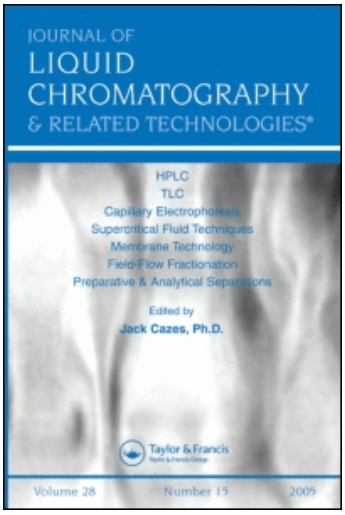

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Separation of High Purity Przewaquinone A by High-Speed Countercurrent Chromatography

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ABSTRACT

High-speed countercurrent chromatography (HSCCC) was used to purify przewaquinone A from the extract of *Salvia miltiorrhiza* Bunge using a two-phase solvent system composed of carbon tetrachloride–methanol–water–*n*-hexane, at an optimum volume ratio of 3 : 3 : 2 : 1. The method yielded 15.3 mg of 98% przewaquinone A from 100 mg of a crude sample containing przewaquinone A, at 16.0% in a single run. The recovery of przewaquinone A is as high as 93.7%. Identification was performed by IR, UV, ^{1}H NMR, ^{13}C NMR, and FAB-MS.

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Key Words: Countercurrent chromatography; *Salvia miltiorrhiza*; Przewaquinone A.

INTRODUCTION

Salvia miltiorrhiza Bunge, Dan-Shen in Chinese, is a well-known traditional Chinese medical herb used for treatment of various kinds of diseases, especially, for coronary disorders.[1] Przewaquinone A is one of the active ingredients of *Salvia miltiorrhiza* Bunge, and some research work has shown its antitumor activities.[2] The chemical structure of przewaquinone A is given in Fig. 1.

The separation and purification of przewaquinone A using conventional methods, such as column chromatography, requires several steps resulting in low recovery of the product. High-speed countercurrent chromatography (HSCCC), being a support-free liquid–liquid partition chromatographic technique, eliminates irreversible adsorption of the sample onto the solid support,[3] and, therefore, the method has been widely used for the preparative separation of natural products. Compared with traditional solid–liquid column chromatography, it yields a higher recovery and efficiency.

The present paper describes a successful preparation of high purity przewaquinone A from a *S. miltiorrhiza* Bunge extract by HSCCC. The method provides greater preparative quantity at a higher sample recovery rate compared with the conventional method.[4]

EXPERIMENTAL

Apparatus

The analytical HSCCC instrument employed was a Model GS 20 analytical high-speed countercurrent chromatograph designed and constructed in Beijing Institute of New Technology Application, Beijing, China. The apparatus holds a pair of column holders symmetrically on the rotary frame at a distance of 5 cm from the central axis of the centrifuge. The multilayer coil separation column was prepared by winding a 50 m long, 0.85 mm I.D. polytetrafluoroethylene (PTFE) tube directly onto the holder hub forming multiple coiled layers with a total capacity of 40 mL. The β value varied from 0.4 at the internal terminal to 0.7 at the external terminal ($\beta = r/R$ where r is the distance from the coil to the holder shaft, and R, the revolution radius or the distance between the holder axis and central axis of the centrifuge). Although the revolution speed of the apparatus could be regulated with a

speed controller in a range between 0 and 2000 rpm, the optimum speed of 1800 rpm was used in the present studies.

Preparative HSCCC was performed using a Model GS10A2 multilayer coil planet centrifuge (Beijing Institute of New Technology Application, Beijing, China) equipped with a PTFE multilayer coil of 110 m in length and 1.6 mm in I.D. with a total capacity of 230 mL. The β value of this preparative column ranges from 0.5 to 0.8. Although the revolution speed of the apparatus could be regulated with a speed controller in a range between 0 and 1000 rpm, the optimum speed of 800 rpm was used in the present studies.

