Available online at www.sciencedirect.com
ScienceDirect
Journal of Pharmaceutical and Biomedical Analysis 43 (2007) 1331–1334
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Determination of free isomeric oleanolic acid and ursolic acid in *Pterocephalus hookeri* by capillary zone electrophoresis

Ping Yang [a,b], Yuqin Li [c], Xia Liu [a,*], Shengxiang Jiang [a]

[a] *Key laboratory for Natural Medicine of Gansu Province, Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences, Lanzhou 730000, China*
[b] *Graduate School of the Chinese Academy of Sciences, Chinese Academy of Sciences, Beijing 100039, China*
[c] *Pharmacy College, Taishan Medicine College, Taishan 271016, China*

Received 23 April 2006; received in revised form 25 October 2006; accepted 28 October 2006
Available online 1 December 2006

Abstract

A rapid capillary zone electophoresis (CZE) method for the quantification of two bioactive terpenes in *Pterocephalus hookeri* was developed. With β-cyclodextrin as an additive, excellent resolution for these hydrophobic isomers can be obtained in less than 11 min. Linear calibration range for oleanolic acid was between 15.6 and 1000 μg/ml ($r = 0.9998$), for ursolic acid between 31.2 and 1000 μg/ml ($r = 0.9992$). The limits of detection for oleanolic acid and ursolic acid were 3.4 and 3.8 μg/ml, respectively. The contents of the free oleanolic acid and ursolic acid in *P. hookeri* were determined with recoveries ranging from 95.2 to 106.0%. The contents of oleanolic and ursolic acids were found to be 0.21 mg/g (RSD 5.49%) and 0.53 mg/g (RSD 3.37%), respectively. After hydrolysis by acid, these values were 1.12 mg/g (RSD 3.29%) and 0.43 mg/g (RSD 3.42%).

Keywords: Capillary zone electophoresis; *Pterocephalus hookeri*; Oleanolic acid; Ursolic acid; β-Cyclodextrin

1. Introduction

Pterocephalus hookeri is a Chinese herbal medicine for the treatment of flu, inflammation, rheumatism [1]. Tian et al. investigated the active components of this plant, and found that it contains ursolic acid (UA), oleanolic acid (OA) and four saponins (hookerosides) [2]. OA and UA are pentacyclic triterpene type compounds, which are found in many plants in the free acid state (the aglycone) or as the aglycone part in triterpenoid saponins. TLC [3], LC–MS [4], HPLC [5] and GC [6] have been used for the separation and determination of OA and UA. HPLC needs a large amount of solvent and long analysis time, while in GC the derivatization procedure is time-consuming.

The OA and UA content in *Ligustrum lucidum Ait* [7] and in *cornel* [8] have been determined by micellar electrokinetic chromatography (MEKC). No baseline separation was obtained for *Ligustrum lucidum Ait*, and the linear calibration range was too narrow for the *cornel* species.

Capillary electrophoresis (CE) has the advantages of excellent separation efficiency, rapid analysis and minimal consumption of both samples and solvents. Additives, e.g. cyclodextrins (CDs) are often added to the background buffer to improve resolution in capillary zone electrophoresis (CZE) by interactions between solute and CDs, solute and capillary wall, CDs and capillary wall, especially in chiral separations [9]. The increased separation efficiency can be attributed to the decreased diffusion due to formation of aggregates and reduced adsorption to the capillary wall [10]. The structures of OA and UA are so similar that no baseline separation has been obtained by ordinary CZE and MEKC. For this reason, CDs were added in CZE background buffer in this study to achieve baseline separation. β-CD known to interact with this class of compounds, was selected as the modifier to develop a simple, efficient, rapid method for the determination of the structural isomers OA and UA in *P. hookeri*.

2. Experimental

2.1. Instruments

Agilent 3D CE system equipped with a DAD detector was used. The data acquisition was carried out with the A.09.03 revisions of the Agilent chemstation software. Uncoated fused-silica capillaries of 75 μm i.d. (Yongnian Photoconductive Fibre

* Corresponding author. Tel.: +86 931 4968203; fax: +86 931 8277088.
E-mail address: gsliuxia@lzb.ac.cn (X. Liu).

