A Comparative Study of the Separation of Oleanolic Acid and Ursolic Acid in *Prunella vulgaris* by High-Performance Liquid Chromatography and Cyclodextrin-Modified Micellar Electrokinetic Chromatography

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A high-performance liquid chromatographic (HPLC) method and a cyclodextrin-modified micellar electrokinetic chromatographic (CD-MEKC) method were developed to separate and determine oleanolic acid (OA) and ursolic acid (UA) in *Prunella vulgaris*. HPLC separations were carried out on a Hedera ODS C_{18} column with methanol -H_2O- acetic acid (85:15:0.3, v/v/v) as mobile phase at a flow-rate of 0.8 ml min^{-1}. CD-MEKC analysis was performed on a CL1030 capillary electrophoresis system with a 6% (v/v) methanol solution (pH = 9.0) containing 10 mM disodium tetraborate, 10 mM sodium dihydrogen phosphate, 50 mM sodium dodecylsulfate (SDS), 15 mM 2-hydroxypropyl-*β*-cyclodextrin (2-HP-*β*-CD) as background electrolyte. The analytical results of HPLC and CD-MEKC were compared with each other. CD-MEKC has better analytical efficiency for two components, and the analytical time (15 min) was shorter than that of HPLC (35 min).

Keywords: HPLC, CD-MEKC, *Prunella vulgaris*, Oleanolic acid, Ursolic acid
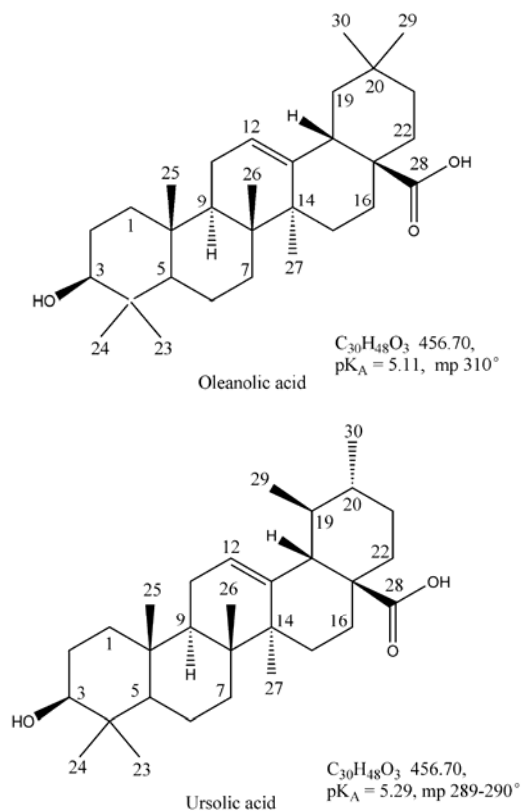
INTRODUCTION

The dry fruit of *Prunella vulgaris* L., which is a traditional Chinese medicine (TCM) used in China for centuries, has been reported to have the effects of anti-HIV [1] and anti-inflammatory activity [2]. Oleanolic acid (OA) and ursolic acid (UA) (Fig. 1), as two main pentacyclic triterpene acids in *P. vulgaris*, possess important pharmacological properties. OA is ascertained to have anti-diabetogenic [3] and hepatoprotective activities [4]. UA is used as a significant antitumorigenesis [5-6] and antioxidant [7] agent.

The main analytical methods of triterpene acids in TCMs are thin layer chromatography (TLC) [8], gas chromatography (GC) [9-11], high-performance liquid chromatography (HPLC) [12,13], capillary zone electrophoresis (CZE) [14] and

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micellar electrokinetic chromatography (MEKC) [15]. TLC is easy to operate, but the precision and accuracy are lower. GC can be employed to determine OA and UA, but it is time-consuming and needs a derivatization reaction. HPLC and MEKC are the most common methods for quantitative analysis of the triterpene acids with high separation efficiency and short analytical time. However, the resolution of the acids is not satisfied due to their similar structures. OA and UA are hydrophobic position isomers. The only difference between them is the configuration of the methyl group on the ring E.

