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Preparative separation of rhein from Chinese traditional herb by repeated high-speed counter-current chromatography

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Abstract

High-speed counter-current chromatography (HSCCC) was repeatedly used for isolation and purification of rhein from *Rheum officinale* Baill (Dahuang) with a two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water (3:7:5:5, v/v), which had been selected by analytical (HSCCC). Using two preparative units of the HSCCC centrifuge, about a 500 mg amount of the crude extract was separated, yielding 6.7 mg of rhein at a high purity of over 97%.

Keywords: Counter-current chromatography; *Rheum officinale*; Preparative chromatography; Plant materials; Pharmaceutical analysis; Rhein

1. Introduction

Rheum officinale Baill (Dahuang) is a useful traditional Chinese herb. Pharmacological tests revealed that rhein not only has a strong antibacterial action on *Bacteroids fragilis*, but also may be useful in cancer chemotherapy as a biochemical modulator [1]. It has been reported that rhein retards the progression of type-2 diabetic nephropathy [2].

The separation of active compounds from natural sources may encounter various problems. For example, the compound of interest is often present only as a minor component in an extremely complex mixture. Rhein is scarce among hydroxyanthraquinones in alcohol extract of *R. officinale* Baill, and strongly adsorbed onto the solid support of conventional silica gel column chromatography [3].

High-speed counter-current chromatography (HSCCC), being a support-free liquid–liquid partition chromatographic technique, eliminates irreversible adsorption of the sample onto the solid support [4], and has been widely used in preparative separation of natural products [5]. Although many hydroxyanthraquinones have been purified from *R. officinale* Baill by HSCCC at high purity [3,6], the preparative separation of rhein had not been reported. The present paper describes the successful preparative separation and purification of rhein from the crude alcohol extract of *R. officinale* Baill by high-speed chromatography.

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2. Experimental

2.1. Apparatus

The analytical HSCCC instrument employed in the present study is a Model GS 20 analytical high-speed counter-current 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 multi-layer coil separation column was prepared by winding a 50 m × 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 the range between 0 and 2000 rpm, an optimum speed of 1800 rpm was used in the present studies. A manual sample injection valve with a 1.0 ml loop was used.

A multi-dimensional CCC system (Fig. 1), set up by Dr. Fuquan Yang [7] in our laboratory, was used with two Model GS10A2 multi-layer coil planet centrifuges (Beijing Institute of New Technology Application, Beijing, China) each equipped with a PTFE multi-layer coil of 110 m × 1.6 mm i.d. with a total capacity of 230 ml. The β-value of the preparative column ranges from 0.5 to 0.8.

Two Model NS-1007 constant-flow pumps (Beijing Institute of New Technology Application, Beijing, China) were used to elute the mobile phase while continuous monitoring of the effluent was achieved with two Model 8823A-UV Monitors (Beijing Institute of New Technology Application, Beijing, China) at 254 nm. Two manual six-port valves, one with a 20 ml loop used as the injection valve and the other without loop used as the switching valve (Tianjin High-New Science & Technology Company, Tianjin, China), were used to introduce the sample into the column. Two portable recorders (Yokogawa Model 3057, Sichuan Instrument Factory, Chongqing, China) were used to draw the chromatogram. A rotary

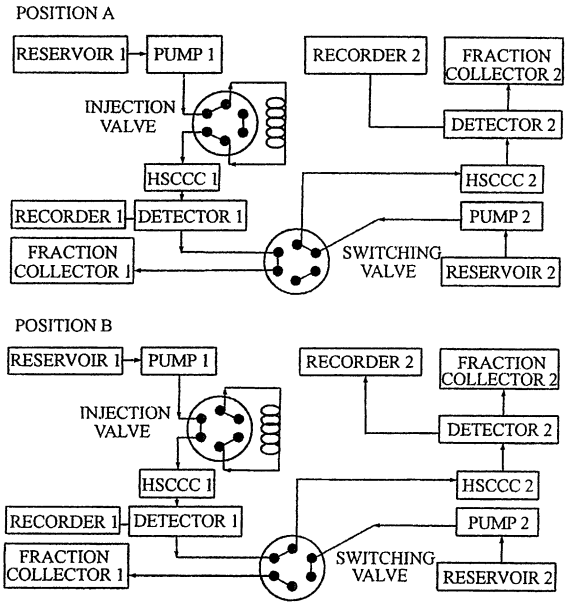

Fig. 1. Schematic diagram of the repeated high-speed counter-current chromatography (HSCCC) system with two sets of high-speed counter-current chromatographs, a six-port injection valve and a six-port switching valve.

evaporator (Model RE-90, Beijing Institute of New Technology Application, Beijing, China) was also used.

