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Short communication

Preparative isolation and purification of hydroxyanthraquinones from *Rheum officinale* Baill by high-speed counter-current chromatography using pH-modulated stepwise elution

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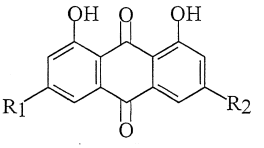
Abstract

Analytical and preparative high-speed counter-current chromatography was successfully used for the isolation and purification of hydroxyanthraquinones from *Rheum officinale* Baill (Dahuang) using pH-modulated stepwise elution. Four major components including chrysophanol, emodin, physcion and aloe-emodin were isolated each at over 98% purity. Published by Elsevier Science B.V. All rights reserved.

Keywords: *Rheum officinale*; Plant materials; Hydroxyanthraquinones; Anthraquinones; Quinones

1. Introduction

Rheum officinale Baill (Dahuang in Chinese), an important traditional Chinese medicinal herb, has a strong antibacterial action and has been used for the treatment of bacterial dysentery. The major active constituents of this herb are hydroxyanthraquinones, including chrysophanol, emodin, physcion, aloe-emodin and rhein and their glucosides. The chemical structures of these five hydroxyanthraquinones are given in Fig. 1. Pharmacological tests revealed that all these components are effective on dysentery bacillus, typhoid bacillus and cholera bacillus, among which aloe-emodin is most potent and rhein has been found to have a strong antibacterial action on *Bacteroides fragilis*. On the other hands, these hydroxyanthraquinones produce some problems in



	R_1	R_2
rhein	H	COOH
emodin	OH	CH_3
aloe-emodin	H	CH_2OH
chrysophanol	H	CH_3
physcion	OCH_3	CH_3

Fig. 1. Chemical structures of hydroxyanthraquinones from *R. officinale* Baill.

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isolation by conventional silica gel column chromatography because they are strongly adsorbed onto the solid support.

High-speed counter-current chromatography (HSCCC), being a support-free liquid–liquid partition chromatography, eliminates irreversible adsorption of sample onto the solid support [1], and therefore is considered as a suitable alternative for the separation of phenolic compounds such as flavonoids and hydroxyanthraquinones [2–4].

The present paper describes HSCCC separation of hydroxyanthraquinones from a crude extract of *R. officinale* Baill. The method uses pH-modulated stepwise elution based on their characteristic acidity which is determined by the number of carboxylic and phenolic hydroxyl groups as well as the position of the phenolic hydroxyl group in the molecule. Analytical HSCCC is first used for selecting a two-phase solvent system and three basic mobile phases suitable for the pH-modulated stepwise elution program. The optimized HSCCC condition thus obtained led to the successful preparative HSCCC separation of hydroxyanthraquinones from a crude extract of *R. officinale* Baill.

2. Experimental

2.1. Apparatus

The analytical HSCCC instrument employed in the present study is a new Model GS 20 analytical high-speed counter-current chromatograph designed and constructed at 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×0.85 mm I.D. PTFE (polytetrafluoroethylene) tube directly onto the holder hub forming multiple coiled layers with a total capacity of 30 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 to 2000 rpm, an optimum speed of 1500 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) equipped with a PTFE multilayer coil of 110 m×1.6 mm I.D. with a total capacity of 230 ml. The β values of this preparative column range from 0.5 to 0.8.

The solvent was pumped into the column with a Model NS-1007 constant-flow pump (Beijing Institute of New Technology Application). Continuous monitoring of the effluent was achieved with a Model 823A-UV Monitor (Beijing Institute of New Technology Application) operating at 254 nm and a Model 333 pH meter (ATI Orion Research, Boston, MA, USA). A manual sample injection valve with a 1.0 ml loop (for the analytical HSCCC) or a 20 ml loop (for the preparative HSCCC) (Tianjin High-New Science and Technology Company, Tianjin, China) was used to introduce the sample into the column respectively. A portable recorder (Yokogawa Model 3057, Sichuan Instrument Factory, Chongqin, China) was used to draw the chromatogram.

The high-performance liquid chromatography (HPLC) equipment used was a Shimadzu LC-10AVP system including LC-10AT liquid chromatograph, SPD-M10AVP photodiode array detector, SCL-10AVP system controller, CTO-10ASVP column oven and DGU-12A degasser (Shimadzu, Kyoto, Japan).

