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Short communication

Determination of bioactive diterpenoids from *Andrographis paniculata* by micellar electrokinetic chromatography

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Abstract

The present paper describes the development of a micellar electrokinetic chromatographic (MEKC) method for simultaneous determination of andrographolide, deoxyandrographolide and neoandrographolide in ethanol extracts of *Andrographis paniculata*. Separations were carried out in a fused-silica capillary tube with UV detection at 214 nm. Good separation was achieved using a 20 m*M* borate buffer, containing 20 m*M* sodium dodecyl sulphate and 10 m*M* sodium cholate, adjusted to pH 8.3 at an operating voltage of 25 kV, temperature of 35°C and a hydrodynamic injection of 5 s. The method was validated with good correlation coefficients obtained (0.9986–0.9989) while relative standard deviation (RSD) of migration time was between 1.14 and 2.42. It is concluded that this method could be used for speedy and accurate qualitative and quantitative analysis of bioactive diterpenoids in andrographis herb and its derived products.

Keywords: *Andrographis paniculata*; Andrographolide; Deoxyandrographolide; Neoandrographolide

1. Introduction

The resurging use of herbs in recent years for healthcare and therapeutic purposes has generated many new suppliers and business activities, which in turn have generated a good deal of problems about quality control and efficacy studies of herbal substances [1]. Andrographis Herb, also known as Kalmegh, is the dried aerial part of *Andrographis paniculata*. It belongs to the Acanthaceae family of the plant kingdom. The herb is commonly used in combating the common cold and respiratory inflammations. It is reported to remove latent heat, counteract hepatotoxicity and induce hypoglycemia [2]. The therapeutic activity of this herb has been attributed to andrographolide and its related diterpenoid compounds, i.e. deoxyandrographolide and neoandrographolide. However, each one of these constituents exhibits varying degrees of anti-inflammatory effects and antibacterial activities [3]. Recent clinical studies reveal that andrographolide in the herb demonstrates better hypoglycemic effects [4], choleretic [5] and hepatoprotective activity against CCl_4 [6], as well as galactosamine and paracetamol intoxication [7] while dehydroandrographolide is a better in vitro inhibitor against the human immunodeficiency virus (HIV) [8]. These different effects imply that qualitative and quantitative control of the

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herbs is of paramount importance to ensure its maximal therapeutic value.

Although a number of methods, including gravimetric, colorimetric, titrimetric, spectrophotometric and high-performance liquid chromatographic (HPLC), have been reported for the quantitative estimation of herbal components, many of these procedures are time consuming, imprecise and require multiple steps of handling. Capillary electrophoresis (CE), on the other hand, has been claimed to be speedy and reliable. This analytical technique has become one of the most popular methods for qualitative and quantitative analysis of herbal drugs. The method is suitable for simultaneous analysis of mixed compounds and offers advantages of excellent separation in terms of efficiency and resolution of analysis. In addition, the amount of the sample and solvent used is minimized, commonly 5–50 nl.

A. paniculata has been previously analyzed by various techniques, such as TLC [2], UV-spectrometry [9,10], chemiluminescence [11], HPLC [9,12–14] and LC [15]. TLC method normally takes 1–3 h to separate components in herbal extracts with comparatively poor sensitivity. As far as UV spectrometry and chemiluminescence are concerned, only andrographolide has been separated from the complex sample matrix and quantified. The results of UV spectrometry were not satisfactory, with a recovery of andrographolide of only 94% and correlation coefficient of 0.994. Wang et al. (1994) used indirect chemiluminescence to quantify the andrographolide concentration in rabbit plasma [11]. By forming a complex of luminol-H_2O_2–Co^{2+}, a stable form of light for detection is produced. However, due to its complexity, this method is not commonly adopted. Besides, overall method repeatability was 2.35% with recovery ranging from 91.5 to 108.8%. A more widespread method using HPLC offers a shorter analysis time. It took less than 3 min for andrographolide to be eluted from a spherical silica (3.9 mm×15 cm) column [9]. Despite this, there remains a need for a technique that can simultaneously resolve and quantify andrographolide, deoxyandrographolide and neoandrographolide. Such quantification of bioactive constituents is of paramount importance in order to ensure safe usage and quality control of the herb as well as its derived products. This paper describes the development of such a technique utilizing the resolving power of micellar electrokinetic chromatography (MEKC).

