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Separation and determination of podophyllum lignans by micellar electrokinetic chromatography

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Abstract

A micellar electrokinetic chromatography method was established for the quantitative analysis of seven podophyllum lignans in *Podophyllum emodi* Wall. var. *chinesis sprague*. The optimum buffer system was 10 m*M* NaH_2PO_4–5 m*M* borate–100 m*M* sodium dodecylsulfate–30% isopropanol (pH 7.20). Voltage was 18 kV and detection at 214 nm. The second derivative chromatogram was used to determine a low-content component and those not fully separated from adjacent ones. The RSD values of migration times and peak areas were <2.2 and $<5.5\%$, respectively. The effects of several CE parameters on the resolutions were studied systematically.

Keywords: *Podophyllum* spp.; Plant materials; Lignans

1. Introduction

Podophyllum emodi Wall. var. *chinesis sprague* (*P. emodi*) has been used to treat snake-bites, ulcer, fractures and rheumatism since the time of the Han dynasty in China [1]. In the 19th century, podophyllum lignans were firstly isolated from *P. emodi* and the same family species called *Podophyllum peltatum* (*P. peltatum*) [2]. The breadth of the pronounced cytotoxic and anticancer of these components has come to be appreciated relatively recently [3]. Initial expectation regarding the clinical utility of podophyllum lignans was tempered largely due to their unacceptable gastrointestinal toxicity. This led to the appearance of etoposide (VP-16) and teniposide (VM26), semi-synthetic products from podophyllum lignans [4]. So far, the main source for these drugs has been *P. emodi*, a native of India, but due to over-exploitation, this species has been declared endangered. One of the directions of the continued research on podophyllum lignans was the development of alternative and renewable sources. Besides chemical total synthesis [5,6], the Chinese species *P. emodi* might be considered as another viable source [1].

Analytical methods described in literature are mainly based on TLC and HPLC [7–9]. Recently, owing to its high resolving power, low solvent consumption and simple pretreatment, CE has been used as an attractive method for separating and monitoring Chinese traditional medicines [10–13]. Two reports using micellar electrokinetic chromatography (MEKC) for the separation of podophyllum lignans have been available. In one paper two

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lignans were identified in the extract of *P. emodi* [14]; while in another, although seven from *P. peltatum* were separated, only podophyllotoxin was quantitatively determined [15]. Since each podophyllum lignans has its own characteristic biological activity [16,17], the knowledge of the quantitative estimation of them is important and valuable.

2. Experimental

2.1. Materials

P. emodi was collected in the northwest of China and identified by Xuan Tian of the Organic Chemistry Research Laboratory, Department of Chemistry of Lanzhou University, China, who isolated seven standard substances (Fig. 1). The physical constants and spectrometric data of these components coincided with those in literatures [18,19]. All chemicals were of analytical reagent grade and purchased from Xian Reagent, China. Deionized water was used throughout.

Stock solutions of 200 m*M* sodium dihydrogen phosphate and 200 m*M* sodium dodecylsulfate (SDS), 100 m*M* sodium tetraborate were prepared by dissolving corresponding substances in water. Micellar buffers were prepared by mixing 1.0 ml sodium dihydrogen phosphate stock solution, 0.5 ml sodium tetraborate stock solution, appropriate volume of SDS stock solution, and then diluting to 20 ml. After pH of the running buffer was adjusted with 0.2 *M* HCl or 0.2 *M* NaOH, organic modifier was mixed into it. These electrolytes were filtered through a 0.45-μm membrane prior to use.

The 1.0000 mg/ml standard solutions of the seven components were prepared in methanol, while various other concentrations of them were prepared by diluting the standard stock solutions with methanol.

