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Short communication

Separation of salidroside from *Rhodiola crenulata* by high-speed counter-current chromatography

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Abstract

High-speed counter-current chromatography (HSCCC) was used to purify salidroside from an extract of *Rhodiola crenulata* with two steps using a two-phase solvent system composed of ethyl acetate–*n*-butanol–water (1:4:5, v/v) in the first run and chloroform–methanol–isopropanol–water (5:6:1:4) in the second run. The method yielded 21.9 mg of salidroside from 1.216 g of the crude sample at 98% purity determined by HPLC analyses. Identification was performed by ^{1}H NMR, ^{13}C NMR, and MS.
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Keywords: Counter-current chromatography; *Rhodiola crenulata*; Salidroside

1. Introduction

Rhodiola crenulata was first used by Tibetans for maintaining body health and treating various diseases in AD 760. As a traditional herbal remedy, *Rhodiola crenulata* has been used by Tibetans in many ways such as clearing heat in the lungs, eliminating toxins from the body, treating various epidemic diseases, edema of limbs, traumatic injuries and burns. Salidroside is one of the most active ingredients of *Rhodiola crenulata*. In recent years, some research work has shown its antitumor activities [1]. Furthermore, the concentration of salidroside in Rhodiola has become one of the standard indexes to appraise the quantity of Rhodiola [2]. Consequently, it becomes increasingly important to prepare high purity salidroside as a reference material.

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 [3], and therefore the method has been widely used for the preparative separation of natural products. Comparing with traditional solid–liquid column chromatography, it yields a higher recovery and efficiency.

The present paper describes a successful preparation of high purity salidroside from *Rhodiola crenulata* by HSCCC in two steps using different two-phase solvent systems. The method provides greater

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preparative quantity at higher sample recovery rate compared with the conventional method [4].

2. Experimental

2.1. Apparatus

The analytical HSCCC instrument employed was a Model GS 20 analytical high-speed counter-current chromatograph designed and constructed in the 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 rev./min, the optimum speed of 1800 rev./min 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 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 rev./min, the optimum speed of 800 rev./min 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). Continuous monitoring of the effluent was achieved with a Model 8823A-UV Monitor (Beijing Institute of New Technology Application) 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, 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, an SPD-M10AVP diode array detector, an SIL-10ADVP auto-injector, an SCL-10AVP system controller, a CTO-10AVP column oven, a DGU-14A degasser and a Class-VP-LC work station (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). *Rhodiola crenulata* was supplied by Tibet Pannil (Lhasa, Tibet Province, China) while salidroside reference material was purchased from the National Institute for the Control of Pharmaceutical & Biological Products, Ministry of Health, Beijing, China.

2.3. Preparation of sample solution

About 0.85 kg of *Rhodiola crenulata* was extracted in a 5-l-capacity glass bottle with 4 l of acetone–water (70:30) at room temperature. The extract was evaporated down to 500 ml under reduced pressure. After this aqueous solution was successively extracted with 500 ml each of diethyl ether, ethyl acetate and *n*-butanol, it was evaporated to dryness under reduced pressure which yielded 62.93 g of the crude sample. The sample solutions were prepared by dissolving the crude sample in a 1:1 (v/v) mixture of each phase used for separation at suitable concentrations according to the analytical or preparative purpose.

2.4. Selection of two-phase solvent systems

The following two solvent systems were prepared:

ethyl acetate–*n*-butanol–water (1:4:5, v/v) and chloroform–methanol–isopropanol–water (5:6:1:4, v/v). Each solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and the two phases were separated shortly before use.

2.5. Separation procedure

HSCCC 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 inlet 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 rev./min (for analytical instrument) or 800 rev./min (for preparative instrument). After hydrodynamic equilibrium was reached, indicated by a clear mobile phase eluting at the tail outlet, 2 ml (for analytical instrument) or 20 ml (for preparative instrument) of sample solution (10 mg/ml) were 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 the 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 ~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.

2.6. HPLC analysis and identification of salidroside

The crude sample, salidroside reference material 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–acetonitrile (19:1, v/v) was eluted at a flow-rate of 1.0 ml/min and the effluent monitored at 280 nm by diode array detection. The concentration of samples was determined by the external standard curve of salidroside.