2. Experimental

2.1. Apparatus

MEKC was carried out on a capillary electrophoretic instrument (model: Beckman P/ACE 5000; Beckman Instruments, Fullerton, CA, USA) equipped with a 50 μm I.D.×57 cm (50 cm from inlet to detector) fused-silica capillary tube (Supelco, Bellefonte, PA, USA). The instrument was connected to a personal computer which controlled the P/ACE instrument. Data analysis was performed using a P/ACE station software developed by Beckman. The capillary electrophoretic equipment was operated at normal mode with the cathode at the detector end. Hydrodynamic injection was used under high pressure (20 p.s.i.). Wavelength of UV–visible detection was set at 214 nm for taking absorbance of diterpenoids in andrographis sample.

2.2. Chemicals

Purified andrographolide (98%) was purchased from Aldrich (Germany). It was used as a reference material for subsequent analysis of diterperoids in the extract of andrographis herb. Sodium cholate was also obtained from Aldrich (Germany). Sodium dodecyl sulfate was obtained from Bio-Rad (Hong Kong). Sodium tetraborate-10-hydrate, ethanol and hydrochloric acid (analytical grade) were obtained from Riedel-de-Haën (Seelze, Germany) and sodium hydroxide was obtained from Pharmacos (Southend on Sea, Essex, UK). Acetonitrile (analytical grade) was purchased from LabScan (Bangkok, Thailand). Milli-Q deionised water was used throughout the study (Millipore, Bedford, MA, USA).

2.3. Sample preparation

Due to the lack of commercially purified materials of standards of deoxyandrographolide and neoandrographolide, they were prepared according to a method outlined by Wu and Chow (1982) [16]. Their identity was confirmed by mass spectrometry and

infrared spectroscopy. Purity was assessed by reversed-phase HPLC using a solvent of 60% methanol in water. Deoxyandrographolide and neoandrographolide were eluted at 9.04 and 9.66 min, respectively, with area ratios greater than 99%, reflecting the high purities obtained.

To prepare ethanol extract of the herb, 63 g of the dried, powered whole plant was divided into nine portions. The portions were extracted consecutively with 150 ml of ethanol for 3 h. The sample solution was collected and dried completely under speed vacuum. A total of 10 mg of the sample was dissolved in 1 ml of ethanol, then diluted down with milli-Q water to a concentration of 1 mg/ml (10% ethanol). Prior to injection, the sample solution was sonicated in an ultrasonic bath for 15 min and filtered through a 0.2 μm cellulose filter.

3. Results and discussion

3.1. Method development

Optimization of the MEKC separation conditions for *A. paniculata* involved variation of buffer composition, concentration, pH, organic solvent and surfactant content. At 10 m*M* borate buffer, two peaks appeared in the electrophoerogram; one that overlapped with the solvent peak which was subsequently identified as andrographolide, while the other was a combination of deoxyandrographolide and neoandrographolide (sample spiking was used to overcome any problems in peak identification) (Fig. 1). Increasing the concentration of borate buffer did not improve the separation of these three components because of their neutral properties whilst the resultant increased joule heating enhanced the current up to 200 μAMP, which should be avoided. Therefore, a buffer concentration of 20 m*M* was used for subsequent separations.

Variation of pH from 7.5 to 9.5 showed a significant effect on andrographolide. Above pH 9.0 its peak was split in two. Under alkaline conditions, andrographolide was hydrolyzed with its lactone ring being cleaved to form andrographolic acid. Therefore, a pH of 8.3 was selected as the optimum.

SDS has been shown to be useful for the enhanced separation of highly hydrophobic compounds while sodium cholate is employed for less hydrophobic compound [17]. Firstly, buffer composition was adjusted to contain 10 m*M* SDS, in order to separate andrographolide from the solvent peak. Further increases in the SDS concentration from 20 to 30 m*M* only increased the migration time of the andrographolide. Therefore, 20 m*M* SDS was selected as the optimum concentration. To this 10 m*M* sodium cholate was added to further improve selectivity.