2.2. Equipment

The separation was performed using a Waters Quanta 4000 capillary electrophoresis apparatus (Waters Chromatography Division, Milford, MA, USA) with a positive power supply. Fused-silica capillaries (manufactured by Waters) of 50 cm (42.5 cm effective length)×50 μm I.D. were used. The temperature of the instrument was maintained at 26±0.5°C with assistance of a laboratory-made electrical heater. The other conditions were voltage 18 kV, injection in the hydrostatic mode at a height of 10 cm for 8 s and detection at 214 nm. The data acquisition was carried out with a Maxima 820

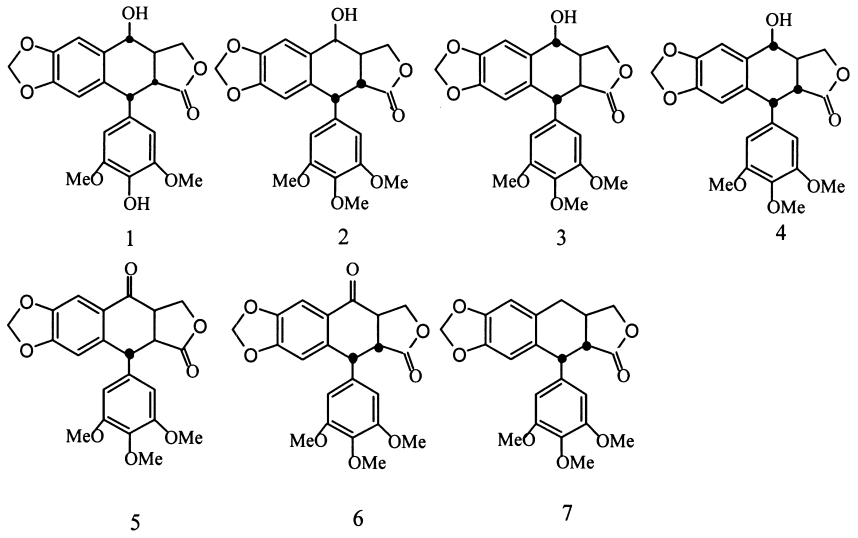

Fig. 1. Structures of seven podophyllum lignans. 1=4′-Demethylpodophyllotoxin, 2=epipodophyllotoxin, 3=picropodophyllin, 4=podophyllotoxin, 5=picropodophyllone, 6=podophyllotoxone, 7=deoxypodophyllotoxin; ● denotes β-H.

Chromatograph workstation. A 5-min wash cycle with 0.1 *M* NaOH followed by 3 min deionized water, and 5 min separation buffer was necessary to condition the capillary.

2.3. Sample preparation

P. emodi root powdered (1.0 g) was extracted with 10 ml methanol in ultrasonic apparatus for 30 min. After centrifuging, the extract was poured out and the residue was extracted two times with the same procedure. Finally, all these extracts were combined together and filtered. The methanol solution was evaporated to 10.00 ml, and then analyzed directly.

3. Results and discussion

The CE chromatogram of a mixture containing seven lignans found in *P. emodi* is shown in Fig. 2 (see Fig. 1 for their corresponding structures). Of these seven components, 1, 2, and 7 were easily separated, while 3, 4, 5, and 6 were with difficulty, because pairs 3 and 4, and 5 and 6 are diastereoisomers. When pH of the running buffer was over 8, component 4 could transform into 3, and 6 into 5 [20]. The experiment showed that when the heat effect inside the capillary was serious, the peaks of the components split into two, implying that the configurations of them changed. This was a very complicated and subtle separation object. In order to optimize the separation conditions, the influences of several CE parameters on the resolution (R_s) were studied carefully.

Table 1
Effect on the separation of pH and voltage

pH	Analysis time (min)	R_s (3–4)	Voltage (kV)	Analysis time (min)	R_s (3–4)
6	45	0.92	14	>60	1.0
6.5	36	0.83	16	43	0.95
7	29	0.76	18	28	0.8
7.5	26	0.74	20	20	0.6
8	24	0.70	22	14	0.2

3.1. Influences of pH and applied voltage

To verify the effect of buffer pH on the separation efficiency, experiments were performed with 10 m*M* NaH_2PO_4–5 m*M* borate–100 m*M* SDS–30% isopropanol as background electrolyte and applied voltage as 18 kV. It was found that when the pH was under 6, all the peaks became low and 3, 4, 5, and 6 merged together. When the pH was adjusted from 6 to 8, R_s value between the most crucial pair 3 and 4 increased by 0.2, while analysis time was prolonged for another 20 min (Table 1). In order to investigate the applied voltage effect on the separation, experiment conditions were 10 m*M* NaH_2PO_4–5 m*M* borate–100 m*M* SDS–30% isopropanol and pH 7.20.