2.2. Reagents

All organic solvents, sodium bicarbonate, sodium carbonate, sodium hydroxide and hydrochloric acid used in this study are of analytical grade and purchased from Beijing Chemical Factory (Beijing, China). Chrysophanol, emodin, physcion, aloe-emodin and rhein standard samples were purchased from National Institute for the Control of Pharmaceutical and Biological Products, Ministry of Health (Beijing, China).

2.3. Extraction of crude hydroxyanthraquinones from R. officinale Baill

About 1 kg of dried *R. officinale* Baill plant was extracted with 1.5 l of ethanol three times at room

temperature. The extracts were combined and evaporated to dryness under reduced pressure which yielded 100 g of dry power. A 40-g amount of this dried extract was refluxed with a solvent mixture consisting of 250 ml of ethanol and 50 ml of 25% hydrochloric acid for 4 h. After cooling and removing the ethanol under reduced pressure, the total amount of hydroxyanthraquinones was extracted with 500 ml of diethyl ether three times. The ether extract was evaporated to dryness to yield 21 g of a crude sample of hydroxyanthraquinones.

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

The two-phase solvent system utilized in the present study was prepared by mixing diethyl ether and distilled water at a suitable volume ratio and thoroughly equilibrating the mixture in a separatory funnel at room temperature. After the two phases were separated, the organic phase was used as stationary phase, and the aqueous phase as the mobile phase. For performing stepwise elution a portion of the aqueous phase was basified by adding 4.0% $NaHCO_3$, 0.7% Na_2CO_3 and 0.2% NaOH, and these three mobile phases at different pH values were successively eluted through the column in an increasing order of pH for the separation.

The sample solutions were prepared by dissolving the crude extract from *R. officinale* Baill in the upper organic phase at suitable concentrations for analytical and preparative purpose.

2.5. Separation procedure

Analytical HSCCC was performed with a Model GS 20 HSCCC instruments as follows: the multilayer coiled column was first entirely filled with the upper organic stationary phase. The lower aqueous mobile phase was then pumped into the head end of the inlet column at a flow-rate of 1.0 ml/min, while the apparatus was run at a revolution speed of 1500 rpm. After hydrodynamic equilibrium was reached, as indicated by a clear mobile phase eluting at the tail outlet, the sample solution (10 mg in 1 ml of upper organic phase) was injected through the sample port. Then, the stepwise elution was started by successively eluting the column with three different mobile phases containing 4.0% $NaHCO_3$, 0.7% Na_2CO_3 and 0.2% NaOH, respectively, in an increasing order of pH as mentioned earlier. The effluent from the tail end of the column was continuously monitored with a UV detector at 254 nm. 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.

Preparative HSCCC was performed with a Model GS10A2 HSCCC centrifuge equipped with a multilayer coil column of 1.6 mm I.D., a total volume of 230 ml and a 20 ml sample loop. The mobile phase was eluted at a flow-rate of 2.0 ml/min at a revolution speed of 800 rpm. The peak fractions were collected with test tubes according to the obtained chromatogram.

2.6. HPLC analyses and identification of CCC peak fractions

The HPLC analyses were performed with a Shimpack CLC-ODS column (150×4.6 mm I.D.) at a column temperature of 40°C. The mobile phase composed of methanol–0.1% H_3PO_4 (80:20, v/v) was isocratically eluted at a flow-rate of 1.0 ml/min and the effluent monitored by a photodiode array detector. Identification of HSCCC peak fractions was carried out by comparing their retention times and UV spectra with those of standard samples.

3. Results and discussion

Based on their characteristic acidity, pH-modulated stepwise elution was performed for HSCCC separation of hydroxyanthraquinones. Using analytical HSCCC a suitable two-phase solvent system was obtained from diethyl ether–water by adding dilute bases to the aqueous mobile phase. Fig. 2 shows the separation of hydroxyanthraquinones from the crude extract of *R. officinale* Baill by analytical HSCCC. In this separation, although rhein was eluted together with impurities in the first peak, the rest of the components were all resolved well and eluted within 2.5 h. The HPLC analysis indicated that major peaks 1, 2, 3, 4 and 5 of the HSCCC chromatogram