The major factor influencing the resolution of neoandrographolide and deoxyandrographolide is temperature. As shown in Fig. 2, peak resolution was significantly improved by increasing temperature. At 35°C, baseline separation was achieved. For capillary zone electrophoresis, increasing temperature results in a loss of resolution. Conversely, increasing temperature has a markedly positive effect on selectivity in MEKC as partitioning is enhanced at higher

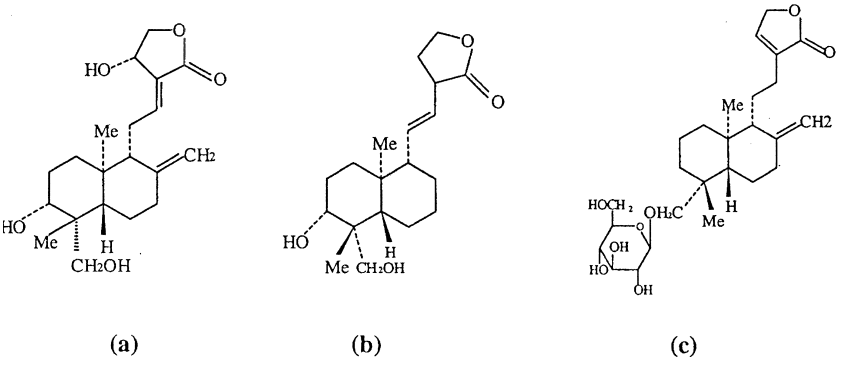

Fig. 1. Chemical structures of the three main diterpenoids in *A. paniculata*. (a) Andrographolide, (b) Deoxyandrographolide and (c) Neoandrographolide.

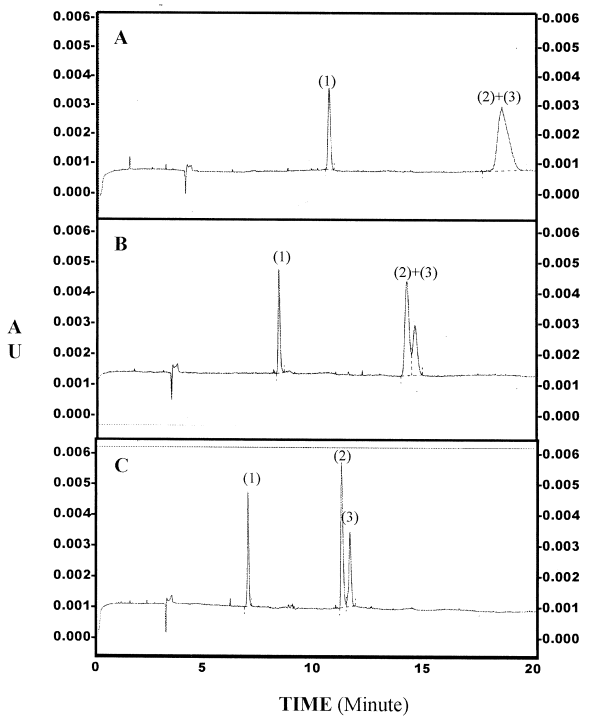

Fig. 2. Effect of temperature on the resolution of bioactive markers of *A. paniculata*. Capillary temperature was maintained at (**A**) 25°C (**B**) 35°C and (**C**) 35°C during electrophoresis. Peak (1), (2) and (3), respectively, represent andrographolide, deoxyandrographolide and neoandrographolide.

temperature [18]. Increasing the operating voltage did result in slight improvements in resolution but also increased overall analysis time. Therefore, 25 kV was selected as the optimum operating voltage.

3.2. Method validation

The precision of CE depends on the repeatability of migration times and corrected peak areas. The major factor in obtaining good repeatability is the correct selection of rinse steps [19]. Freshly prepared standard solutions of andrographolide, deoxyandrographolide and neoandrographolide at concentrations of approximately 20.0 ppm (10% v/v ethanol) were injected 10 times within the same day. Separate vials were used for each injection. As detailed in Table 1, the RSDs of migration time were 1.41–2.42% while that of corrected areas were 4.81–5.49% indicating good repeatability.

Linearity of the system was determined by preparing five different standard concentrations in the range of 0.5–20 ppm for andrographolide, deoxyandrographolide and neoandrographolide. Calibration curves of corrected peak area versus concentration were plotted for each of the analytes. The correlation coefficients calculated were between 0.9986 and 0.9989 indicating good linearity (data not shown).

3.3. Sample analysis

The amounts of the three diterpenoids determined using the optimized MEKC method in different parts of the plant are shown in Table 2. The root of *A. paniculata* contains relatively small amounts of the three diterpenoids (0.61, 0.68 and 0.70% for andrographolide, deoxyandrographolide and neoandrographolide, respectively). The quantified levels of diterpenoids in the stem were nearly twice that of the root. The leaf portion of *A. paniculata* contained 1.00% andrographolide, a smaller amount than had been determined by Sharm et al. (1992) [9] but still higher than the other diterpenoids; the leaf contained 0.61% deoxyandrographolide and only 0.26% neoandrographolide.

