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Short communication

Preparative isolation and purification of acteoside and 2′-acetyl acteoside from *Cistanches salsa* (C.A. Mey.) G. Beck by high-speed counter-current chromatography

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Abstract

High-speed counter-current chromatography (HSCCC) was applied to the separation and purification of phenylethanoid glycosides (PhGs) acteoside and 2′-acetylacteoside from *Cistanches salsa* (C.A. Mey) G. Beck with a quaternary two-phase solvent system composed of ethyl acetate–*n*-butanol–ethanol–water (4:0.6:0.6:5, v/v). HPLC analyses of the CCC fractions revealed that the two main PhGs were over 98% purity. Their chemical structures were identified by ^{1}H NMR, ^{13}C NMR and MS.

Keywords: Counter-current chromatography; *Cistanches salsa*; Pharmaceutical analysis; Plant materials; Preparative chromatography; Acteoside; Acetylacteoside; Phenylethanoids; Glycosides

1. Introduction

Cistanches salsa (C.A. Mey) G. Beck, one species of *Cistanches* which belongs to *Orobanchaceae* family, is a parasitic plant native in the northwest of China. The stem of this plant is an important traditional Chinese medicine and used for kidney deficiency, female infertility, morbid leucorrhea, neurasthenia and senile constipation due to colonic inertia. The major active constituents of this herb are phenylethanoid glycosides (PhGs) [1–3]. The preparative separation and purification of PhGs from plant materials by classical methods is tedious and usually requires multiple chromatography steps on silica gel, polyamide, cellulose, Dianion HP-20, Sephadex columns, etc. Because of their phenolic hydroxyl groups, PhGs have a tendency of being strongly adsorbed onto the solid support during separation.

High-speed counter-current chromatography (HSCCC), being a support-free liquid–liquid partition chromatography, eliminates irreversible adsorp-

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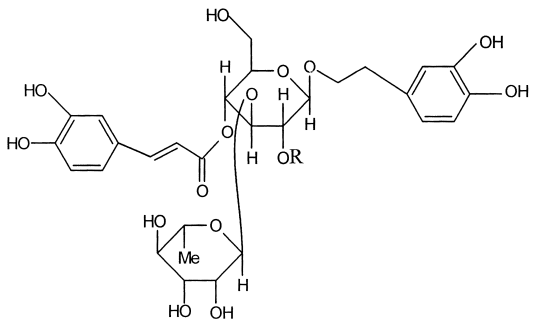
tion of sample onto the solid support [4], and has been widely used in preparative separation of natural products. The present paper describes the successful preparative separation and purification of acteoside and 2′-acetylacteoside, whose chemical structures are given in Fig. 1, from the stems of *C. salsa* (C.A. Mey) G. Beck by HSCCC.

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 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.



(1) Acteoside R=H

(2) 2'-Acetylacteoside R=Ac

Fig. 1. Chemical structures of acteoside and 2′-acetylacteoside from *C. salsa* (C.A. Mey) G. Beck.

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×1.6 mm I.D. with a total capacity of 230 ml. The β value ranges from 0.4 at the inner layer to 0.7 at the outer layer.

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 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 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, a 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.

2.3. Preparation of sample

Raw stems of *C. salsa* (C.A. Mey) G. Beck (ca. 8.0 kg) were percolated 8 times each with 10 l of

75% aqueous ethanol at room temperature, and the extract was concentrated to dryness. The residual was suspended in 5 l of water and extracted three times with 5 l water-saturated light petroleum (b.p. 60-90°C), ethyl acetate and *n*-butanol successively which yielded 105 g of light petroleum extract, 95 g of ethyl acetate extract and 210 g of *n*-butanol extract after being combined and evaporated to dryness under reduced pressure. Portions of the above *n*-butanol extract containing PhGs as major components were subjected to HSCCC.

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

The solvent systems utilized in the present study were prepared by mixing ethyl acetate–*n*-butanol–ethanol–water (4:0.6:0.6:5, 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 *n*-butanol extract of *C. salsa* (C.A. Mey.) G. Beck in the lower aqueous phase at suitable concentrations according to the analytical or preparative purpose.