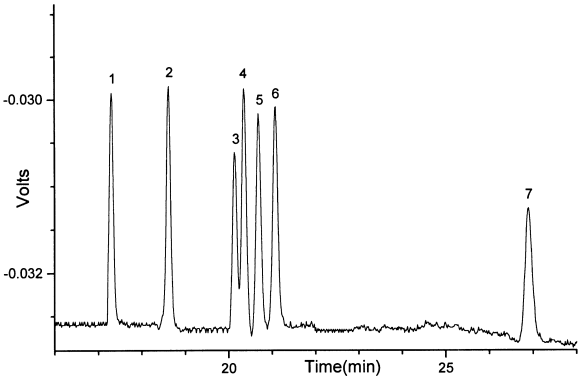

Fig. 2. Chromatogram of a lignan standard mixture. See Fig. 1 for peak identification. Conditions: 50 cm fused-silica capillary (50 μm I.D.); voltage, 18 kV; buffer, 10 m*M* NaH_2PO_4–5 m*M* borate–100 m*M* SDS–30% isopropanol (pH 7.20); detection, 214 nm; current, 44 μA.

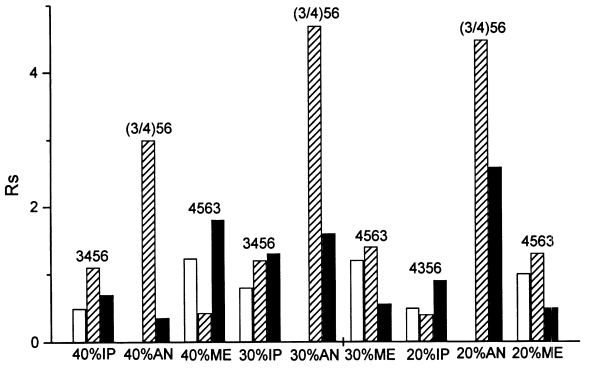

Fig. 3. Effect of organic modifiers on the resolution of the adjacent peaks. The number above column bars shows the migration order corresponding to the organic modifier written on the *x*-axis. IP = isopropanol, AN = acetonitrile, ME = methanol. Other conditions as for Fig. 2.

When applied voltage was varied from 22 to 14 kV, R_s value between 3 and 4 increased by 0.8 while analysis time was prolonged for over 40 min. It can be seen from Table 1 that pH 7.20 and 18 kV were the most effective to use.

3.2. Effect of organic modifiers

From Fig. 3 it was observed that the presence of organic modifiers is crucial for improving separation. Under any of the analysis conditions we tried, component 1 always eluted first, 2 second and 7 last, while the migration order of 3, 4, 5, and 6 changed with the kind and concentration of the modifiers. With 30–40% isopropanol as a modifier, the order was 3, 4, 5, and 6; while with 20% isopropanol, the order was 4, 3, 5, and 6. When acetonitrile was used as a modifier no matter what the SDS concentration, 3 and 4 always merged into one peak — the order being 3/4, 5, and 6. The order changed into 4, 5, 6, and 3 with methanol as a modifier. Ethanol, acetone and *N*,*N*-dimethylformamide were also used to improve the resolution, but no better result were obtained. Fig. 3 reveals an optimum at 30% isopropanol or 20–30% methanol. However, when 20–30% methanol was used, component 1 could not be separated from an unknown in a real sample — 30% isopropanol was therefore adopted.

3.3. Effect of SDS concentration

Fig. 4 shows the effect of SDS concentration on the separation of the four adjacent peaks. It can be seen that the surfactant played an important role in a successful separation. With 30% isopropanol as a modifier, all R_s values increased with increasing SDS concentration. When SDS concentration was below 60 m*M*, 3, 4, 5, and 6 were separated very poorly no matter which organic modifier was used. When it was over 100 m*M*, there was no significant increase in the R_s values. So 100 m*M* SDS was selected.