Cyclodextrins (CDs, *α*-, *β*-, *γ*-) are torus-shaped, enzymatically synthesized, non-reducing oligosaccharides consisting of *D*-glucopyranose units bonded through *α*-l,4-1inkages. The cavities of CDs are relatively hydrophobic while the external faces are hydrophilic. Recently, a CD-MEKC mothod greatly expands the applications of CE [16,17]. Although OA and UA are not chiral molecules, the

Fig. 1. Chemical structures of two pentacyclic triterpene acids.

hydrophobic cavities of CDs can form inclusion complexes with the analytes, which improves the separation of components with hydrophobic, isomerous or chiral properties significantly [18].

In this work, for the fist time, a comparative study was carried out and the factors affecting the separation of OA and UA, including the detection wavelength and composition of mobile phase in HPLC and pH value, organic modifier composition, SDS and CDs concentration in CD-MEKC were investigated. In addition, the two methods have been compared with each other and validated for determination of the acids in samples.

EXPERIMENTAL

Materials and Reagents

A total of three sets of plant materials of *P. vulgaris* were purchased from Hubei (sample A), Yunnan (B) and Anhui (C) provinces of China, respectively. OA and UA were provided by the National Institute for the Control of Pharmaceuticals and Biological Products (Beijing, China). 2-HP-*β*-CD was

obtained from Sigma (St. Louis, MO, USA). Methanol and acetic acid were of HPLC grade (Hanbon Technologies, Jiangsu, China). All other reagents and solvents were of analytical reagent grade and used without further purification unless otherwise noted. All aqueous solutions were prepared using newly double-distilled water.

Apparatus and Conditions

HPLC analysis was performed on a Shimadzu LC-2010 apparatus equipped with a Shimadzu SPD-M10A photodiode array detector (Shimadzu Corporation, Kyoto, Japan). Separations were carried out at 30 °C on a Hedera ODS C_{18} column (5 μm, 250 × 4.6 mm, Hanbon Technologies, Jiangsu, China). The mobile phase consisted of a mixture, methanol -H_2O- acetic acid (85:15:0.3, v/v/v). The optimum wavelength for determination was 210 nm, and the flow-rate was 0.8 ml min^{-1}.

CD-MEKC analysis was carried out on a CL1030 capillary electrophoresis system (Beijing Cailu Scientific Inc., Beijing, China) equipped with a UV-Vis detector that can perform wavelength scanning from 190 to 740 nm. An uncoated fused-silica capillary (75 μm × 58 cm) with an effective length of 50 cm was used to separate the analytes, which were injected into the capillary by hydrodynamic flow at a height differential of 10 cm for 5 s. The applied voltage was 20 kV, and the detection wavelength was 205 nm. At the beginning of experiment, the capillary was purged with 0.5 M NaOH for 5 min, followed by 0.1 M NaOH for 5 min, deionized water for 5 min and then running buffer for 5 min. Between runs, the capillary was flushed with 0.1 M NaOH for 2 min followed by deionized water for 2 min and then running buffer for 2 min.

Preparation of Standard Solutions

Stock solutions of OA (0.72 mg ml^{-1}) and UA (1.56 mg ml^{-1}) were prepared in methanol. Standard solutions of OA and UA at various concentrations were prepared by appropriate diluting the stock solutions. Calibration graphs were plotted subsequently for linear regression analysis of the peak area with concentration.

Preparation of Sample Solution

The pulverized samples (approximately 2.0 g for three sets, respectively) were accurately weighed and soaked with 50 ml petroleum ether at 60 °C for 1 h. After filtration, the residue

was extracted with 50 ml 95% ethanol for 20 min in an ultrasonic bath and it was repeated twice. The extracts were combined, filtered and concentrated. Finally, the residue was dissolved with running buffer and diluted to 10 ml. The solution was then filtered through a 0.45 μm syringe filter (Type Millex-HA, Millipore, USA) before injection.

