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Short communication

Simultaneous determination of key bioactive components in *Hedyotis diffusa* by capillary electrophoresis

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Abstract

A capillary zone electrophoresis (CZE) method based on systematic one-variable-at-a time approach was developed for the analysis of four important bioactive components (geniposidic acid, ursolic acid, quercetin and *p*-coumaric acid) in the extract of *Hedyotis diffusa* (HD). Separations were carried out in a fused-silica capillary tube with peak detection at 214 nm. Good separation was achieved using a 20 mM borate buffer containing 5% acetonitrile as organic modifier and pH adjusted to 10.0. Operating voltage was 15 kV and temperature was maintained at 25 °C while hydrodynamic injection was 5 s. A good linearity, with correlation coefficients in the ranges of 0.997–0.999 was obtained in the calibration curves of each standard. Relative standard deviation (R.S.D.) of migration time was between 0.32 and 0.70% and deviation of corrected peak area was between 8.84 and 11.99%. These results indicate that this method could be used for rapid and simultaneous analysis of the bioactive components in HD and other herbal products.

Keywords: *Hedyotis diffusa*; Capillary zone electrophoresis; Organic modifier; Geniposidic acid; Ursolic acid; Quercetin; *p*-Coumaric acid

1. Introduction

Traditional Chinese medicines have been used to treat human diseases in China for centuries. In the last three decades, pharmaceutical explorations of bioactive components in traditional medicines have attracted lots of scientific attention [1]. Because most traditional Chinese medicines consist of complex constituents, a proper analytical technique for their rapid and simultaneous analysis is urgently required [2].

Hedyotis diffusa (HD), also called 'Ped-Hue-Juwa-Chji-Cao' in Chinese, is a notable annual herb widely distributed in northeast Asia. The herb has been commonly used for the treatment of various diseases, including rheumatism, arthritis, appendicitis, sore throat, urethral infection, contusions and for eliminating the invasion and extension of malignancies [3–5]. Recent study by Gupta et al. [6] found that aqueous extract of HD effectively inhibited the proliferation of eight cancer cell lines and induced significant increase of apoptosis; yet exhibited minimum toxic effect on normal pancreatic cells. There were other reports indicating that methanol extracts of this herb exerted antimicrobial activities and antifungal activities [7].

Because this herb shows a lot of beneficial effect to human health, it has been used to augment the therapeutic effects of other herbs. Whatever the uses, effectiveness of treatment for a disease is dose dependent. Therefore, it is necessary to determine the amount of bioactive component in a pharmaceutical product. It has been demonstrated that the major active components in HD are ursolic acid, oleanolic acid, *p*-coumaric acid, geniposidic acid and quercetin [8–10]. These components represent different chemical nature and can be used as quality markers for HD. However, there is no suitable method currently available for simultaneous determination of these components in the herb. A simple, reliable and reproducible method for the analysis of bioactive components in HD is vital no matter whether for pharmacokinetic studies or for quality control of the herb.

Capillary electrophoresis (CE) has become one of the most popular methods for qualitative and quantitative analysis of

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herbal products. The method is suitable for simultaneous analysis of mixed compounds and offers advantages of excellent separation in terms of efficiency and resolution of analysis. As there is no report on the determination of the major active substances in HD by CE, this paper aims to describe a CZE method for quick and simultaneous determination of four key bioactive components of different chemical nature in the herb.

2. Experimental

2.1. Apparatus

A capillary electrophoretic system (Beckman P/ACE 5000) purchased from Beckman Instruments (Fullerton, CA, USA) was used throughout the whole study. The system and the method of data analysis were the same as previously described [11]. The CE system was operated in a normal mode with the cathode at the detector end. Prior to running any standard or sample solutions through the system, the capillary was conditioned with 0.1 M NaOH for 15 min followed by Milli-Q water for 15 min as described previously but with some modifications [12]. In between each injection, the capillary was flushed again with 0.1 M NaOH, followed by Milli-Q water and the running buffer in use for 2 min in order to regenerate and equilibrate the capillary. The operating conditions for the system were set at a voltage of 20 kV, temperature of 25 °C and hydrodynamic injection under high pressure at 20 psi for 5 s with UV–vis detection at wavelength 214 nm.