The final identification of salidroside was carried out by MS, ^{1}H NMR and ^{13}C NMR spectra.

3. Results and discussion

A series of experiments was performed to determine suitable two-phase solvent systems for HSCCC. The following polar two-phase solvent systems were tested: ethyl acetate–1-butanol–water at various volume ratios of 2:1:3, 1:2:3, 1:4:5; acetic acid–1-butanol–water (1:4:5); 1-butanol–water; 1-butanol–1-propanol–water (2:1:3); and chloroform–methanol–water (7:13:8). Among those ethyl acetate–1-butanol–water (1:4:5) gave the best partition coefficient value, and therefore was used for the first CCC separation. The second solvent system, chloroform–methanol–2-propanol–water (5:6:1:4), was found in the monograph by Hostettmann et al. [5].

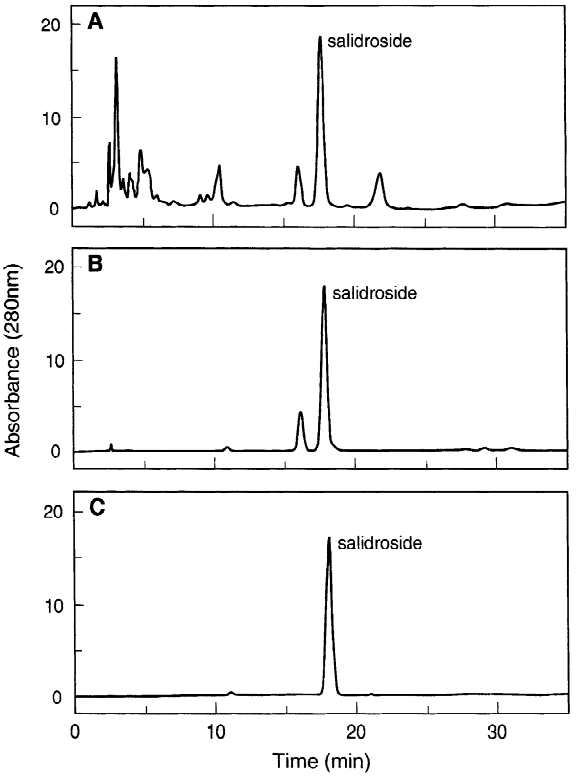

Fig. 1. The results of HPLC analysis of the crude sample, peak c fraction (Fig. 2) and the salidroside fraction (Fig. 3). (A) The crude sample; (B) peak c; (C) the salidroside fraction shown in Fig. 3. Column, Inertsil ODS-3 column (150×4.6 mm I.D.); column temperature, 35 °C; mobile phase, water–acetonitrile (19:1, v/v); flow-rate, 1.0 ml/min.

The crude sample was analyzed by HPLC (Fig. 1A), which indicated that it contained salidroside at 1.87%. HSCCC was used for purification of salidroside in two steps: in the first run using a solvent system composed of ethyl acetate–*n*-butanol–water (1:4:5, v/v) four fractions (peaks a, b, c and the residue in the column) were obtained (Fig. 2). HPLC analysis indicated that peak c contained 7.68 mg of salidroside at about 47.7% purity (Fig. 1B). In the second run, the fraction corresponding to peak c was subjected to HSCCC using a solvent system composed of chloroform–methanol–isopropanol–water (5:6:1:4, v/v) (Fig. 3) which yielded salidroside at a high purity of 98% by HPLC analyses (Fig. 1C). This two-step HSCCC separation yielded 21.9 mg of salidroside from 1.216 g of the crude sample. The salidroside recovery was as high as 94.4%.