RESULTS AND DISCUSSION

HPLC Analysis

Detection wavelength. The influence of detection wavelengths was investigated from 200 to 225 nm. At wavelengths below 210 nm, the baseline became unstable because methanol has strong absorption which could cause interference to detection. Meanwhile, at wavelengths above 215 nm, the adsorption of analytes diminished significantly. Thus, the detection wavelength was chosen at 210 nm, as OA and UA have better absorption and sensitivity at this wavelength.

Mobile phase. The volume ratio of methanol in mobile phase significantly affected the retention time and resolution of OA and UA. When the volume ratio exceeded 90% (v/v), OA and UA would be eluted quickly without being separated from each other. However, the retention time of triterpene acids was more than 50 min when the content decreased to 80%. In addition, the pH value of mobile phase also had obvious effect on the separation. Acetic acid was added to the mobile phase in order to improve the separation of OA and UA by restraining the ionization of 17-COOH and enhancing the effect of different configuration of C_{29}/C_{30}. The mobile phase, namely methanol-water-acetic acid (85:15:0.3, v/v/v), was selected in these experiments, so that the resolution of two triterpene acids became 1.38. Baseline separation could not be obtained even when far-UV grade acetonitrile was used.

Linearity, limits of detection and reproducibility. The peak area (y/μV s) and the concentration (x/μg ml^{-1}) were fit to the linear functions with the following regression equations: OA, $y = 520019 + 3759.50x$ ($R^2 = 0.99990$); UA, $y = 482474 + 3518.58x$ ($R^2 = 0.99993$). Good linear relationships between the peak area and concentration were obtained in range of 18-360 μg ml^{-1} and 39-780 μg ml^{-1} for OA and UA, respectively. The limit of detection (LOD) under the proposed HPLC conditions was determined at a signal-to-noise ratio (S/N) of 3, which found to be 3.5 μg ml^{-1} for OA and 3.9 μg ml^{-1} for UA, respectively.

Five injections of standard mixture (180 μg ml^{-1} for OA and 390 μg ml^{-1} for UA, respectively) were performed to test the analytical reproducibility. The resulting relative standard deviation values (R.S.D.) for peak areas and retention times were 1.39%, 0.56% and 1.28%, 0.49% for OA and UA, respectively.

CD-MEKC Analysis

Mobility calculation. The velocity of analytes can not be expressed by the migration time or apparent electrophoretic mobility in the separation systems. The effective electrophoretic mobility, avoiding the influence of electroosmotic flow, has been taken as the revised velocity of analytes [19].

In this experiment, the effective electrophoretic mobility (μ_{eff}) of the analytes was used to express the separation results of triterpene acids according to the equation described by Fu [20] as follows:

$$\mu_{ef} = \frac{L_{ef} \cdot L_t}{V \cdot t} \cdot \frac{t - t_0}{t_{mc} - t_0}$$

where t, t_0 and t_{mc} are the migration time of analyte, electroendosmotic flow marker (methanol in this experiment), and micellar (sudan Ⅲ used for marker), respectively, L_{ef} is the effective length of capillary between injection and detection, L_t is the total length of capillary and V is the applied voltage.

With high reliability of qualitative analysis, μ_{eff} was employed to identify the peaks of the triterpene acids in TCMs.

Effect of borate-phosphate concentration on CE separation. The influence of several different concentrations of disodium tetraborate and sodium dihydrogen phosphate on the separation of standard mixture solution was investigated. It was found that the baseline separation can not be achieved by only changing the borate or phosphate concentrations from 5 to 40 mM. Moreover, when buffer concentration exceeded 20 mM, the migration time of triterpene acids increased significantly, because the values of zata potential and electroosmotic flow decreased as ionic strength increased. Finally, both sodium dihydrogen phosphate and disodium tetraborate concentrations were 10 mM used in the buffer.

Effect of SDS concentration on CE separation. MEKC, as a newly fast-developing CE mode, is applicable to the separation of non-charged compounds. SDS, a typical anionic

surfactant, was taken as micellar in MEKC. In this study, electrolyte systems containing SDS ranging from 10 to 90 mM were investigated. It was apparent that the effective electrophoretic mobilities and resolution of triterpene acids increased as SDS concentration changed from 10 to 90 mM. However, the electric current and joule heat increased rapidly when the concentration of SDS was beyond 60 mM. Taking migration time and resolution into comprehensive consideration, a buffer solution with 50 mM SDS was selected.

