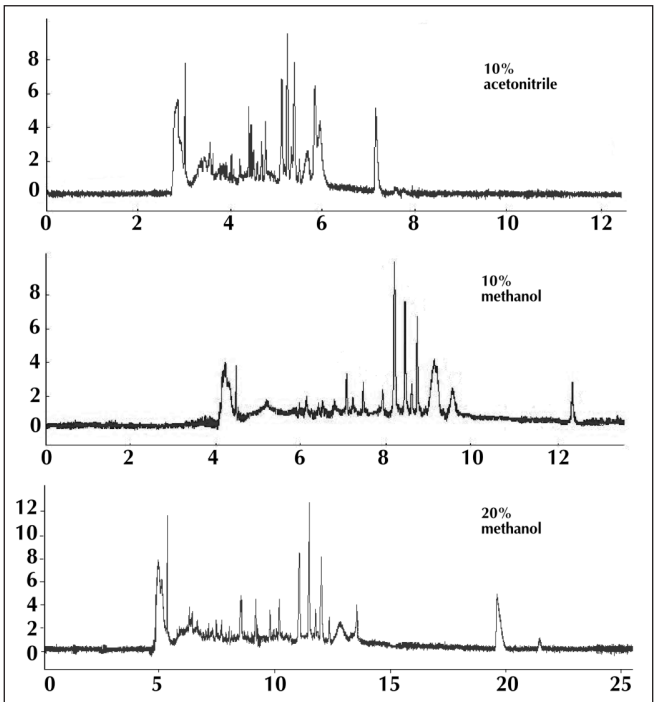

Figure 3. Chromatograms of the sample by CE analysis. Method: buffer, 200 mM boric acid (pH 8.0) containing 10% methanol, 20% methanol, and 10% acetonitrile; applied voltage, 30 kV; injection time, 5 s; temperature, 20°C; UV wavelength, 328 nm; capillary, 50 cm (effective length 42 cm) × 50-µm i.d.

tion speeds separate the components. HPLC is a solid–liquid partition chromatography, and separation on HPLC depends on the partition coefficient of different components in the two phases. However, because of the different principles of the two methods, they have distinct characteristics, and there are some advantages and disadvantages between them: different retention time and different common peaks. The first superior aspect of CE over HPLC lay in the shorter analytic time, which is suitable for traditional Chinese medicine (TCM) analysis, especially time-consuming analyses such as fingerprint. In HPLC fingerprint, the running time is 60 min, while in CE analysis, 14 min is enough. Therefore, CE, as a new method in fingerprint development, can reduce the running time greatly. Secondly, the cost of the column and the solvent in CE is much lower than in HPLC. As we all know, various impurities (such as chlorophyll) in TCM will impair a chromatography column and shorten its life, while that will not happen to a CE column because it is hollow and easy to wash. Furthermore, the buffer is cheaper than the organic solvent used in HPLC. So CE showed advantages over HPLC in low cost per analysis. On the other hand, the volumes of sample injected were very small in CE, which leads to a higher concentration demanded of the sample. Here CE showed slightly less sensitivity than HPLC. This is why, with the same sample preparation, there were 15 common peaks in the fingerprint with HPLC but only 10 common peaks with CE. Another problem caused by the small sample volume of CE was the relatively low stability of separation, which caused the high RSD of relative retention times and relative peak area. However, the precision of CE was preferable for fingerprint.

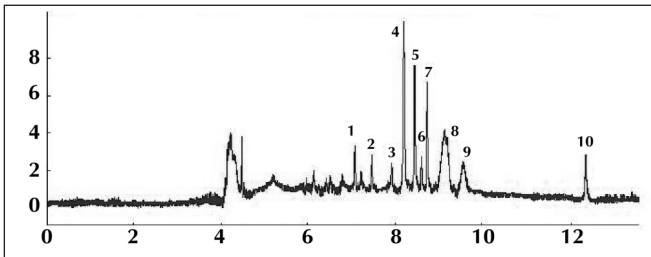

Figure 4. The electropherograms of the sample. Method: buffer, 200 mM boric acid (pH 8.0) containing 10% methanol; applied voltage, 30 kV; injection time, 5 s; temperature, 20°C; UV wavelength, 328 nm; capillary, 50 cm (effective length 42 cm) × 50-µm i.d.

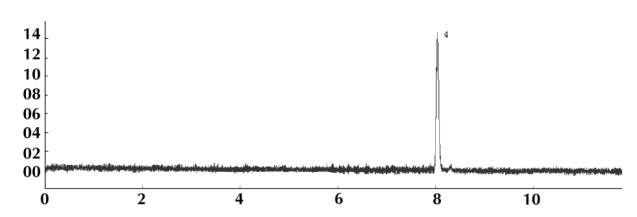

Figure 5. Chromatograms of chlorogenic acid standard sample by CE analysis. Method: buffer, 200 mM boric acid (pH 8.0) containing 10% methanol; applied voltage, 30 kV; injection time, 5 s; temperature, 20°C; UV wavelength, 328 nm; capillary, 50 cm (effective length 42 cm) × 50-µm i.d.

