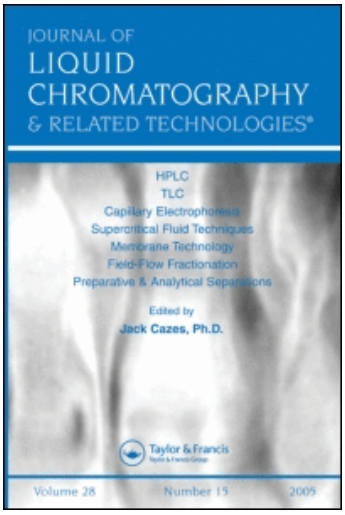

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STUDIES OF CRUDE ETHANOL EXTRACT OF ROOTS OF SALVIA PRZEWALSKII MAXIM BY MICELLAR ELECTROKINETIC CAPILLARY CHROMATOGRAPHY

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ABSTRACT

A crude ethanol extract of roots of *Salvia przewalskii* Maxim was studied by micellar electrokinetic chromatography. Optimum conditions for the separation were established. Three peaks belonging to cryptotanshinone, dihydrotanshinone I and tanshinone IIA were identified by known standards. The three substances are valuable pharmaceutical ingredients of the plant.

INTRODUCTION

The air dried roots of *Salvia przewalskii* Maxim is a Chinese traditional herb. Many chemists have studied the physiologically active constituents of this herb and have isolated more than 30 substances and have determined their structures.[1] The tanshinone species are major components in *Salvia przewalskii*[2] and have acquired considerable importance in the treatment of heart disease and inflammation.[1,3-5]

Tanshinone IIA plays a major part in the treatment of heart disease.[6] Cryptotanshinone and dihydro-tanshinone I exhibited stronger inhibition agains *staphylococcus aureus* and its drug resistant species.[7] Tanshinone IIA and cryptotanshinone were found to have an inhibitive effect against $H_{37}RV$.[7]

Thin layer chromatography (TLC)[3-6] and high performance liquid chromatography (HPLC)[5] have been used for the separation and determination of tanshinone species. TLC and HPLC are not only limited in separation power, but also time consuming, since a number of prior steps are often required. For HPLC, the chromatographic column is easily contaminated and hard to clean.

Micellar electrokinetic capillary chromatography (MECC) was developed by Terabe et al.[8] in 1984 and Cohen et al.[9] in 1987. It is the most common mode of operation of capillary electrophoresis (CE). In MECC, resolution occurs because of a combination of kinetic and thermodynamic phenomena. So it is a highly efficient and fast technique, the application of which in the biological and pharmaceutical fields has developed rapidly in recent years.[10-12] For MECC, very complicated samples can be analysed directly without much pretreatment, because any contaminants in the CE tube can be rinsed away with a suitable solvent after each analysis.

The application of MECC to the analysis of roots of *Salvia przewalskii* Maxim has not hitherto been reported. In this work, roots of *Salvia przewalskii* Maxim were analysed directly after ethanolic extraction without further purification, and three peaks belonging to cryptotanshinone, dihydrotanshinone I and tanshinone IIA were identified by known standards. The effects of the addition of N,N-dimethyl-formamide (DMF) and tetra ethylenemglycol (TEG) in the electrolyte on separation selectivity are also discussed.

EXPERIMENTAL

Instrumentation

The CE system employed was a Quanta 4000 (Waters Chromatography Division of Millipore, Milford, MA, USA) with a positive power supply. Waters AccuSep fused silica capillaries (60 cm x 75 μm I.D.) were used throughout. Direct UV detection was achieved with the use of a Hg lamp and a 254 nm optical filter. A window for on column detection was created 7.6 cm from the end of the capillary.