The high-performance liquid chromatography (HPLC) equipment used was a Shimadzu LC-10AVP system including two LC-10ATVP solvent delivery units, an SPD-M10AVP UV-Vis photodiode array detection (DAD) system, a Model 7726 injection valve with a 20 μl loop, an SCL-10AVP system controller, a CTO-10ASVP column oven, a DGU-12A degasser, and a Class-VP-LC workstation (Shimadzu, Kyoto, Japan).

2.2. 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). Rhein (90% standard) was purchased from National Institute for the Control of Pharmaceutical & Biological Products (Beijing, China).

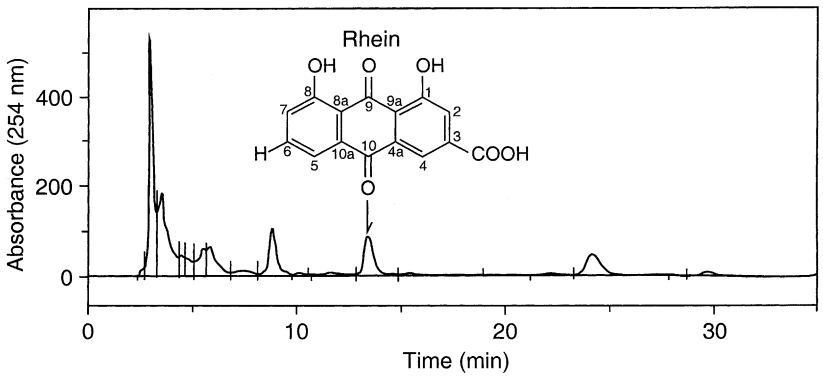

Fig. 2. HPLC analyses of the crude ethanol extract from *Rheum officinale* Baill with the chemical structure of rhein. HPLC conditions: Supelcosil ODS column (250 mm × 4.6 mm i.d.), column temperature: 35 °C. Mobile phase: methanol–0.5% aqueous H_3PO_4 (60:40, v/v), flow-rate: 1.0 ml/min, monitored at 254 nm by a DAD.

2.3. Preparation of sample

About 1 kg of dried roots of *R. officinale* Baill was ground, and a 150 g amount of this dried powder was extracted (refluxed) with 1000 ml of ethanol, and then concentrated to dryness under reduced pressure, yielding 40 g of a crude sample in which rhein purity is determined by HPLC (Fig. 2).

2.4. Preparation of two-phase solvent system and sample solutions

The solvent system utilized in the present study was prepared by mixing *n*-hexane–ethyl acetate–methanol–water (9:1:5:5, 1:1:1:1 or 3:7:5:5, v/v), and thoroughly equilibrating the mixtures in a separatory funnel at room temperature, two phases being separated shortly before use.

The sample solutions were prepared by dissolving the crude extract in the lower phase at suitable concentrations according to the analytical or preparative purpose.

2.5. Separation procedure

Analytical HSCCC was performed with a Model GS 20 HSCCC instruments as follows: the multi-player 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, while the apparatus was run at a revolution speed of 1800 rpm. After hydrodynamic equilibrium was established, as indicated by a clear mobile phase eluting at the tail outlet, the sample solution (15 mg in 1 ml of lower phase) 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 fractions were collected according to the chromatogram. 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 after the separation was completed.

The repeated HSCCC separation was performed as follows: the switching valve shown in Fig. 1 is initially set in position A, and HSCCC systems 1 and 2 are simultaneously filled with the upper stationary phase using pumps 1 and 2, respectively (Fig. 1). Both apparatuses are rotated at 800 rpm, while the lower phase is eluted through HSCCC systems 1 and 2 using their respective pumps at a flow-rate of 2.0 ml/min. After hydrodynamic equilibrium is reached in each column, as indicated by a clear mobile phase eluting at the tail outlet, the sample solution (500 mg in 20 ml of lower phase) is injected into HSCCC 1 through the injection valve while pump 2 is stopped. The effluent from the outlet of HSCCC 1 is continuously monitored with UV detector 1 at 254 nm, and collected according to the chromatogram. When the target peak appears, the effluent from HSCCC 1 is cut and introduced into the HSCCC 2 column by turning the switching valve to position B. After the target peak is

completely introduced from HSCCC 1 to HSCCC 2 columns, the switching valve is returned to position A, while restarting pump 2 to resume the elution of the target peak with detector 2 and recorder 2.

2.6. HPLC analyses and identification of HSCCC peak fractions

The crude alcohol extract of *R. officinale* Baill, rhein (standard) and HSCCC peak fractions were each analyzed by HPLC. The analyses were performed with a Supelcosil ODS column (250 mm × 4.6 mm i.d.) at column temperature of 35 °C. The mobile phase, composed of methanol–0.5% aqueous H_3PO_4 (60:40, v/v), was isocratically eluted at a flow-rate of 1.0 ml/min and the effluent monitored at 254 and 410 nm by a DAD detector.