0731-7085/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2006.10.042

Factory, Hebei Province, China) with total length of 62.5 cm and effective length of 54 cm were used. A Sartorius PB-10 pH meter (Germany) was used for pH measurements. Injection of samples was carried out by applying pressure of 5000 Pa for 3 s. All operations were performed at 25.0 ± 0.5 °C.

2.2. Materials and reagents

OA was obtained from Drug and Biological Products Examination Center, China. UA was obtained from SIGMA, USA. *P. hookeri* was purchased from a local Chinese herbal store in Lanzhou, China. It was identified by Prof. Ma Zhigang (College of Pharmacy, Lanzhou University), and deposited in Key Laboratory for Natural Medicine of Gansu Province, Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences. All other chemicals were of analytical grade. Deionized water was used throughout.

Stock solutions 100 mM borax, 20 mM β-CD were prepared in water. The buffer (pH 9.53) used in the sample preparation contained 50 mM/l borax and 8 mM/l β-CD.

Stock standard solutions of OA (2 mg/ml) and UA (2 mg/ml) were prepared in ethanol, solutions of lower concentrations were prepared by dilution of the stock solutions with appropriate amount of buffer, standard solution–buffer (50:50, v/v).

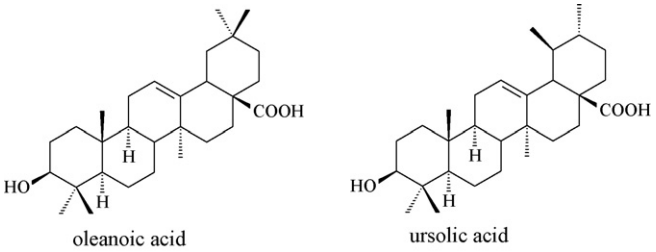
2.3. Sample preparation

Sample A: The dry *P. hookeri* was ground into fine powder. 5.0 g of the powder was extracted with anhydrous ether in a Soxhlet apparatus for 1.5 h. The solution was filtered and evaporated. The residue was dissolved in 25 ml of ethanol–buffer (80:20, v/v).

Sample B: 2 g of the above powder was refluxed for 1 h in 40 ml ethanol and 2 ml HCl. The extracted solution was filtered. The sediment and the colander were washed with ethanol. All solutions were combined, and then ethanol was removed by rotary evaporation. The residue was suspended in 20 ml of water, and extracted three times with 20 ml ether in a separating funnel. The extracts were combined and the ether was eliminated, then the residues were dissolved in 10 ml of ethanol–buffer (80:20, v/v).

3. Results and discussion

The structures of the two isomeric compounds are presented in Scheme 1. In order to obtain successful separation, the effect of concentration, pH of the running buffer, β-CD concentration, organic modifier and the applied voltage were investigated.



Scheme 1. Structures of oleanoic acid (OA) and ursolic acid (UA).

3.1. Method optimization

Generally, triangular peaks in CE can be overcome by choosing a buffer system with a better match of the mobility in the system, and in order to decrease the effect on the peak shape, the ionic strength of this buffer system should be higher than the strength of the samples [11]. In our experiments, because the analytes bear negative charge in this buffer solution, they have the opposite migration direction regarding electroosmotic flow (EOF). When the borax concentration was less than 50 mM, the chromatographic peaks were asymmetrical. When the borax concentration was above 50 mM, the peaks were symmetrical but the current was over 100 μA, creating a large heat effect. The optimum borax concentration was 50 mM.

The addition of β-CD improves the resolution of the analytes due to different complex constants of OA and UA with β-CD. However, higher β-CD concentration increases the viscosity of the solutions and this would increase the separation time and may also decrease the resolution. This implies that between these two parameter extremes there will be an optimum condition to allow maximum separation of the two isomers. The optimum β-CD concentration was 8 mM.

This is based on the fact that the effective mobility of the analytes is a function of β-CD concentration. Since the organic modifier competes with the solutes for the β-CD cavity, the effective β-CD concentration in the buffer can be calculated using Eq. (1) as suggested by Zukowski et al. [12].

$$[\beta\text{-CD}]_e = \frac{[\beta\text{-CD}]}{1 + K_m[E]} \quad (1)$$

E denotes the organic modifier. K_m describes the affinity of the organic modifier to the β-CD cavity. The K_m for β-CD in ethanol was 0.93 at 25 °C [13]. For determining the mobility of OA (μ_A), UA (μ_B) at a number of different effective β-CD concentrations, a series of data is shown in Table 1.