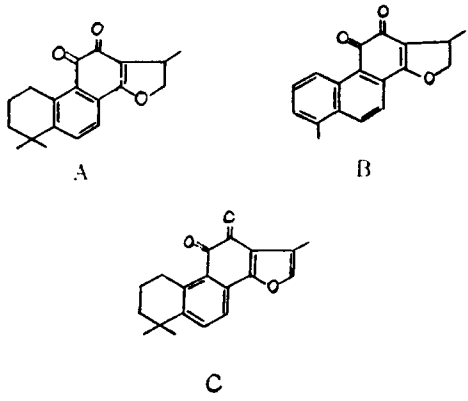
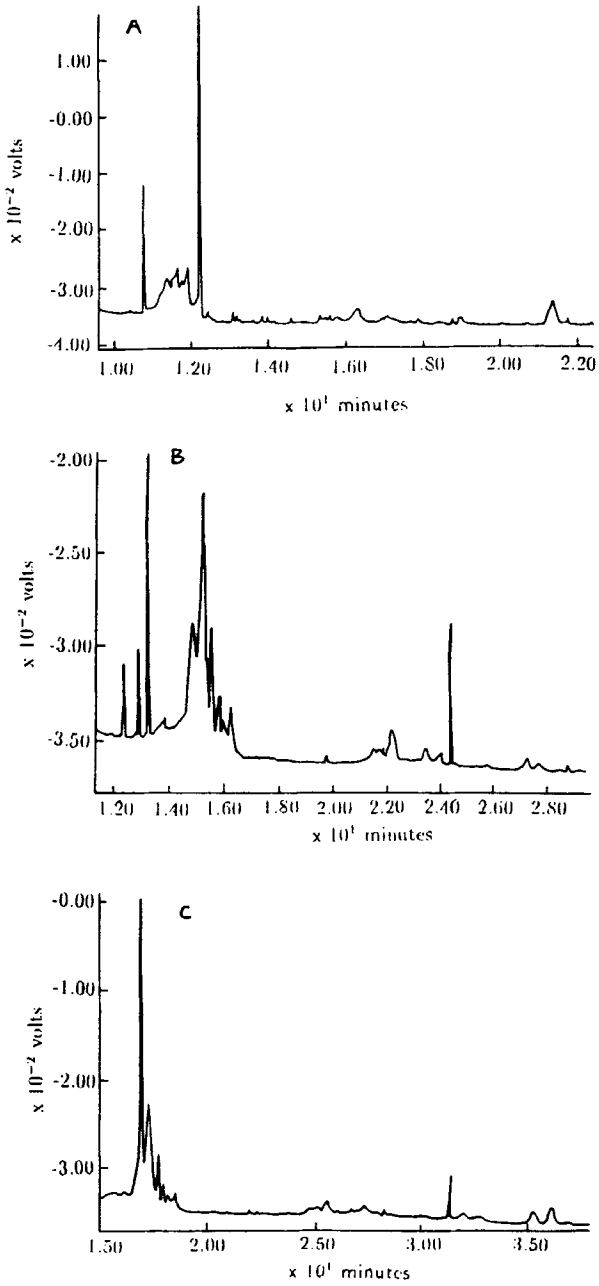


Figure 1. Chemical structures of cryptotanshinone (A), dihydrotanshinone I (B) and tanshinone IIA (C).

Hydrostatic sampling mode was selected for injection. Data acquisition was carried out with a Maxima 820 chromatography workstation (Waters) with a system interface module connecting the CE system to the station. Data acquisition rate was 20 points s^{-1}. Collection of electropherographic data was initiated by a signal cable connection between the Quanta 4000 and the system interface module (SIM).

Materials and Reagents

The sample of the roots of *Salvia przewalskii* Maxim was collected from Gansu, China. The air dried roots of *Salvia przewalskii* Maxim (1 kg) were extracted four times with ethanol (95%) at room temperature. After evaporation, the crude material (50 g) was chromatographed on silica gel column eluted with a gradient of benzene -- ethyl ether (10:1-1:1-1:5-ethyl ether) and three fractions were obtained. Then, from the three fractions, the pure compounds, cryptotanshinone, dihydrotanshinone I and tanshinone IIA, were obtained by recrystallization in methanol.[13] The crude ethanol extract of *Salvia przewalskii* Maxim and the three compounds isolated from the extract were dissolved in N,N-dimethylformamide to give appropriate concentrations for their separation by MECC. The chemical structures of cryptotanshinone, dihydrotanshinone I and tanshinone IIA are illustrated in Figure 1.



Sodium tetraborate, sodium dodecyl sulfonate (SDS) (chemical grade), N,N-dimethylformamide (DMF) and tetra ethylene glycol (TEG) were purchased in China.

The run buffer solutions were prepared as to contain 25.0 mmol L^{-1} of sodium tetraborate, 10 mmol L^{-1} of SDS, 10% (v/v) of DMF and 0.2% (v/v) of TEG, whose pH was adjusted to 9.5 by addition of appropriate volume of concentrated KOH solution. Unless otherwise specified, all chemicals were of analytical reagent grade. All solutions were prepared using filtered, degassed and deionized distilled water.