Most impurities have been successfully removed and sharp, well-resolved peaks obtained for the three diterpenoids when analyzing the whole plant extract of *A. paniculata* (Fig. 3). The ethanol extract of *A.*

Table 1
Repeatability assay by the Relative Standard Deviation (%RSD) of marker migration times and peak areas for ten mixed standard injections

Analyte	Mean migration time (min)	%RSD of migration time	Mean corrected peak area	%RSD of corrected area
Andrographolide	6.44	1.14	2255.80	4.81
Deoxyandrographolide	10.84	2.42	557.00	4.39
Neoandrographolide	11.26	2.41	484.80	5.49

Table 2
Percentage content of andrographolide, deoxyandrographolide and neoandrographolide in *A. paniculata* by capillary electrophoresis

Test sample	Yield of ethanol extract (%w/w)	Andrographolide [Mean[a] ± SD (%w/w)]	Deoxyandrographolide [Mean[a] ± SD (%w/w)]	Neoandrographolide [Mean[a] ± SD (%w/w)]
Whole plant	7.83	3.54±0.25	1.05±0.22	0.83±0.19
Leaf	6.16	1.00±0.08	0.61±0.01	0.26±0.01
Stem	0.88	1.11±0.20	1.27±0.09	1.37±0.00
Root	3.25	0.61±0.04	0.68±0.10	0.70±0.14

[a] Values were obtained in three separate experiments, $n=3$.

paniculata whole plant contained a relatively high percentage of andrographolide (3.54%), with lower levels of the other diterpenoids determined, i.e. deoxyandrographolide at 1.05% and neoandrographolide at 0.83% (Table 2).

4. Conclusion

Andrographis herbal substances have been used in the treatment of a huge range of ailments from common cold to cancer. They are brought into market in various commercial forms. Crude drugs may be entire as leaves and whole plant; or may be cut, broken, or sliced, as in other herbs. They may be more or less matted together by hydraulic pressure or powdered and then molded into different dosage forms. From the above findings, it is concluded that different parts of the plant contained different amounts of the bioactive diterpenoids and whole plant is recommended for extraction or medicinal use as it contains the highest levels of andrographolide. The described MEKC method for qualitative and quantitative determination of diterpenoids in *A. paniculata* is simple, rapid, precise and sensitive. Good separation was obtained for all three diterpenoids in crude alcohol extract of *A. paniculata* and may be of value in standardizing the bioactive components for preparation of formulations containing this herb. Hopefully, with more data accumulated, a form of quality control involving standardization of pharmacologically active constituents in *A. paniculata* using this method could be adopted for routine uses.

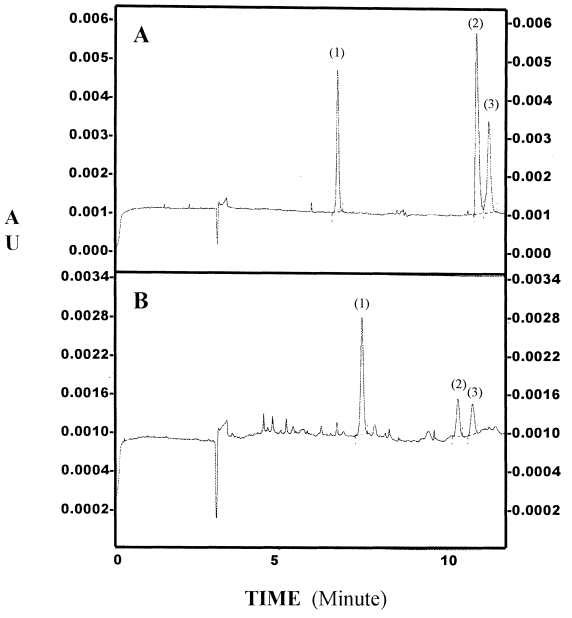

Fig. 3. Electropherograms of mixed standard solution of diterpenoid (panel **A**) and whole plant ethanol extract of *Andrographis* paniclate (panel **B**). Peak (1), (2) and (3) in each panel are, respectively, andrographolide, deoxyandrographolide and neoandrographolide.

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