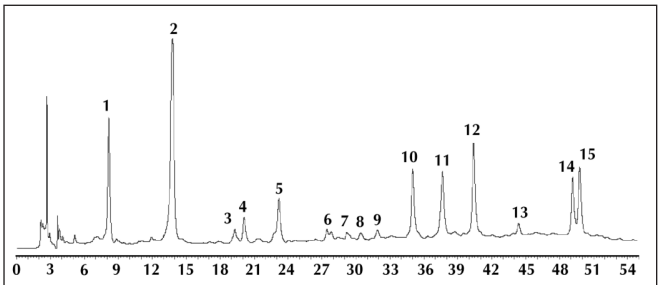

Figure 6. Chromatograms of sample by HPLC analysis. Method: an Alltech ODS column (4.6 × 250 mm, 5 µm); mobile phase, solvent A (CH_3CN) and solvent B (0.1% acetic acid–water); the gradient mode, 0 ~ 30 min, 90–78% B; 30–40 min, 78–74% B; 40–55 min, 74–67% B; the flow-rate was 1.0 mL/min. The wavelength was 328 nm.

Conclusion

The constituents in TCM are complicated. Because each method has its own advantages and disadvantages, it is necessary to develop more approaches in fingerprint development. In this study, CE and HPLC methods have been developed for the fingerprint of *M. tenacissima*. Both of them were effective in showing the distribution of the components. CE showed advantages over HPLC in low cost per analysis, rapidity, and a minimal use of organic solvents. Nevertheless, HPLC was still a robust method for fingerprint analysis as a result of its high precision, simple operation, good popularization, and ability to express more chemical information.

This study has demonstrated a simple and rapid CE procedure that makes possible the development of the fingerprint of *M. tenacissima*.

Acknowledgments

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References

1. Editorial Committee of the Pharmacopoeia of People's Republic of China. The Pharmacopoeia of People's Republic of China, Part 1. Chemical Industry Press, Beijing, China 2005.
2. State Administration of Traditional Chinese Medicine. Selected Works of Chinese Bencao (No. 4). Scientific and Technical Publishers, Shanghai, 1999.
3. S.X. Qiu, S.Q. Luo, L.Z. Lin, and G.A. Cordell. Further polyoxypregnanes from Marsdenia tenacissima. *Phytochemistry* **41:** 1385 (1996).
4. Drug Administration Bureau of China. Requirements for Studying Fingerprint of Traditional Chinese Medicine Injections (Draft). Shanghai, 2000.
5. H.M. Lu, Y.Z. Liang, and S. Chen. Identification and quality assessment of Houttuynia cordata injection using GC–MS fingerprint: A standardization approach. *J. Ethnopharmacol.* **105:** 436 (2006)
6. X. Di, Kelvin K.C. Chan, H.W. Leung and C.W. Huie. Fingerprint profiling of acid hydrolyzates of polysaccharides extracted from the fruiting bodies and spores of Lingzhi by high-performance thin-layer chromatography. *J. Chromatogr. A* **1018:** 85 (2003)
7. L.H. Zhao, C.Y. Huang, Z. Shan, B.R. Xiang, and L.H. Mei. Fingerprint analysis of Psoralea corylifolia L. by HPLC and LC–MS. *J. Chromatogr. B* **821:** 67 (2005).
8. W. Tiansong. Capillary electrophoresis and fingerprint of traditional Chinese medicine. *Chinese Traditional Patent Medicine* **6:** 397–99 (2000).
9. F.A. Tomas-Barberan. Capillary electrophoresis: a new technique in the analysis of plant secondary metabolites. *Phytochem. Anal.* **6:** 177–92 (1995).
10. I.M. Johansson, E.C. Hung, J.D. Henion, and J.Z. Weigenbaum. Capillary electrophoresis-atmospheric pressure ionization mass spectrometry for the characterization of peptides: Instrumental considerations for mass spectrometric detection. *J. Chromatogr.* **554:** 311 (1991).
11. G.M. Mclaughlin, J.A. Nolan, J.L. Lindahl, R.H. Palmieri, et al. Pharmaceutical drug separations by HPCE: Practical guidelines. *J. Liq. Chromatogr.* **15:** 927 (1992).
12. C. Schwer and E. Kenndler. The influence of organic solvents on the electroosmotic velocity and the potential. *Anal. Chem.* **63:** 1801 (1991).

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