Procedure

At the beginning of each day, the capillary was rinsed with sufficient deionized water for about 15 minutes. It was also flushed with 0.5 mol L^{-1} KOH solution after being used for three or four days.

Experiments were performed to optimize the CE system. Unless otherwise specified, the standard conditions used were: 10 KV applied voltage, 15 s hydrostatic loading time and a working electrolyte consisting of 25.0 mmol L^{-1} of sodium tetraborate, 10 mmol L^{-1} of SDS, 10% (v/v) of DMF and 0.2% (v/v) of TEG. Before each run, the capillary was purged for 3 minutes under vacuum. All operations were at room temperature.

RESULTS AND DISCUSSION

Effect of pH

The pH of the buffer solution containing 25.0 mmol L^{-1} of sodium tetraborate, 10 mmol L^{-1} of SDS and 10% (v/v) of DMF was varied by addition of concentrated HCl or concentrated KOH solution.

Figure 2. (left) Effect of DMF concentration on MECC of the crude ethanol extract of the roots of *Salvia* (*przewalskii* Maxim. Buffers: 25.0 mmol L^{-1} sodium tetraborate (pH 9.5) containing 10 mmol L^{-1} SDS and a) no DMF (b) 10 % (v/v) DMF and (c) 15% (v/v) DMF; capillary: 60 cm x 75 μm; voltage: 10 KV; gravity injection: 10cm for 15 s; detection: absorption at 254 nm.

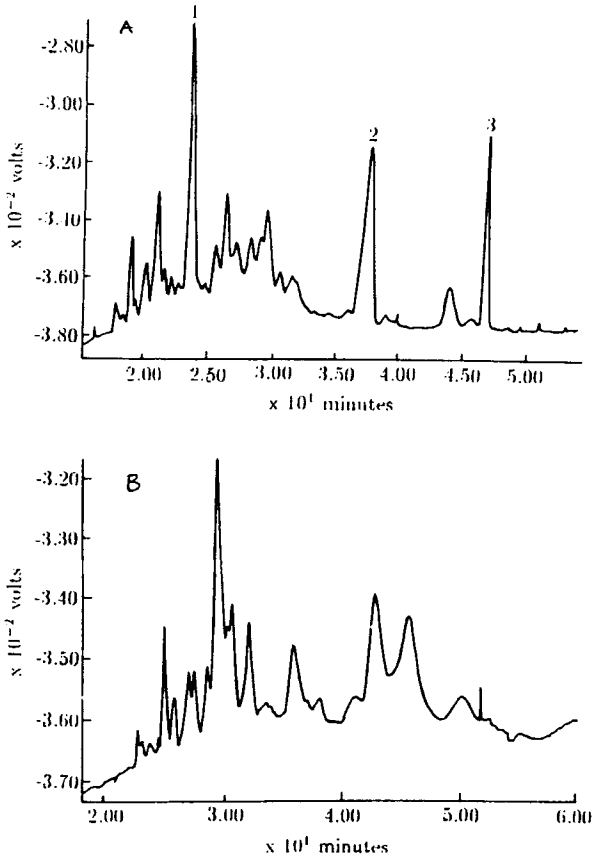


Figure 3. Effect of TEG concentration on MECC of the crude ethanol extract of roots of *Salvia przewasskii* Maxim.
Buffers: 25.0 mmol L^{-1} sodium tetraborate (pH 9.5) containing 10 mmol L^{-1} SDS, 10% (v/v) DMF and (a) 0.2% (v/v) TEG and (b) 0.5% (v/v) TEG; capillary: 60 cm x 75 μm; voltage: 10 KV; gravity injection: 10 cm for 15 s; detection: absorption at 254 nm. Peaks: 1 = cryptotanshinone, 2 = tanshinone IIA, 3 = dihydrotanshinone I, other peaks are not identified.

Figure 4. (right) MS spectra of the three substances. A: tanshinone IIA. B: dihydrotanshinone. C: cryptotanshinone, M^+: peak of molecular ion.