The solvent was pumped into the column with a Model NS-1007 constant-flow pump (Beijing Institute of New Technology Application, Beijing, China). Continuous monitoring of the effluent was achieved with a Model 8823A-UV Monitor (Beijing Institute of New Technology Application, Beijing, China) at 254 nm. A manual sample injection valve with a 2.0 mL loop (for the analytical HSCCC) or a 20 mL loop (for the preparative HSCCC) (Tianjin High-New Science & Technology Company, Tianjin, China) was used to introduce the sample into the column, respectively. A portable recorder (Yokogawa Model 3057, Sichuan Instrument Factory, Chongqing, China) was used to draw the chromatogram. The high-performance liquid chromatography (HPLC) equipment used was a Shimadzu LC-10A system including two LC-10ATVP pumps, a SPD-M10AVP diode array detector, a SIL-10ADVP auto-injector, a SCL-10AVP system controller, a CTO-10AVP column oven, a DGU-14A degasser, and a Class-VP-LC work station (Shimadzu, Kyoto, Japan).

Reagents

All organic solvents used for HSCCC were of analytical grade and purchased from Beijing Chemical Factory, Beijing, China. Acetonitrile used for HPLC analysis was of chromatographic grade and purchased from Tianjin Huaxi Special Reagent Factory, Tianjin, China. *Salvia miltiorrhiza* Bunge extract was purchased from Guangzhou SHR biotechnology Co. Ltd., Guangzhou, Guangdong Province, China.

Preparation of Sample Solution

About 11 g amount of *S. miltiorrhiza* Bunge extract was chromatographed on a silica (100–200 mesh) column, eluted with dichloromethane–ethyl acetate (9/1, v/v). The collected fraction was analyzed by HPLC, and the przewaquinone A fraction was evaporated to dryness under reduced pressure, which

yielded 518.8 mg of the crude sample. The sample solutions were prepared by dissolving the crude sample in the lower phase solvent used for separation, at suitable concentrations according to the analytical or preparative purpose.

Preparation of Two-Phase Solvent System

The following solvent system was prepared: The carbon tetrachloride–methanol–water–*n*-hexane (3 : 3 : 2 : 1, v/v/v/v) mixture was thoroughly equilibrated in a separatory funnel at room temperature, and the two phases were separated shortly before use.

Separation Procedure

High-speed countercurrent chromatography was performed as follows: the multilayer coiled column was first entirely filled with the upper phase. The lower phase was then pumped into the head end of the column at a flow-rate of 1.0 mL/min (for analytical instrument) or 2.0 mL/min (for preparative instrument), while the apparatus was run at 1800 rpm (for analytical instrument) or 800 rpm (for preparative instrument). After hydrodynamic equilibrium was reached, indicated by a clear mobile phase eluting at the tail outlet, 2 mL (for the analytical instrument) or 20 mL (for the preparative instrument) of sample solution (5 mg/mL) was injected through the sample port. The effluent from the tail end of the column was continuously monitored with a UV detector at 254 nm. Each peak fraction was collected according to the chromatogram. After all desired peaks were eluted, the rotation and elution were stopped. Then, the column contents were collected into a graduated cylinder by N_2 pressured at approximately 0.5 Mpa. The retention of the stationary phase relative to the total column capacity was computed from the volume of the stationary phase collected from the column.

High Performance Liquid Chromatographic Analysis and Identification of Przewaquinone A

The crude sample and each peak fraction from HSCCC were analyzed by HPLC. The analyses were performed with an Inertsil ODS-3 column (150 × 4.6 mm I.D.) at a column temperature of 35°C. The mobile phase, composed of water and acetonitrile (45 : 55, v/v), was eluted at a flow-rate of 1.0 mL/min and the effluent monitored at 254 nm by a PAD detector. The concentration of the high purity przewaquinone A was determined by the area