In an attempt to simulate the experimental results, Eq. (2) is proposed to explain the influence of β-CD concentration (x) on the effective mobility of the analytes (y).

$$y = \frac{A_1 - A_2}{1 + e^{(x-x_0)/dx}} + A_2 \quad (2)$$

where A_1 and A_2 are the effective mobilities of the analytes at the lowest and highest β-CD concentration investigated; x_0 and dx are constants. Fig. 1 shows the experimental and the simulation results. The simulating parameters are listed in Table 2. A high correlation relationship (correlation coefficients >0.99) between the experiment and simulating results was obtained. As Eq. (2) shows, two extremes (maximum or minimum) of the effective mobility for the analytes were reached at the lowest and highest β-CD concentrations. The effective mobility of each analyte changed in the range of the maximum and minimum effective mobility values with the change of $e^{(x-x_0)/dx}$. This can be explained by the change of the solubility of the analytes in the β-CD-buffer mobile phase, which resulted in the change of

Table 1
The effective β-CD concentration and the corrected mobility of A and B

[β-CD] (mM/l)[a]	2	4	6	8	10
$[\beta\text{-CD}]_e$ (mM/l)[a]	0.77	1.54	2.31	3.08	3.86
μ_A (10^{-9} m^2 s^{-1} v^{-1})[b]	−7.6795	−7.6047	−7.2952	−7.1327	−7.0409
μ_B (10^{-9} m^2 s^{-1} v^{-1})[b]	−9.4904	−8.7663	−8.1900	−7.7391	−7.7137

[a] [β-CD] and $[\beta\text{-CD}]_e$ stand for the β-CD concentration and the effective β-CD concentration, respectively.
[b] μ_A and μ_B stand for the corrected mobility of OA and UA at different β-CD concentration.

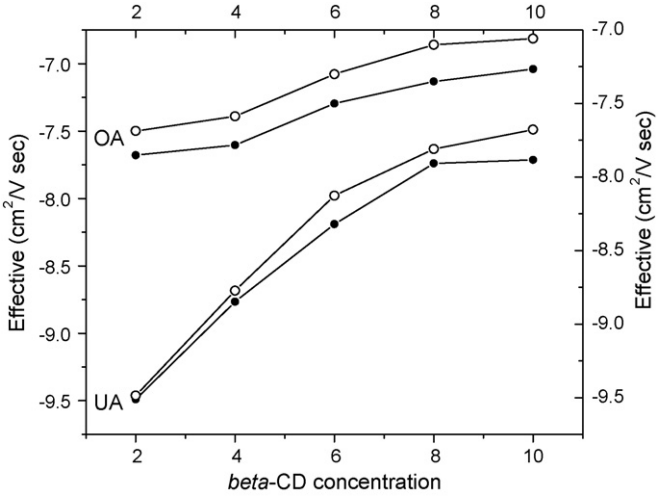


Fig. 1. Effect of β-CD concentration on the mobility of OA and UA. Buffer: 50 mM Borax, 2–10 mM β-CD, 10% ethanol; capillary, 62.5 cm (54 cm to detector) × 75 μm i.d.; applied voltage, 20 kV; cartridge temperature, 25 °C; detection, 214 nm; standard mixture solution, 62.5 μg/ml of OA, 62.5 μg/ml of UA. (◯) Simulating results; (●) experimental results.

the partition behavior of the analytes between the mobile phase and β-CD pseudostationary phase. This is also the explanation for the change of migration time of the analytes with the change of β-CD concentration.

The pH of the electrophoretic medium is also a governing factor in separation. But in this experiment, the change in pH in the range 8.0–10.0 only slightly affected the resolution and the number of theoretical plates (*N*). This phenomenon can be attributed to the similar electric charge of their aggregates in the studied pH range. The selected pH was 9.53.

Organic solvents can alter selectivity and improve resolution. But the addition of organic modifier reduces the electroosmotic velocity and results in an increase of the migration time. Therefore, the buffer system containing different concentrations of ethanol was investigated. The resolution increased with increasing ethanol concentrations, but above 10%, the peak of UA was overlapped with the unknown peak (UK) in the electropherogram of Sample A (see Fig. 2). Considering the total analysis time and resolution of all peaks, 10% ethanol was chosen as the optimum.