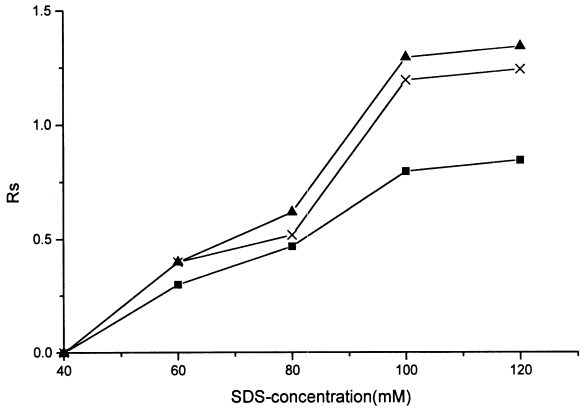

Fig. 4. Effect of SDS concentration on the resolution of the adjacent peaks. ▲: 5–6; ■: 3–4; ×: 4–5. Other conditions as for Fig. 2.

3.4. Linearity and reproducibility

Under the selected conditions, the linear relationships between the concentration of components 1, 2, 6, and 7 and the corresponding peak areas are shown in Table 2. Because 3 and 4 was not baseline separated and the content of 5 in a real sample was very low, the second derivative chromatogram was used to determine these three components. The linear relationships between their concentrations and the corresponding peaks height of the second derivative chromatogram (see Fig. 5) are shown in Table 3. The

Table 2
Calibration data

No.[a]	Linear range (μg/ml)	Slope	Slope error	Intercept	Intercept error	Standard error	Coefficient $n=6$	Detection limit ($S/N=3$) (μg/ml)
1	20–500	124.41	5.71	−30.98	1525.03	2042.57	0.9968	4
2	20–1000	170.80	3.06	−879.11	1404.77	2314.80	0.9995	1.5
6	20–500	114.48	4.30	2265.69	699.61	686.22	0.9986	3
7	20–500	227.84	4.29	425.07	802.61	631.52	0.9998	5

n: The number of data points.
[a] See Fig. 2 for the substances identification.

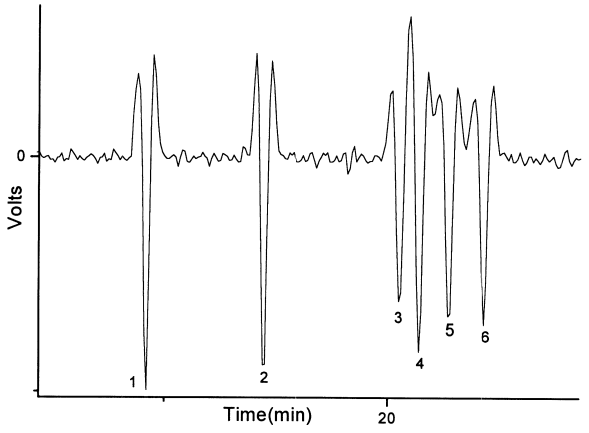

Fig. 5. Part of the second derivative chromatogram of Fig. 2.

method was validated for the reproducibility of the migration times and the peak areas of the substances. For a given sample, RSD values of the migration times and the peak areas for five replicate injections were below 2.2 and 5.5%, respectively. The detection limits of the components were shown in Tables 2 and 3.

4. Applications

Methanol solution of sample extracts were injected and separated under the optimum conditions described above. A chromatogram of a real sample is shown in Fig. 6a. From the figure it was observed that except for components 3 and 4, all the others were successfully separated in 28 min. The components 1, 2, 6, and 7 were determined by the normal