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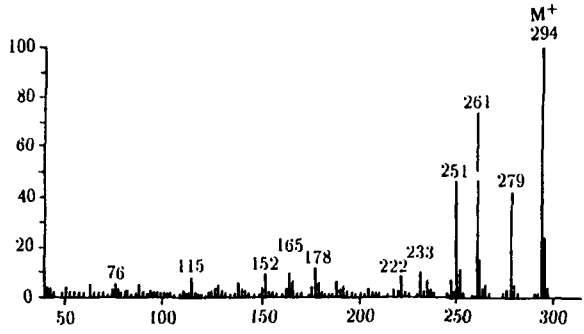

A

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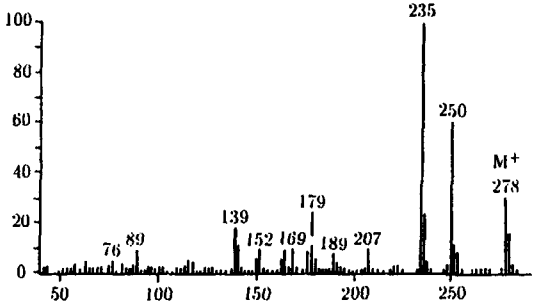

B

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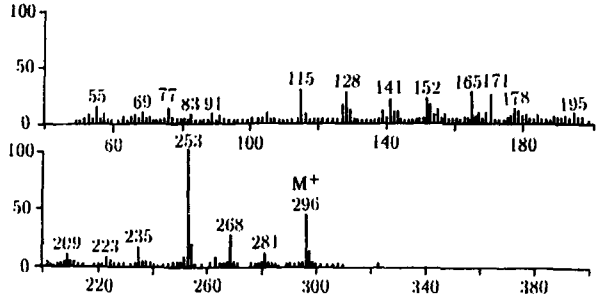

C

The effect of pH on the separation of the crude ethanol extract of the roots of *Salvia przewalskii* Maxim was investigated. It was observed that, at lower pH, poor separation was obtained, whereas when the pH was increased from 9.2 to 9.8 many peaks were separated. As the pH of the buffer was further increased, results of the separation did not improve. For subsequent work, the pH of 9.5 was employed.

Effect of DMF

MECC of the ethanol extract of the roots of *Salvia przewalskii* Maxim uses SDS as micelles. In MECC the separation mechanism involves partitioning between the aqueous phase and the micellar phase. Therefore, any manipulation to the buffer system will have some effect on the distribution coeffecient and thus on the separation selectivity.

Organic modifiers are often used in MECC to decrease the affinities of hydrophobic solutes for the micellar phase. In addition, organic solvents reduce electroosmotic flow and subsequently expand the migration window.[14-17] As a result, resolution of highly hydrophobic compounds in MECC is enhanced. Addition of DMF to the SDS buffer improved the separation, as shown in Figure 2.

The best separation was observed when 10% (v/v) of DMF was added to the buffer, and this concentration was used in subsequent experiments.

Effect of TEG

In order to obtain a better separation, some additives to the buffer containing 25.0 mmol L^{-1} of sodium tetraborate, 10 mmol L^{-1} of SDS and 10% (v/v) of DMF were tried. β-Cyclodextrin (β-CD) was added to the buffer at different concentrations (0-20 mmol L^{-1}), but no improvement of the separation wasobserved. Addition of tetra ethylene glycol (TEG) to the buffer improved the separation considerably, as shown in Figure 3.

The best separation was obtained when 0.2% (v/v) of TEG was added to the buffer. Using this buffer (25.0mmol L^{-1} sodium tetraborate + 10 mmol L^{-1} SDS + 10% (v/v) DMF + 0.2% (v/v) TEG), many peaks were separated and the three tanshinone compounds were also separated.

Identification of the Components of Interest in Roots of *Salvia przewalskii* Maxim

The three peaks belonging to cryptotanshinone, dihydrotanshinone I, and tanshinone IIA were identified by comparing their migration times with those of standards. Pure standards were also added to the samples so that the peak heights of related compounds were increased in order to improve their detectability. The ratio of absorbances at different wavelengths between the sample and the standard in UV detection mode was compared. Figure 4 showed the MS spectra of the three substances. By the above methods, the three components of interest in roots of *Salvia przewalskii* Maxim were identified.

CONCLUSION

The successful separation of the main tanshinone substances in the crude ethanol extract of the roots of *Salvia przesalskii* Maxim shows that MECC is a useful method for the determination of pharmaceutical compounds in natural product isolation procedures. The addition of modifiers to the SDS buffers, such as organic solvents and TEG greatly improved the separations.

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