The separation voltage directly determines the migration time and influences the resolution. Attempts were made to optimize the separation conditions by using different applied voltages ranging from 12 to 22 kV. At the higher voltage, the resolution was decreased to the point that the OA and UA peaks could not be resolved. Based on these results, 20 kV was selected as the optimum voltage to accomplish a good compromise.

According to the factors mentioned above, the best resolution was obtained with an electrolyte of 50 mM borax, 10% ethanol

Table 2
Simulation parameters of Eq. (2)

A_1[a]	A_2[a]	x_0 (%)	dx (%)	R^2
OA				
−7.7066	−7.0486	5.5750	1.0285	0.9953
UA				
−10.170	−7.6168	3.7098	1.7008	0.9963

[a] 10^{-9} m^2 s^{-1} v^{-1}.

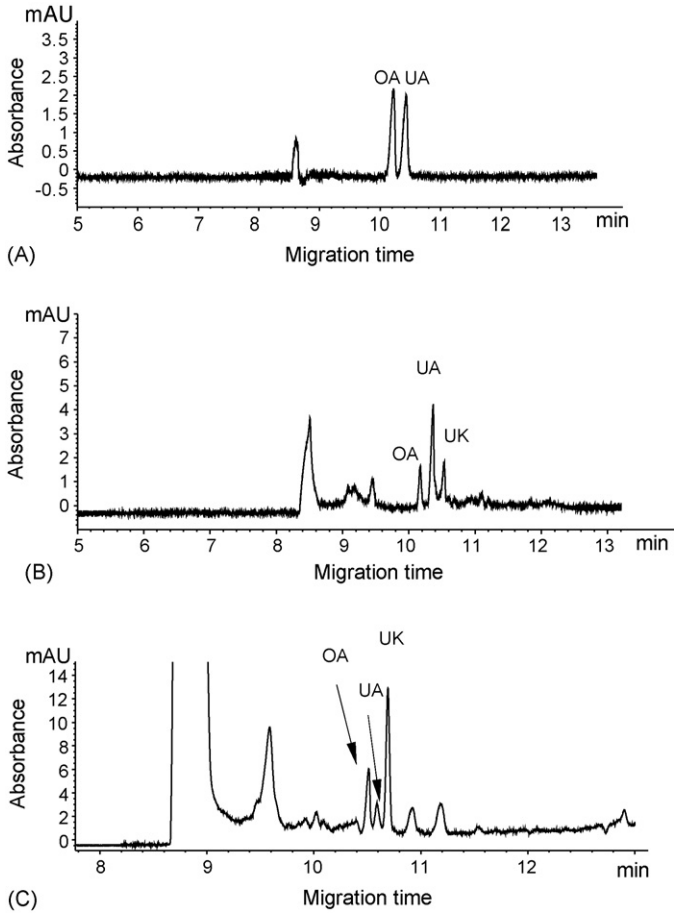


Fig. 2. Electropherograms of the standards mixture solution and the real samples (peak UK: unknown). (A) Standard mixture. (B) The extract of *Pterocephalus hookeri*. (C) The extract of *Pterocephalus hookeri* after hydrolysis. Buffer: 8 mM β-CD; 10% ethanol (pH 9.53). Other conditions are the same as in Fig. 1.

Table 3
Contents of the free analytes in the *Pterocephalus hookeri* (Sample A) and the aglycone after hydrolysis (Sample B)

Sample	OA (mg/g)	RSD (%)	UA (mg/g)	RSD (%)
A	0.21	5.49	0.53	3.37
B	1.12	3.29	0.43	3.42

($n = 3$, mg/g).

(v/v), 8 mM β-CD (pH 9.53) and 20 kV applied voltage. A typical electropherogram obtained from a standard mixture under the optimum conditions is shown in Fig. 2A. The two analytes were well separated within 11 min; the method is faster than the HPLC method [5].