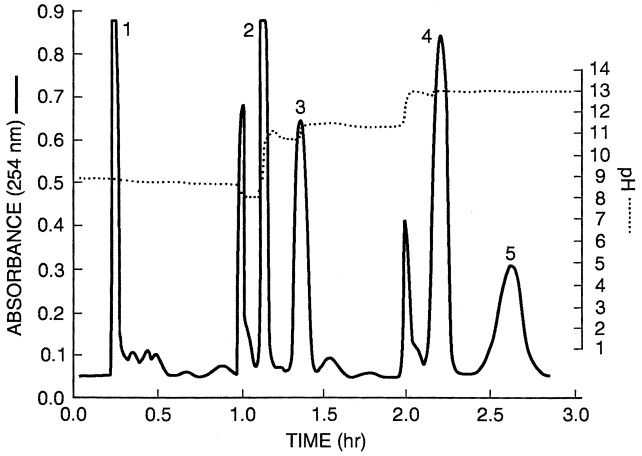

Fig. 2. Chromatogram of the crude sample of hydroxyanthraquinones from *R. officinale* Baill by analytical HSCCC. Solvent system: diethyl ether–basic water; stationary phase: upper organic phase; mobile phase: 35 ml of 4.0% $NaHCO_3$ and 55 ml of 0.7% Na_2CO_3 and 80 ml of 0.2% NaOH; flow-rate: 1.0 ml/min; revolution speed: 1500 rpm; sample size: 10 mg dissolved in 1 ml stationary phase; retention of the stationary phase: 50%. Peaks: 1=rhein+unknown; 2=emodin; 3=aloe-emodin; 4=chrysophanol; 5=physcion.

corresponded to rhein, emodin, aloe-emodin, chrysophanol and physcion, respectively.

The optimized two-phase solvent system and pH modulated stepwise elution program selected by analytical HSCCC could be directly used in preparative HSCCC while the rotation speed and the flow-rate were modified according to the column mass and tube diameter, respectively. Fig. 3 shows the result obtained from 300 mg of the crude hydroxyanthraquinone extract of *R. officinale* Baill by preparative HSCCC using pH-modulated stepwise elution. Emodin, aloe-emodin, chrysophanol and physcion were all well resolved. After separation, each purified compound was extracted from the peak fraction with ethyl acetate after acidification by concentrated hydrochloric acid. HPLC analyses given in Fig. 4 indicated that the purity of all four major hydroxyanthraquinones thus obtained was over 98%.

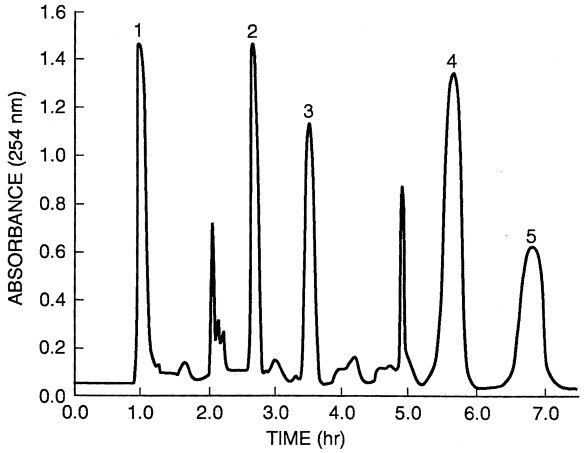

Fig. 3. Chromatogram of the crude sample of hydroxyanthraquinones from *R. officinale* Baill by preparative HSCCC with a diethyl ether–aqueous base system. Stationary phase: upper organic phase; mobile phase: 120 ml of 4.0% $NaHCO_3$, 240 ml of 0.7% Na_2CO_3 and 480 ml of 0.2% NaOH; flow-rate: 2.0 ml/min; revolution speed: 800 rpm; sample size: 300 mg dissolved in 20 ml stationary phase; retention of the stationary phase: 50%. Peaks: 1=rhein+unknown; 2=emodin; 3=aloe-emodin; 4=chrysophanol; 5=physcion.

The results of our studies clearly demonstrated that the HSCCC procedure using the pH-modulated stepwise elution program with a suitable two-phase solvent system is cost-effective and very efficient for the separation of hydroxyanthraquinones from a crude extract of *R. officinale* Baill.