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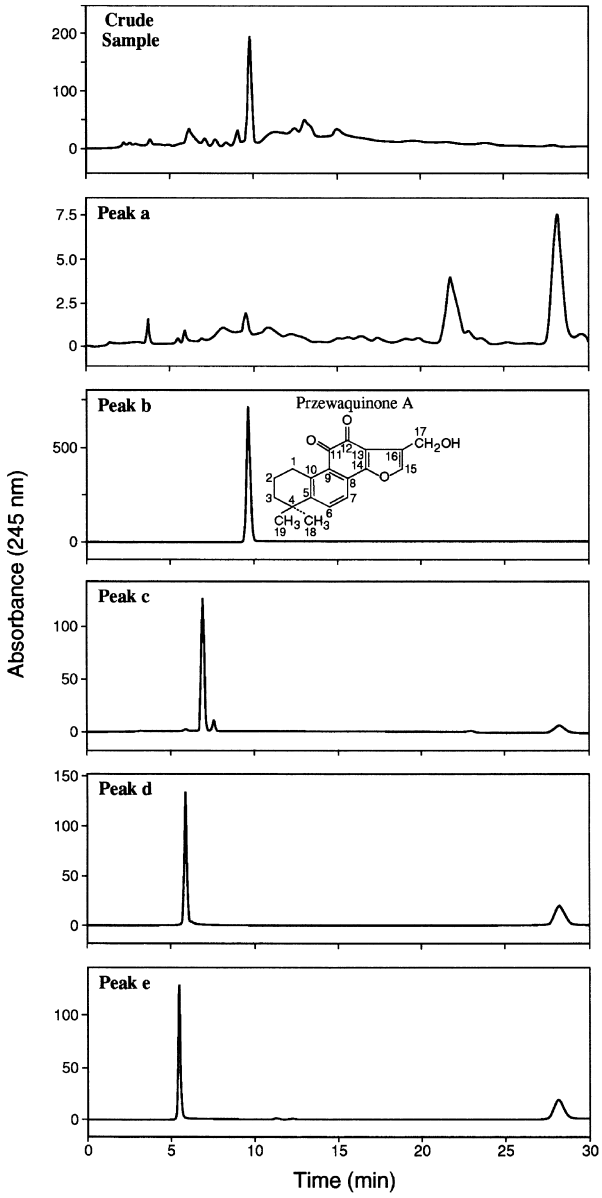

Figure 1. The results of HPLC analysis of the crude sample and the przewaquinone A fraction. (A) The crude sample; (B) przewaquinone A. Column: Inertsil ODS-3 column (150 × 4.6 mm I.D.); column temperature 35°C; mobile phase: water/acetonitrile (45 : 55, v/v); flow-rate: 1.0 mL/min.