Identification of the target compound (rhein) was based on retention time referenced with a pure standard of rhein together with MS, ^{1}H NMR; and ^{13}C NMR spectra.

3. Results and discussion

The HPLC analysis of the crude extract of *R. officinale* Baill indicated that it contained several compounds as shown in Fig. 2. Rhein purity of crude extract is 1.4% based on external standard curve as determined by HPLC.

In order to achieve an efficient resolution of target compounds, the two-phase solvent system of *n*-hexane–ethyl acetate–methanol–water was examined using analytical HSCCC by varying the mutual volume ratio. This solvent system can be applied to a broad spectrum of samples with a moderate degree of polarity, since hydrophobicity of the solvent system is easily adjusted by changing the relative volume ratio between *n*-hexane and ethyl acetate. The results illustrated in Fig. 3 indicated that the volume ratio of 3:7:5:5 was most suitable for the HSCCC run for purification of rhein.

Fig. 4 shows the result obtained from 500 mg of the crude extract of *R. officinale* Baill by preparative

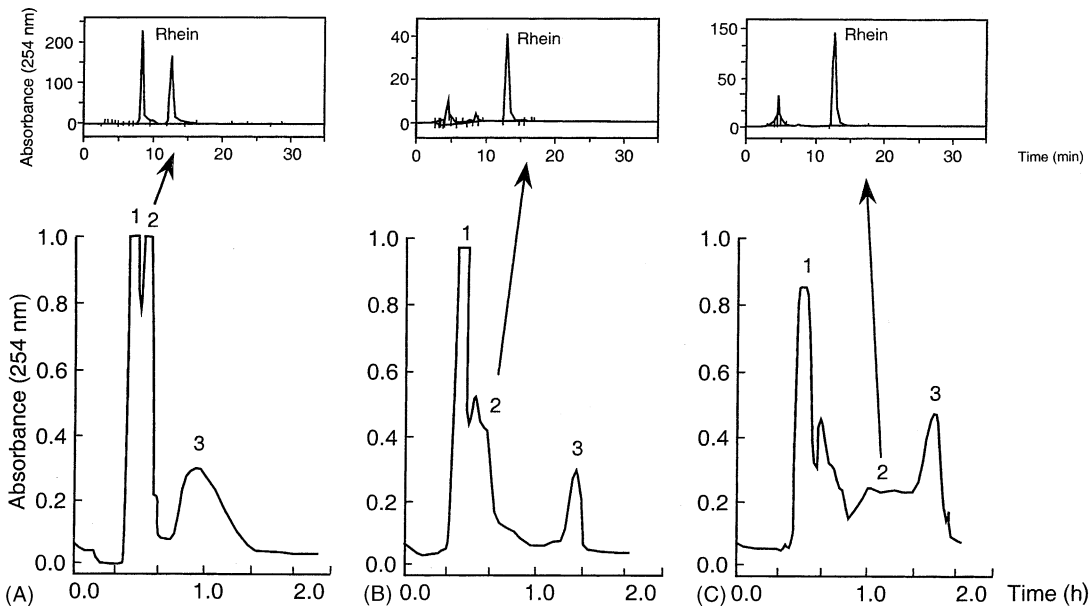

Fig. 3. Chromatogram of the crude ethanol extract of *Rheum officinals* Baill by analytical HSCCC. Solvent systems: (A) *n*-hexane–ethyl acetate–methanol–water (9:1:5:5, v/v); (B) *n*-hexane–ethyl acetate–methanol–water (1:1:1:1, v/v); (C) *n*-hexane–ethyl acetate–methanol–water (3:7:5:5, v/v); stationary phase: upper organic phase; mobile phase: lower aqueous phase; flow-rate: 1.0 ml/min; revolution speed: 1800 rpm; sample: 15 mg dissolved in 1.0 ml lower phase. Peak 2 of A, B and C contains rhein. HPLC conditions: Supelcosil ODS column (250 mm × 4.6 mm i.d.), column temperature: 35 °C. Mobile phase: methanol–0.5% aqueous H_3PO_4 (60:40, v/v), flow-rate: 1.0 ml/min monitored at 254 nm by a DAD.