Effect of organic modifiers on CE separation. Different concentrations of methanol, ethanol and acetonitrile were added to the running buffer to investigate the effect of organic modifiers on the separation of OA and UA. The two acids can not be completely separated when ethanol and acetonitrile were used. As the concentration of methanol increased from 0 to 6% (v/v) in the buffer, the separation was improved increasingly and the baseline became more stable. The probable reason was that methanol can alter selectivity, reduce viscosity of background electrolyte and improve the solubility of the hydrophobic triterpene acids. When the concentration of methanol exceeded 6%, the resolution showed a little change.

Effect of pH value on CE separation. pH value may be a key factor affecting the peak shape and efficiency in CE system. In this experiment, the effect of pH value on the separation of triterpene acids (pK_A of the acids is given in Fig. 1) was studied from 6.0 to 10.0. When a running buffer of pH 9.0 was used, two acids existed in ionic and two sharp and symmetric peaks were achieved. As pH increased, the effective electrophoretic mobility of triterpene acids decreased and resulted in increased migration time. However, the resolution of triterpene acids was slightly improved in the experiments.

Effect of HP-β-CD concentration on CE separation. It is known that the triterpene acids can form inclusion complexes with CDs [18] and the stability of complexes has obvious effects on the resolution and the elution order of triterpene acids. In this work, we tested electrolyte systems containing HP-β-CD ranging from 5 to 25 mM. It was found that the resolution of two triterpene acids changed dramatically with increasing HP-β-CD concentration, and it was 2.58 when 15 mM HP-β-CD was used in the buffer.

Linearity, limits of detection and precision. The triterpene acids were separated in the buffer of 6% (v/v) methanol pH 9.0 containing 10 mM disodium tetraborate, 10 mM sodium dihydrogen phosphate, 50 mM SDS and 15 mM HP-β-CD within 15 min. The good linear relationships between the peak area (μV s) and concentration (μg ml^{-1}) were obtained in the ranges of 18-180 μg ml^{-1} and 39-390 μg ml^{-1} for OA and UA, respectively. The detection limits of OA and UA were 2.6 μg ml^{-1} and 2.8 μg ml^{-1}, respectively. The peak area (y) and the concentration (x) were fit to the linear functions as: $y_{OA} = 1296 + 87.19x_{OA}$ ($R^2 = 0.99890$); $y_{UA} = 2870 + 83.98x_{UA}$ ($R^2 = 0.99880$).

The precision study was comprised of repeatability and reproducibility studies. These were developed in five different samples. The repeatability was established by analyzing each sample five times on the same day. The reproducibility was determined by analyzing each sample (one time/day) on a 6-day period. The R.S.D. of repeatability and the reproducibility both by HPLC and CD-MEKC were less than 2.70% and 2.90%, respectively.

Sample Analysis

The analytical results of OA and UA in *P. vulgaris* obtained from three provinces are summarized in Table 1.

Table 1. Contents of OA and UA in *P. vulgaris* from Three Provinces of China

	HPLC				CD-MEKC			
	OA		UA		OA		UA	
	Content (mg g^{-1})	R.S.D (n = 5)(%)	Content (mg g^{-1})	R.S.D (n = 5)(%)	Content (mg g^{-1})	R.S.D (n = 5)(%)	Content (mg g^{-1})	R.S.D (n = 5)(%)
A	1.02	1.79	2.32	1.83	1.11	2.31	2.44	2.52
B	0.95	1.84	2.09	1.95	1.02	2.55	2.25	2.67
C	0.96	1.65	2.23	1.66	1.03	2.78	2.34	2.49

A stands for Hubei province; B stands for Yunnan province; C stands for Anhui province.

From the results, it can be seen that the contents of OA and UA in *P. vulgaris.* from Hubei province were higher than the others. The chromatograms and electropherograms of standard mixure and sample B are given in Figs. 2-3.