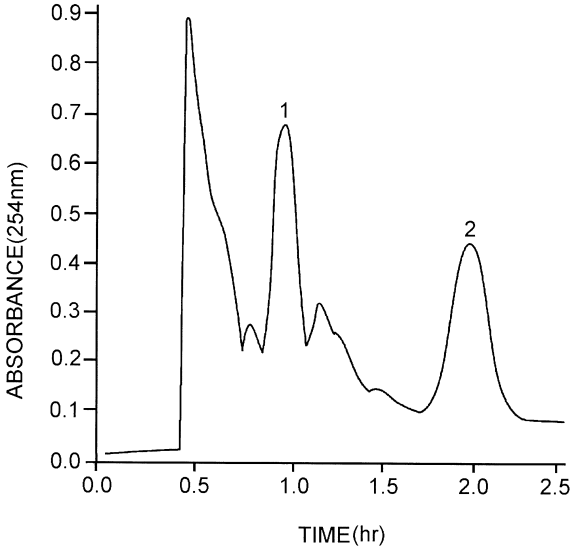


Fig. 2. Chromatogram of the crude *n*-butanol extract from *C. salsa* (C.A. Mey) G. Beck by analytical HSCCC. Solvent system: ethyl acetate–*n*-butanol–ethanol–water (4:0.6:0.6:5, v/v); stationary phase: upper organic phase; mobile phase: lower aqueous phase; flow-rate: 1.0 ml/min; revolution speed: 1800 rpm; sample size: 40 mg dissolved in 1 ml lower aqueous phase; retention of the stationary phase: about 35%. 1: acteoside; 2: 2′-acetylacteoside.

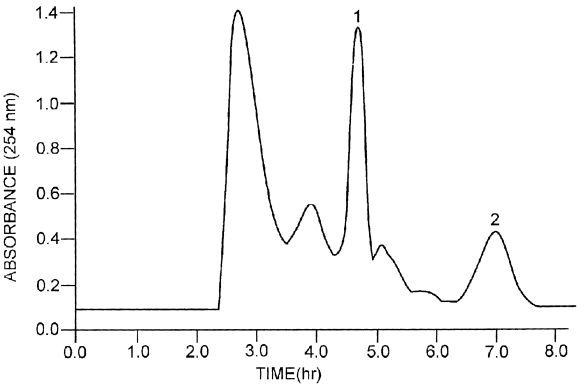


Fig. 3. Chromatogram of the crude *n*-butanol extract of *C. salsa* (C.A. Mey) G. Beck by preparative HSCCC. Solvent system: ethyl acetate–*n*-butanol–ethanol–water solvent system (4:0.6:0.6:5, v/v); stationary phase: upper organic phase; mobile phase: lower aqueous phase; flow-rate: 1.5 ml/min; revolution speed: 800 rpm; sample size: 200 mg dissolved in 5 ml aqueous phase; retention of the stationary phase: 26%. 1: acteoside; 2: 2′-acetylacteoside.

2.5. Separation procedure

Analytical HSCCC was performed with a Model GS 20 HSCCC instrument 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

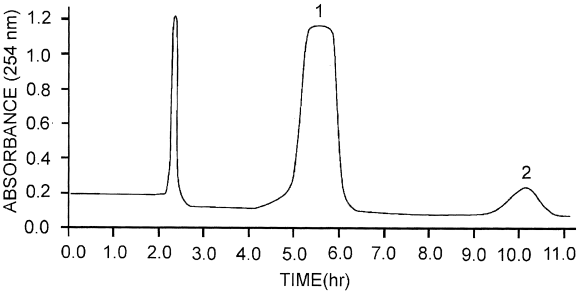


Fig. 4. Chromatogram of combined peak fractions of acteoside and 2′-acetylacteoside in Fig. 3 by preparative HSCCC. HSCCC conditions are the same as those in Fig. 3. Retention of the stationary phase: 26%. 1: acteoside; 2: 2′-acetylacteoside.

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 (40 mg in 1 ml of lower aqueous 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. 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 instrument as follows: the multilayer coiled column was first entirely filled with the upper organic phase as stationary phase. Then the lower aqueous phase was pumped into the head end of the column at a flow-rate of 1.5 ml/min, while the apparatus was rotated at 800 rpm. After hydrodynamic equilibrium was reached, as indicated by a