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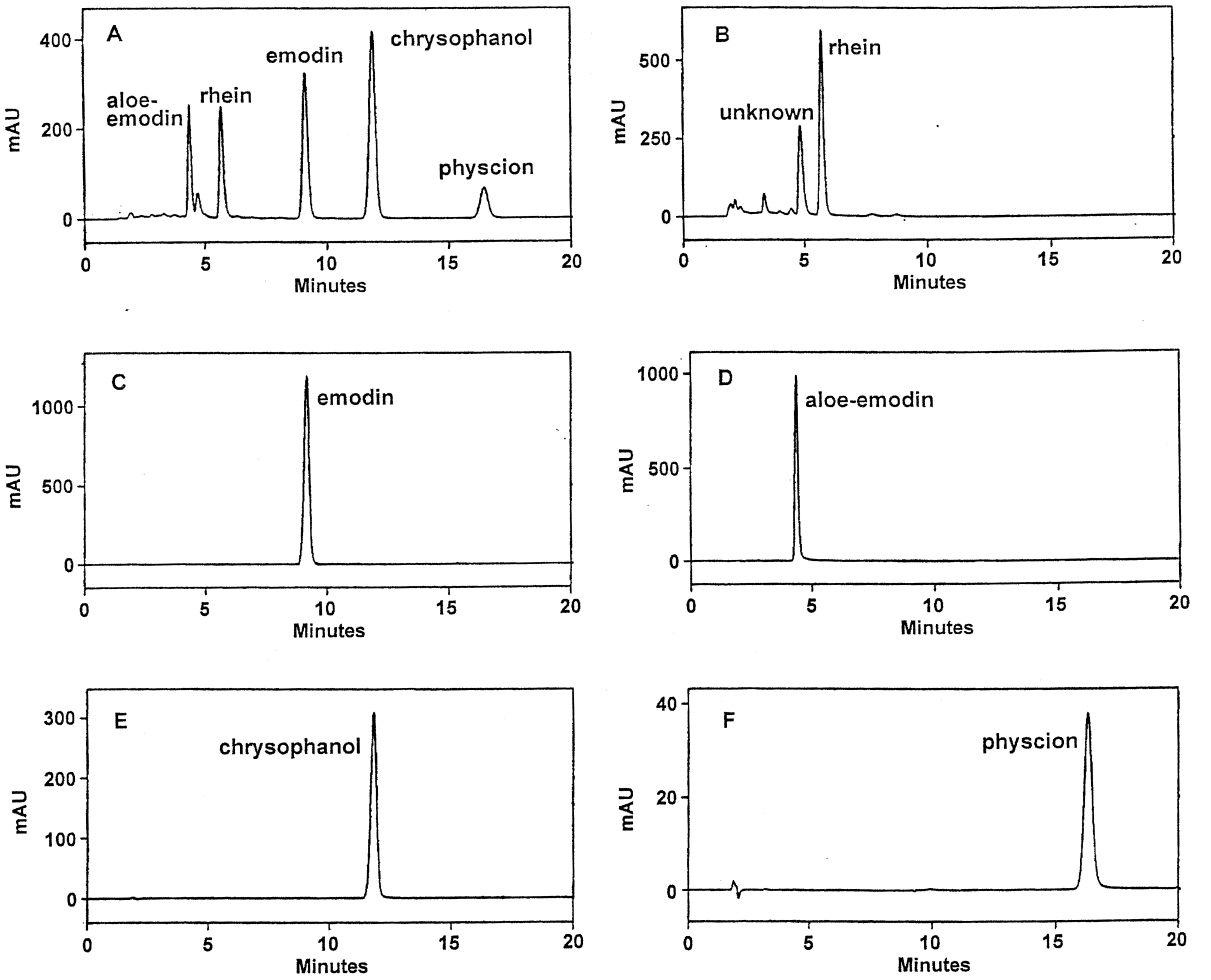

Fig. 4. The result of HPLC analyses of the crude sample and CCC fractions of hydroxyanthraquinones from *R. officinale* Baill. (A) Crude sample; (B) peak 1; (C) peak 2; (D) peak 3; (E) peak 4; and (F) peak 5 of the preparative HSCCC separation shown in Fig. 3. Column: Shim-pack CLC-ODS column (150×4.6 mm I.D.); column temperature: 40°C; mobile phase: methanol–0.1% H_3PO_4 (80:20, v/v); flow-rate: 1.0 ml/min; UV wavelength: 410 nm.

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