2.2. Chemicals

All chemicals were of analytical grade. Purified ursolic acid, quercetin and *p*-coumaric acid were purchased from Sigma Chemical Co. (St. Louis, Mo, USA) while geniposidic acid was obtained from Wako Pure Chemical Industries (Osaka, Japan). These chemicals were used as reference standards for the analysis of components in the methanol extract of HD. Sodium tetraborate used as buffer electrolyte was also purchased from Sigma Chemical Co. Both sodium hydroxide and hydrochloric acid for pH adjustment were ordered from Riedel-de Haën (Seelze, Germany). Organic modifiers, such as methanol, ethanol, propanol, butanol, acetone and acetonitrile were all bought from Lab-Scan. Milli-Q deionised water with resistance larger than 18 MΩ was used throughout the study (Millipore, Bedford, MA, USA).

2.3. Sample preparation

A single lot of dry HD was purchased through a local Chinese medicinal shop (Yunnan Medicine Company, Kunming, China). The whole dry plant was homogenized by milling. Only particles with a size <2 mm were used and 10 g of the fine particles was refluxed with 200 ml of methanol for 1 h in a Soxhlet apparatus. After cooling, the extract was filtered through a 0.2 μm cellulose filter and then dried by speed vacuum. The dried extract was stored at −20 °C until use.

3. Results and discussion

3.1. Method development

Earlier work from our group demonstrated that successful CE separation of a mixture of eleven bioactive components required optimization of the separation condition that involves proper use of pH, buffer concentration, right choice of organic solvent and surfactant concentration [13]. Although three out of the four components described here had already been covered, the method was not suitable for simultaneous analysis of all four bioactive components in HD. Hence, a specific condition for analysis of the herb is needed. The structures of GA, UA, QU and CA, as shown in Fig. 1, indicate that they are anionic substances. Hence, sodium borate was chosen as the background electrolyte. A condition for the best separation of these bioactive compounds was established by systematic one-variable-at-a time approach. Optimization of the pH, the right choice of buffer and borate concentration were investigated first. Further improvement was achieved by adding a right organic modifier and properly setting of the running voltage.

pH of a buffer is the most important parameter as it determines the ionization and mobility of a solute. When pH increased, electrophoretic mobility of standard decreased and results in increased migration time. Consequently, it gave a higher resolution [12]. Fig. 2 shows that electrophoretic mobility of all standards increased to different degree when pH increased. Between pH 8.0 and 9.0, peaks of geniposidic acid and ursolic acid could not be resolved from each other. At pH 9.5, quercetin and *p*-coumaric acid overlapped. But at pH 10, quercetin and *p*-coumaric acid were separated again. As pH of buffer at 10 provided satisfactory resolution, it was chosen as the optimum condition for resolving the standards.

The effect of different borate concentrations ranging from 10 to 50 mM, were investigated on the separation of stan-