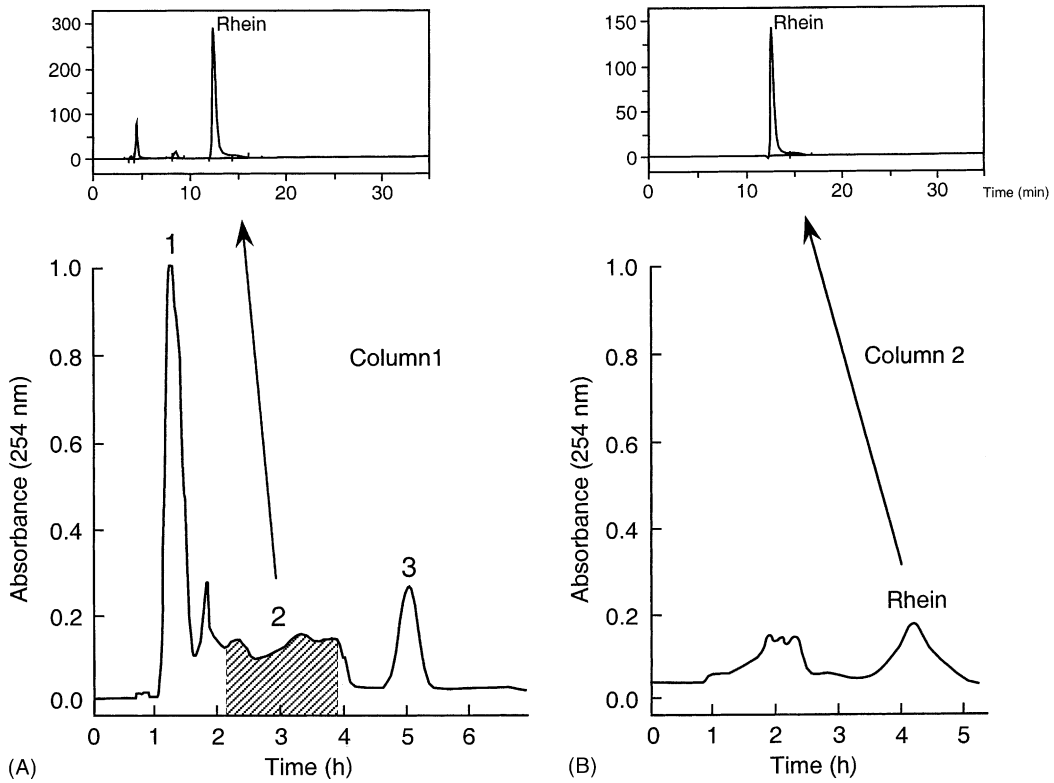

Fig. 4. Chromatogram of the crude ethanol extract from *Rheum officinale* Baill by high-speed counter-current chromatography (HSCCC). Solvent system: *n*-hexane–ethyl acetate–methanol–water (3:7:5:5, v/v); stationary phase: upper organic phase; mobile phase: lower aqueous phase; flow-rate: 2.0 ml/min; revolution speed: 800 rpm; sample: 500 mg dissolved in 20 ml of lower phase. (A) Chromatogram obtained by HSCCC 1 (peak 2 of Fig. 4A contains rhein). (B) Chromatogram of the cut fraction (the shaded part of the peak 2 in Fig. 4A) obtained by HSCCC 2. HPLC conditions: Supelcosil ODS column (250 mm × 4.6 mm i.d.), column temperature: 35 °C. Mobile phase: methanol–0.5% H_3PO_4 (60:40, v/v), flow-rate: 1.0 ml/min monitored at 254 nm by a DAD detector.

HSCCC. Fig. 4A shows the chromatogram obtained from HSCCC 1 and recorder 1. Peak 2 containing a large amount of rhein was cut and introduced into the HSCCC 2 column. The chromatogram in Fig. 4B was obtained by the cut fraction of HSCCC 1 (the shaded part of the peak 2 in Fig. 4A) introduced into and eluted from the HSCCC 2 column. This separation yielded 6.7 mg of rhein at over 97% purity based on HPLC analysis.

The structural identification of rhein was carried out by MS, ^{1}H NMR and ^{13}C NMR spectra as follows: the EI-MS: *m*/*z* 284, 256, 239, 228, 211, 155, 142. It showed the molecular ion at *m*/*z* 284, which is in agreement with the molecular formula $C_{15}H_8O_6$ of rhein.

^{1}H NMR (500 MHz, DMSO) δ ppm: 7.389 (1H, dd), 7.745 (1H, dd), 7.728 (1H, d), 7.835 (1H, dd), 8.139 (1H, d), 11.871 (2H, s), 13.742 (1H, s). The results were similar to those in reference [8].

^{13}C NMR [(500 MHz, dimethyl sulfoxide (DMSO)] δ ppm: 161.106 (C-1), 124.304 (C-2), 165.256 (C-3), 119.147 (C-4), 128.729 (C-5), 138.646 (C-6), 123.861 (C-7), 161.334 (C-8), 181.223 (C-9), 180.758 (C-10), 132.925 (C-4a), 118.582 (C-8a), 118.155 (C-9a), 133.413 (C-10a), 190.937 (3-COOH).

The results of our studies demonstrated that HSCCC is a useful method for the preparative separation of rhein from a crude alcohol extract of *R. officinale* Baill.

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