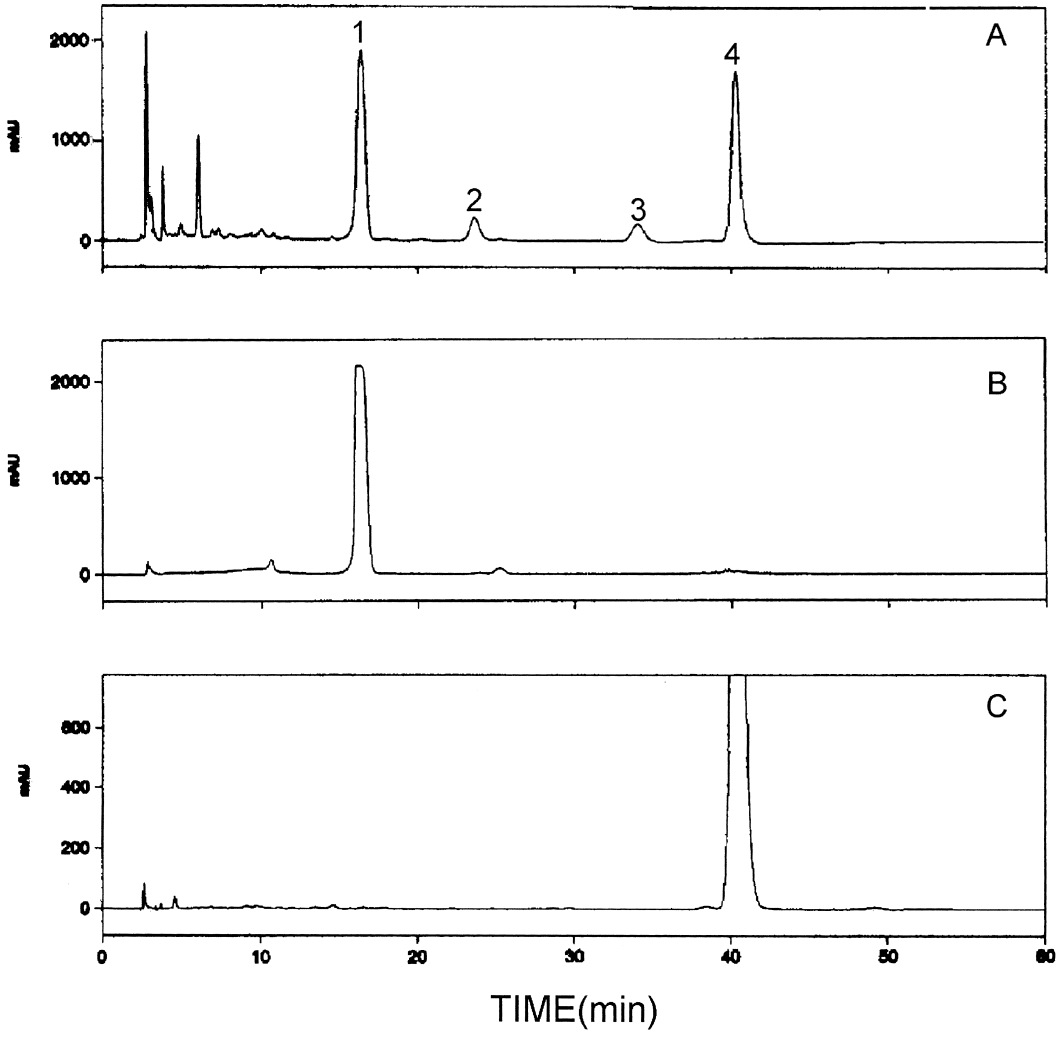


Fig. 5. The result of HPLC analyses of the crude *n*-butanol extract from *C. salsa* (C.A. Mey) G. Beck and repurified CCC fractions shown in Fig. 4. (A) Crude sample; (B) Peak 1; (C) Peak 2. Column: Phenomenex ODS column (250 mm×4.6 mm I.D.); column temperature: 40°C; mobile phase: acetonitrile–1% aqueous acetic acid (18:82, v/v); flow-rate: 1.0 ml/min. 1: acteoside; 2: unknown 1; 3: unknown 2; 4: 2′-acetylacteoside.

clear mobile phase eluting at the tail outlet, the sample solution was injected through the injection valve. The effluent from the outlet of the column was continuously monitored with a UV detector at 254 nm. Each peak fraction was collected according to the chromatogram.

2.6. HPLC analyses and identification of CCC peak fractions

The crude *n*-butanol extract of *Cistanches salsa* (C.A. Mey) G. Beck and each peak fraction from HSCCC were analyzed by HPLC. The analyses were performed with a Phenomenex ODS column (250× 4.6 mm I.D.) at a column temperature of 40°C. The mobile phase composed of acetonitrile–1% aqueous acetic acid (18:82, v/v) was isocratically eluted at a flow-rate of 1.0 ml/min and the effluent monitored by a DAD.

Identification of HSCCC peak fractions was carried out by MS, ^{1}H NMR and ^{13}C NMR spectra.

3. Results and discussion

The crude *n*-butanol extract of *C. salsa* (C.A. Mey) G. Beck was first analyzed by HPLC. The result indicates that the crude sample contains four compounds including acteoside, 2′-acetylacteoside and two unknown compounds in small amounts (see Fig. 5A).

In order to achieve an efficient resolution of target compounds, the two-phase solvent system of EtOAc–*n*-BuOH–EtOH–water was examined using analytical HSCCC by varying the mutual volume ratios. The results indicated that the volume ratio of 4:0.6:0.6:5 could separate acteoside and 2′-acetylacteoside well (Fig. 2).

Fig. 3 shows the result obtained from 228 mg of the crude *n*-butanol extract of *C. salsa* (C.A. Mey) G. Beck by preparative HSCCC. After this separation, the fractions containing acteoside and 2′-acetylacteoside were collected, combined and separated again with the same solvent system (Fig. 4). The second separation yielded fractions each at over 98% purity as determined by HPLC (Fig. 5B and C). The structural identification of these fractions was carried out by MS, ^{1}H NMR and ^{13}C NMR spectra (Fig. 1). The results of our studies clearly demonstrated that HSCCC is very successful in the preparative separation of acteoside and 2′-acetylacteoside from the crude *n*-butanol extract of *C. salsa* (C.A. Mey) G. Beck.

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