The recoveries of the triterpene acids were determined by the method of standards addition. Suitable amounts of the two acids were spiked into sample A.

The mixture was extracted and analyzed by using the proposed procedures. Table 2 shows the recoveries of OA and UA applying HPLC and CD-MEKC methods. The recoveries
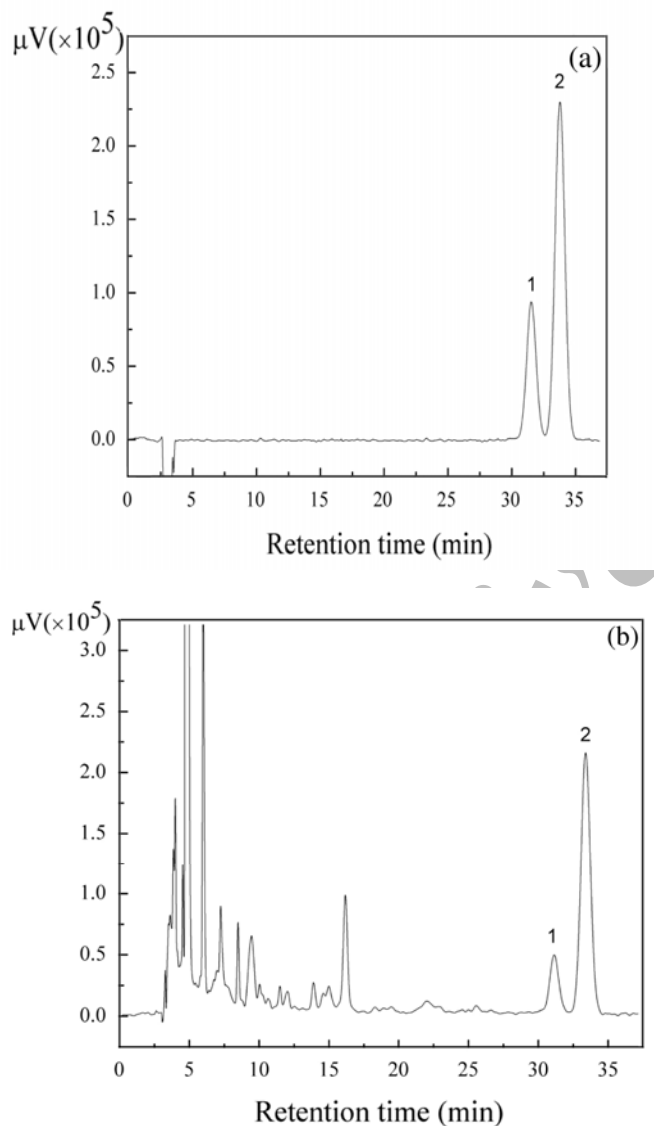
Fig. 2. HPLC chromatograms of standard mixture (a) (0.19 mg ml^{-1} for OA and 0.38 mg ml^{-1} for UA, respectively) and sample B (b) (1-OA, 2-UA). Column: Hedera ODS C$_{18}$: 250 mm × 4.6 mm i.d. (5 μm); flow-rate: 0.8 ml min^{-1}; temperature: 30 °C; detection, 210 nm; mobile phase: methanol-H$_2$O -acetic acid (85:15:0.3, v/v/v).
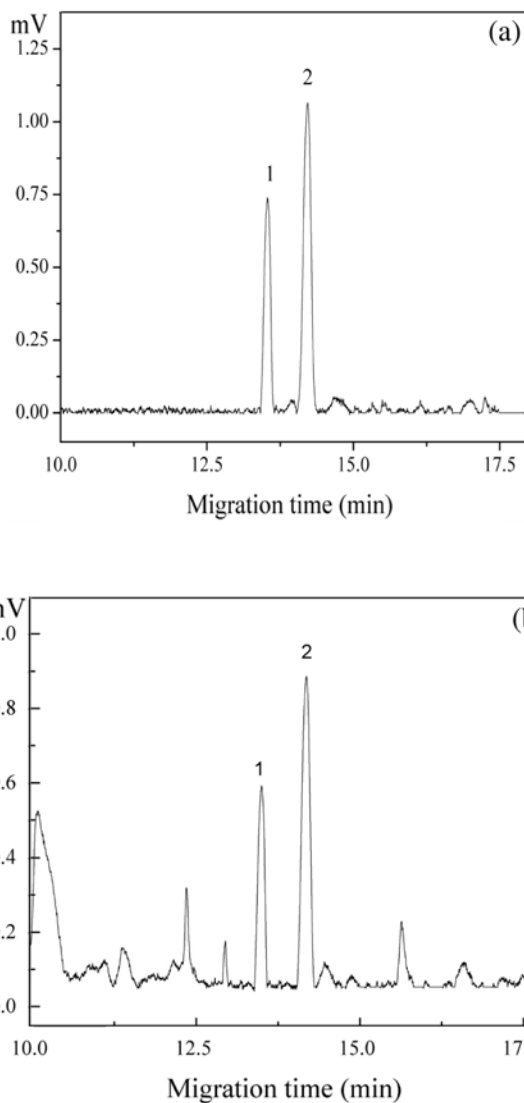
Fig. 3. CD-MEKC electropherograms of standard mixure (a) and sample B (b) (1-OA, 2-UA). Buffer: 6% (v/v) methanol (pH = 9.0) containing 10 mM disodium tetraborate, 10 mM sodium dihydrogen phosphate, 50 mM SDS and 15 mM HP-*β*-CD. capillary, 58 cm (50 cm to detector) × 75 μm i.d.; applied voltage, 20 kV; detection, 210 nm.