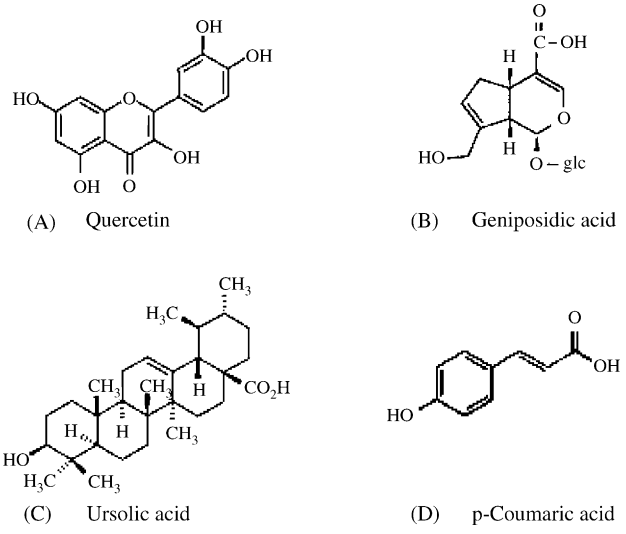

Fig. 1. Chemical structure of important bioactive components of *Hedyotis diffusa*. Four compounds representing iridoids, flavonoids and phenolic compounds were used as standard marker: (A) quercetin; (B) geniposidic acid; (C) ursolic acid and (D) *p*-coumaric acid.

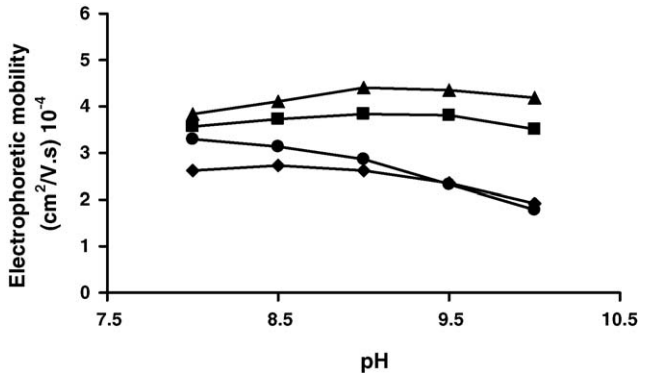

Fig. 2. Effect of pH on the separation of standards. Symbols: (▲) ursolic acid; (■) geniposidic acid; (●) quercetin and (♦) *p*-coumaric acid.

dards. When the borate concentration was increased from 10 to 30 mM, electrophoretic mobility of individual standard was also increased. The resolution of peaks decreased at higher borate concentration. Borax buffer at 20 and 30 mM gave a better resolution than that at 10 mM. However, 20 mM borax gave a shorter migration time. Consequently, 20 mM borax at pH 10.0 was chosen as the ideal concentration for the mobile phase.

Addition of organic solvents to the buffer allows a better separation of analytes that are not water soluble. In the presence of organic modifier, electro-osmotic flow could be reduced by increasing the viscosity of a buffer system [14–16]. In this study, effects of adding 5% organic modifiers to 20 mM borax at pH 10.0 on migration time were studied. For all organic modifiers used, methanol gave the longest analysis time. Except methanol, when carbon chain of organic modifier increased, analysis time slightly increased. Contrary to alcohol, both acetone and acetonitrile gave a shorter analysis time. Although the electropherograms of borax buffer containing acetone or acetonitrile were similar, the later was chosen as organic modifier for further study.

In order to obtain a better resolution of the analytes, various concentrations of acetonitrile as organic modifier were further investigated. Electrophoretic mobility of the analytes was not greatly affected in the presence of acetonitrile. Nevertheless, addition of 5% acetonitrile gave the shortest migration time and produced a better resolution. Therefore, acetonitrile at 5% was chosen to make the buffer.

3.2. Method validation

Precision of the peak parameters, such as migration time and corrected peak area determines reliability of an analytical