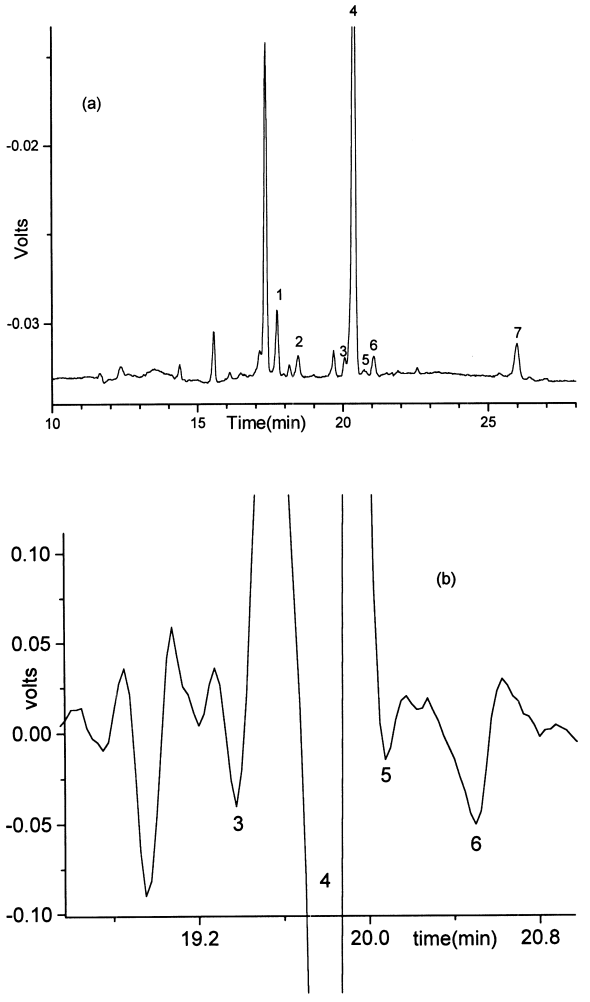

Fig. 6. (a) Chromatogram of methanol root extracts of *P.emodi*. (b) Part of the second derivative chromatogram of (a). For peak identification see Fig. 1. Other conditions as for Fig. 2.

Table 3
Calibration data

No.[a]	Linear range (μg/ml)	Slope	Slope error	Intercept	Intercept error	Standard error	Coefficient ($n=6$)	Detection limit ($S/N=3$) (μg/ml)
3	5–500	0.0021	$1.41\cdot10^{-4}$	−0.01424	0.03191	0.02827	0.9980	5
4	5–1000	0.0031	$1.11\cdot10^{-4}$	0.03704	0.03374	0.03781	0.9978	1.2
5	5–500	0.0019	$1.29\cdot10^{-4}$	0.06258	0.02547	0.03222	0.9955	4

n: The number of data points.
[a] See Fig. 2 for the substances identification.

Table 4
Contents of the seven lignans in sample extracts

Components	No.	Content (%)	RSD (%) ($n=4$)	Added (μg/ml)	Recovery (%)
4′-Demethylpodophyllotoxin	1	0.094	3.3	150	108
				100	95
				50	92
Epipodophyllotoxin	2	0.025	4.1	50	107
				25	92
				10	91
Picropodophyllin	3	0.072	3.9	100	104
				70	106
				30	93
Podophyllotoxin	4	0.581	2.1	450	98
				300	96
				150	103
Picropodophyllone	5	0.009	7.7	30	106
				15	108
Podophyllotoxone	6	0.040	4.5	60	107
				40	92
				20	92
Deoxypodophyllotoxin	7	0.100	3.1	150	108
				100	93
				50	95

chromatogram, while 3, 4, and 5 by the second derivative one (see Fig. 6b). Peaks were identified by spiking the sample with the standards stock solutions. The analytical results are summarized in Table 4. The recovery of the method was determined with the addition of the standard substances in a real sample. Table 4 shows that the values were satisfactory.

5. Conclusion

The developed MEKC method was successfully used to separate and determine seven podophyllum lignans in *Podophyllum emodi* Wall. var. *chinesis sprague*. Meanwhile, the derivative chromatogram performed very well in estimating the low-content component and those not baseline resolved. From the satisfactory results, it can be concluded that the MEKC method combined with the derivative chromatogram has great potential in the separation and estimation of complicated samples of natural products. The results of the quantitative analysis also show that the Chinese species *P. emodi* is a high yielding source of the drugs, etoposide and teniposide.

Acknowledgements

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