Table 2. Recoveries of OA and UA in *P. vulgaris* Applying HPLC and CD-MEKC Methods

Method	Components	Cotent (mg g^{-1})	Added (mg g^{-1})	Found (mg g^{-1})	Recovery (%)
HPLC	OA	1.02	0.32	1.33	96.9
			0.52	1.55	101.9
			0.96	1.97	99.0
	UA	2.32	0.77	3.13	105.2
			1.12	3.48	103.6
			1.53	3.84	99.3
CD-MEKC	OA	1.11	0.32	1.41	93.8
			0.52	1.62	98.1
			0.96	2.09	102.1
	UA	2.44	0.77	3.25	105.2
			1.12	3.57	100.9
			1.53	3.92	96.7

by HPLC were 96.9-101.9% for OA and 99.3-105.2% for UA, respectively. The recoveries by CD-MEKC were 93.8-102.1% and 96.7-105.2% for two acids, respectively.

Comparison of the HPLC and CD-MEKC Methods

In this section the proposed HPLC and CD-MEKC methods, which can give similar results for OA and UA in *P. vulgaris*, are compared with each other. OA and UA could not be well separated from each other by common HPLC on reversed phase and the method appeared to suffer from complexity, whereas the CD-MEKC method described here is robust, cost-effective and simple, while retaining sufficient analytical efficiency. The number of theoretical plates (10^4) obtained with CD-MEKC (9.19 and 5.81 for OA and UA, respectively) were far higher than that with HPLC (1.35 and 1.22 for OA and UA, respectively). In addition, it took more than 35 min for a HPLC analysis, but only 15 min for CD-MEKC analysis.

The separation of two triterpene acids by MEKC was improved by addition of HP-*β*-CD to the borate-phosphate background electrolyte. The concentrations of HP-*β*-CD and SDS affected the sepration a lot. From Table 1, it can be seen that the contents of triterpene acids determined by CD-MEKC were higher than those determined by HPLC. The reason may be that some other components in samples could also form inclusion complexes with CDs applying a CD-MEKC method.

It was possible that the chromatographic properties of the components were dramatically affected in CD-MEKC method, which resulted in the larger measured values of peak areas of triterpene acids. Furthermore, the limits of detection was lower than that of HPLC.

CONCLUSIONS

In this paper, a baseline separation of OA and UA was achieved by using MEKC with HP-β-CD as chiral selector, and both methods were validated for determination of triterpene acids in several samples. Compared with HPLC, CD-MEKC has the advantages of rapidity and high efficiency. It can be used for quantitative study and for quality control of TCMs.

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