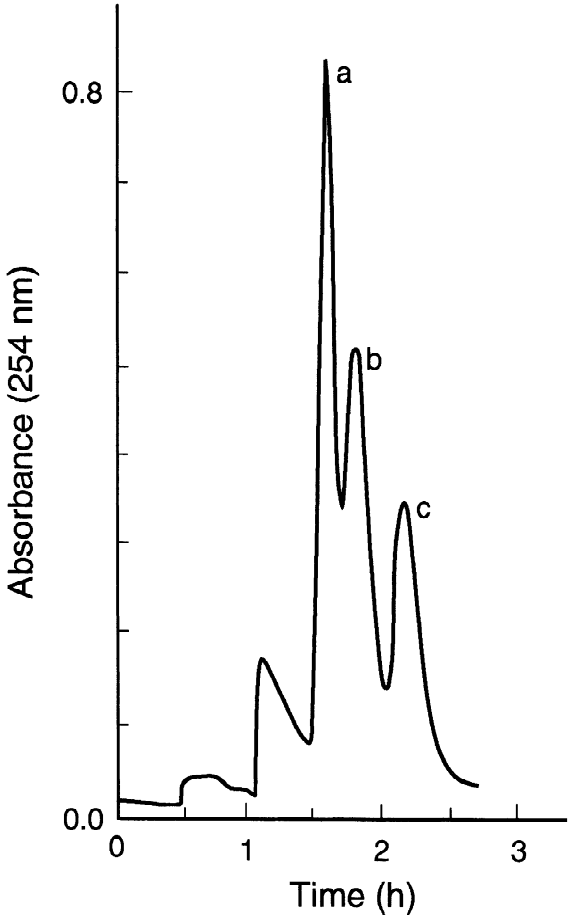

Fig. 2. Preparative HSCCC separation of the crude sample. Peak c contained salidroside (7.68 mg, 47.7% pure). Solvent system, ethyl acetate–*n*-butanol–water (1:4:5, v/v); stationary phase, upper phase; mobile phase, lower phase; flow-rate, 2.0 ml/min; revolution speed, 800 rev./min; sample, 200 mg dissolved in the mixture of 10 ml of each phase; retention of the stationary phase, 30.4%. Peak c, salidroside.

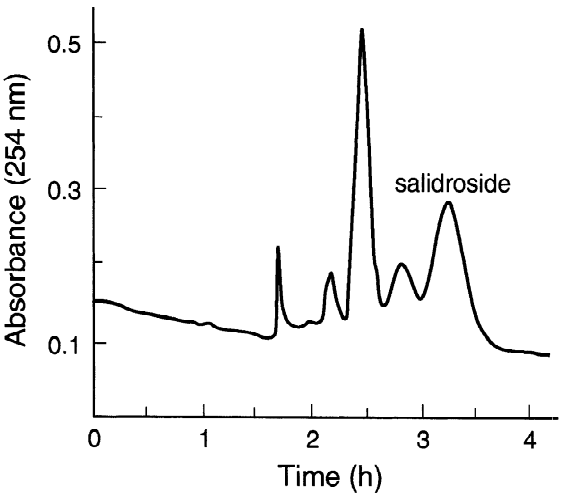

Fig. 3. Preparative HSCCC separation of peak c. The last peak contained salidroside (21.9 mg, 98% pure). Solvent system, chloroform–methanol–isopropanol–water (5:6:1:4, v/v); stationary phase, upper phase; mobile phase, lower phase; flow-rate, 2.0 ml/min; revolution speed, 800 rev./min; sample, 46.1 mg dissolved in the mixture of 1 ml of each phase; retention of the stationary phase, 30.4%.

^{1}H NMR, ^{13}C NMR, and MS data gave the structural identification: FAB-MS *m/z* 301 $[M+H]^{+}$; ^{1}H NMR (600 MHz, $C^{2}H_{3}O^{2}H$) δ ppm ^{1}H-NMR (600 MHz, $[^{2}H_{6}]$DMSO) δ ppm 7.060–6.676 4H H-2,3,5,6, 4.285–4.000 H-5′, 3.862–3.839 2H, H_{2}-6′, 3.699–3.640 (2H, H-3′,4′), 3.352–3.245 (H-2′), 3.185–2.808 (4H, H_{2}-7,H_{2}-8); ^{13}C-NMR (600 MHz, $C^{2}H_{3}O^{2}H$) δ ppm 156.8 C-1, 130.9 C-4, 130.7 (C-3, C-5), 116.1 (C-2, C-6), 104.4 (C-1′), 78.1 (C-5′), 77.9 (C-3′), 75.1 C-2′, 72.1 (C-8), 71.7 C-4′, 62.8 C-6′, 36.4 (C-7). Comparing the above with Ref. [6], the obtained product is confirmed as salidroside.

The results of our studies clearly show that HSCCC is a very useful tool in the preparative separation of salidroside, which yields a highly pure sample at a high recovery rate.

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