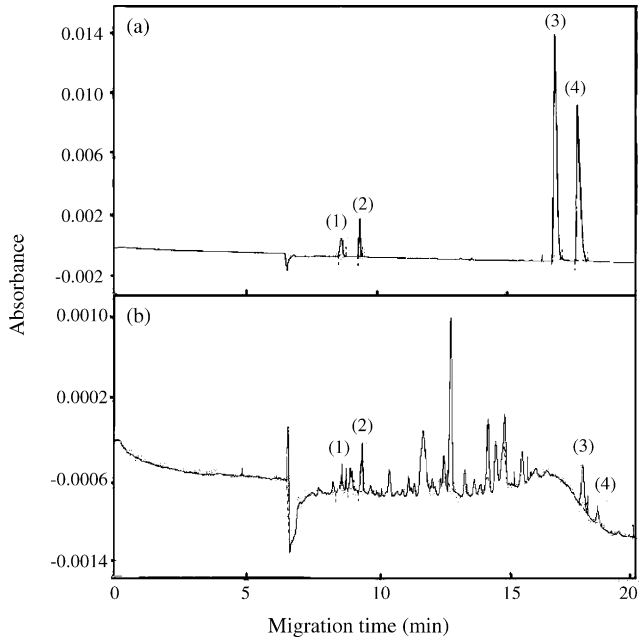

Fig. 3. Electropherograms of mixed standard markers and methanol extract of a herbal sample of *Hedyotis diffusa*. Resolved bioactive markers of standard are shown in panel (a) and extract of the bark of HD sample is shown in panel (b). Peak labeled with (1) ursolic acid; (2) geniposidic acid; (3) quercetin and (4) *p*-coumaric acid.

method as this is important for peak identification and quantitative analysis of the sample [17]. Six repeated analyses of the standards in terms of migration time and corrected peak area were performed under an optimum condition for operating the CZE. Table 1 reveals that the R.S.D. of migration time ranged from 0.3 to 0.7%, indicating that the migration time showed good reproducibility. Thus, the condition used was suitable for qualitative and quantitative determination of the active components. Different concentration from 1 to 50 ppm of standard solution was used to investigate the linearity of the corrected peak areas of each analyte. Correlation coefficient (r^2) at 0.995 or greater is regarded acceptable [18]. The correlation coefficients of calibration curves of ursolic acid, geniposidic acid, quercetin and *p*-coumaric acid were 0.998, 0.999, 0.999 and 0.997, respectively, indicating that the calibration curves are reliable for quantitative analysis.

3.3. Sample analysis

Using the optimized CZE condition established above, amount of ursolic acid, geniposidic acid, quercetin and *p*-

Table 1
Relative standard deviation (R.S.D.) of migration times of markers and their peak areas of six injections of mixed standards (50 ppm)

Marker	Mean migration time (min)	R.S.D. of migration time (%)	Mean corrected peak area	R.S.D. of corrected peak area (%)
Ursolic acid	8.58	0.32	248.2	10.70
Geniposidic acid	9.35	0.43	880.0	8.84
Quercetin	16.93	0.38	6133.6	11.99
p-Coumaric acid	17.79	0.70	4776.4	10.68

Experimental conditions: 20 mM borate and 5% acetonitrile at pH 10; operating voltage was 15 kV at 20 °C with a capillary tube (effective length = 50 cm).

Table 2
Content of some bioactive components present in the methanol extract of *Hedyotis diffusa* by CE

Component	Amount (mg/g)[a]
Ursolic acid	1.004
Geniposidic acid	1.182
Quercetin	0.110
p-Coumaric acid	0.067

[a] $n = 3$.

coumaric acid in a herbal sample of HD was determined. The electropherogram of the standards and the methanol extract of HD are shown in Fig. 3. Peak identification was carried out by spiking the sample with each standard. Although peak identification was ambiguous in comparison to the sample of mixed standards, peaks eluted at 8.64, 9.42, 17.85 and 18.41 min were confirmed to be ursolic acid, geniposidic acid, quercetin and *p*-coumaric acid, respectively, by comparing the UV–vis spectra of the standards and solutes. The amounts of these bioactive components found in the herb of HD are shown in Table 2.

4. Conclusions

In conclusion, an analytical method based on capillary zone electrophoresis for rapid and simultaneous determination of four important bioactive components in HD has been successfully established in this study. The method is simple, efficient, sensitive, accurate and reliable. It can be used for quantitative study and for quality control of the herb by means of analyzing the bioactive components.

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