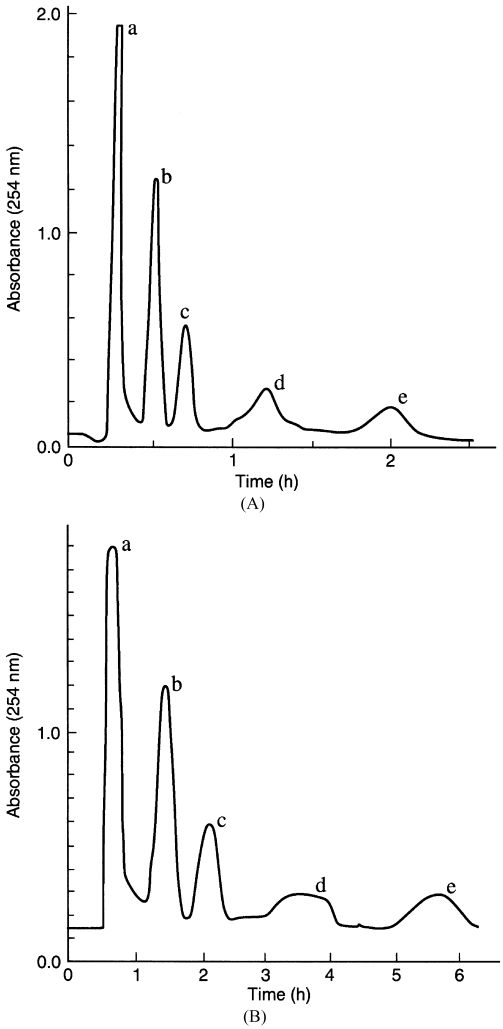

Figure 2. (A) Analytical HSCCC separation of the crude sample. Solvent system: carbon tetrachloride–methanol–water–*n*-hexane (3 : 3 : 2 : 1, v/v/v/v); stationary phase: upper phase; mobile phase: lower phase; flow rate: 6.0 mL/min; revolution speed: 1800 rpm; sample: 10.6 mg dissolved in 2 mL of the lower phase; retention of the stationary phase: 67.5%. Peak b: przewaquinone A. (B) Preparative HSCCC separation of the crude sample. Solvent system: carbon tetrachloride–methanol–water–*n*-hexane (3 : 3 : 2 : 1, v/v/v/v); stationary phase: upper phase; mobile phase: lower phase; flow rate: 2.0 mL/min; revolution speed: 800 rpm; sample: 105.7 mg dissolved in 20 mL of the lower phase; retention of the stationary phase: 77.6%. Peak b: 98% przewaquinone A, 16.2 mg.

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percentage in HPLC analysis, which was used as reference material in the crude sample analysis by the external standard curve method. The final identification of przewaquinone A was carried out by IR, UV, ^{1}H NMR, ^{13}C NMR, and FAB-MS.

RESULTS AND DISCUSSION

The crude sample was analyzed by HPLC (Fig. 1 top), which indicated that it contained przewaquinone A at 16.0%. In order to achieve an efficient separation of the target compounds, analytical HSCCC was used in choosing a suitable two-phase solvent system. The results indicated that carbon tetrachloride–methanol–water–*n*–hexane (3 : 3 : 2 : 1, v/v/v/v) gave the best separation for przewaquinone A [Fig. 2(A)]. With the same solvent system used in the analytical HSCCC, preparative HSCCC was performed for purification of przewaquinone A [Fig. 2(B)]. Six fractions (peak a, b, c, d, e, and the residue in the column) were obtained. High performance liquid chromatographic analysis indicated that the fraction corresponding to peak b contained przewaquinone A at about 98% purity [Fig. 1(B)]. This HSCCC separation yielded 15.3 mg of przewaquinone A from 100 mg of the crude sample. The przewaquinone A recovery was as high as 93.7%.

FAB-MS, UV, IR, and ^{1}H NMR, ^{13}C NMR data gave the structural identification: FAB-MS m/z 311.0$[M+H]^{+}$; UV λ_{max} MeOH 222, 250, 266, 350, 459 nm; IRvmax (KBr) 1670, 3419, 3448 cm^{-1}; ^{1}H-NMR (600 MHz,$CDCl_3$) δppm 7.672–7.581 (1H, H-6), 7.392 (1H, H-7), 7.261 (1H, H-16), 4.672 (2H, H_2-17), 3.208–3.186 (2H, H_2-3), 1.822–1.792 (2H, H_2-1), 1.681–1.662 (2H, H_2-2), 1.323 (6H, Me-18 and Me-19); ^{13}C-NMR (600 MHz, $CDCl_3$) δppm 182.8 (C-11), 176.0 (C-12), 163.3 (C-14), 151.2 (C-10), 145.1 (C-5), 140.5 (C-15), 133.7 (C-6), 126.9 (C-8), 126.4 (C-9), 125.8 (C-13), 120.6 (C-7, C-16), 55.2 (C-17), 37.8 (C-3), 34.8 (C-4), 31.8 (C-18, C-19), 30.0 (C-1), 19.1 (C-2). Comparing the above with Refs.,[1,4,5] the obtained product is confirmed as przewaquinone A.

The results of our studies clearly showed that HSCCC is a very useful tool for the preparative separation of przewaquinone A, which yields a highly pure sample at a high recovery rate.

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