3.2. Linearity, repeatability and detection limit

The linearity between the peak-area (y) and the concentration (x, μg/ml) of standard solutions were investigated, the calibration curves exhibited excellent linear behavior over the concentration range (15.6–1000 μg/ml for OA, 31.2–1000 μg/ml for UA). The linear regression equation was $y = 1.36 + 0.17x$ ($r = 0.9998$) for OA and $y = 3.09 + 0.15x$ ($r = 0.9992$) for UA, respectively. The detection limits were 3.4 μg/ml for OA and 3.8 μg/ml for UA based on signal-to-noise ratio of 3.

The repeatability of the migration time and peak area of OA and UA in the experiment was determined by repeated ($n = 5$) injection of a standard mixture solution of 62.5 μg/ml of OA and 62.5 μg/ml of UA under the optimum conditions. The RSD of intra-day precision was less than 3.72% (OA) and 1.52% (UA); The RSD of inter-day precision was less than 4.58% (OA) and 3.09% (UA).

3.3. Application and recovery

The optimum conditions were applied to the separation and determination of Samples A and B. The electropherograms are shown in Fig. 2(B and C). The peaks were identified by comparison with the UV spectra and the migration times of the two standards, and by spiking the sample solution with standards. The contents of the analytes found in the *P. hookeri* samples together with RSD are given in Table 3. It was found that free OA and UA were all present in the *P. hookeri*. Since different preparation methods were applied for Samples A and B, this leads to different results. The content of UA decreases after hydrolysis (Sample B), although it should be unchanged according to ref. [2]. However, after hydrolysis, the ratio of content of OA and UA was greatly increased OA–UA (72:28) in comparison with the ratio OA–UA (28:72) before hydrolysis, which is in good agreement with the previous literature that one of the four saponins yielded a common aglycone OA by hydrolysis [2].

The recovery of the method was determined with the standard addition method (added: 125, 62.5 and 31.2 μg/ml) for OA and UA in the sample. The results were 95.2–106%.

4. Conclusion

A method based on β-CD CE was developed for direct, robust and rapid qualitative and quantitative analysis of isomeric OA and UA in *P. hookeri*. The CD pseudostationary buffering systems resulted in good separation of OA and UA. The phenomenon that organic solvents may affect the formation of the micelles and cause an increase in the CMC in MEKC was avoided. This improves the peak shapes and the selectivity of the separation.

Acknowledgement

This work was supported by the Scientific Foundation of Chinese Academy of Science (no. KGCX2-SW-213-08).

References

[1] Jiangsu New Medical College, A Dictionary of Chinese Traditional Medicine, vol. 1, Shanghai Science and Technology Press, Shanghai, China, 1986, p. 2688.
[2] J. Tian, F.E. Wu, M.H. Qiu, R.L. Nie, Phytochemistry, 32 (1993) 1535–1538.
[3] K. Krohn, M.S. Rao, N.V. Raman, M. Khalilullah, Phytochem. Anal. 12 (2001) 374–376.
[4] N. Ladislav, A. Mohammed E., H. Hoda, M. Irena, G. Daniel, J. Pharm. Biomed. 31 (2003) 961–968.
[5] J.H. Chen, Z.H. Xia, R.X. Tan, J. Pharm. Biomed. 32 (2003) 1175–1179.
[6] G. Janicsák, K. Veres, M. Kállai, I. Máthé, Chromatographia 58 (2003) 295–299.
[7] H.X. Liu, Y.H. Shi, D.X. Wang, G.L. Yang, A.M. Yu, H.Q. Zhang, J. Pharmaceut. Biomed. 32 (2003) 479–485.
[8] G.Q. Zhang, Y.P. Qi, Z.Y. Lou, C.H. Liu, X.F. Wu, Y.F. Chai, Biomed. Chromatogr. 19 (2005) 529–532.
[9] H.X. Liu, A.M. Yu, F.Q. Liu, Y.H. Shi, L.K. Han, Y.F. Chen, J. Pharm. Biomed. 41 (2006) 1376–1379.
[10] R. Zhang, H.X. Zhang, D. Eaker, S. Hjerten, J. Capillary Electrophor. 4 (1997) 105–112.
[11] T. Schmitt, H. Engelhardt, J. High Resolut. Chromatogr. 16 (1993) 525–529.
[12] J. Zukowski, D. Sybiliska, J. Jurczak, Anal. Chem. 57 (1985) 2215–2219.
[13] Y. Matsui, K. Mochida, Bull. Chem. Soc. Jpn. 52 (1979) 2808–2814.