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Short communication

Preparative isolation of osthol and xanthotoxol from Common Cnidium Fruit (Chinese traditional herb) using stepwise elution by high-speed counter-current chromatography

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Abstract

Preparative high-speed counter-current chromatography (HSCCC) was successfully used for isolation and purification of osthol and xanthotoxol from *Cnidium monnieri* (L.) *Cusson* (Common Cnidium Fruit) using stepwise elution with a pair of two-phase solvent systems composed of *n*-hexane–ethyl acetate–methanol–water at (1:1:1:1, v/v), and (5:5:6:4, v/v), which had been selected by analytical high-speed counter-current chromatography. Using a preparative unit of the HSCCC centrifuge, about a 308 mg amount of the crude extract was separated, yielding 88.3 mg of osthol and 19.4 mg of xanthotoxol at a high purity of over 98%.

Keywords: Counter-current chromatography; Preparative chromatography; Pharmaceutical analysis; *Cnidium monnieri*; Osthol; Xanthotoxol

1. Introduction

Common Cnidium Fruit is a useful traditional Chinese herb: It can be used to various purposes such as drying dampness and killing worms, expel lingcold and the wind, relieving asthma and increasing sperm secretin. It contains coumarins which are known to have a variety of bioactivities including anticoagulant, estrogenic, dermal photosensitizing, antimicrobial, vasodilator, molluscacidal, antithelmintic, sedative and hypnotic, analgesic and hypothermic activities. Pharmacological tests revealed that coumarin derivatives improve ocular blood flow, particularly in the choroids and retina [1]. Osthol is a selective antiproliferative agent in vascular smooth muscle cells [2], and causes hypotension in vivo, inhibits platelet aggregation and smooth muscle contraction in vitro. Also it may interfere with calcium influx and with cyclic nucleotide phosphodiesterases [3]. Osthol also can prevent anti-Fas antibody-induced hepatitis by inhibiting the Fas-mediated apoptotic pathway [4].

The separation of these active compounds from natural sources, however, may encounter various problems. 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 [5], and has been widely used in preparative separation of natural products [6,7]. The present paper describes the successful preparative separation and purification of osthol and xanthotoxol from the crude extract of Common Cnidium Fruit by stepwise high-speed counter-current chromatography.

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

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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. PTFE (polytetrafluoroethylene) 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.

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 value of the preparative column ranged 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 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 10 ml loop (for the preparative HSCCC) (Tianjin High-New Science & Technology Co., 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 plot the chromatogram. A rotary evaporator (Model RE-90, Beijing Institute of New Technology Application) 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). Methanol used for HPLC analysis was of chromatographic grade and purchased from Tianjin Huaxi Special Reagent Factory (Tianjin, China). The crude extract of Common Cnidium Fruit was purchased from Guilin Natural Ingredients (Guilin, China).

2.3. 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 (1:1:1:1, v/v) or (5:5:6:4, v/v), and thoroughly equilibrating the mixture 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.

2.4. 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 phase. The lower 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 1800 rpm. After hydrodynamic equilibrium was established, as indicated by a clear mobile phase eluting at the tail outlet, the sample solution (8 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.

Preparative HSCCC was performed with a Model GS10A2 HSCCC instrument as follows: the multilayer coiled column was first entirely filled with the upper phase

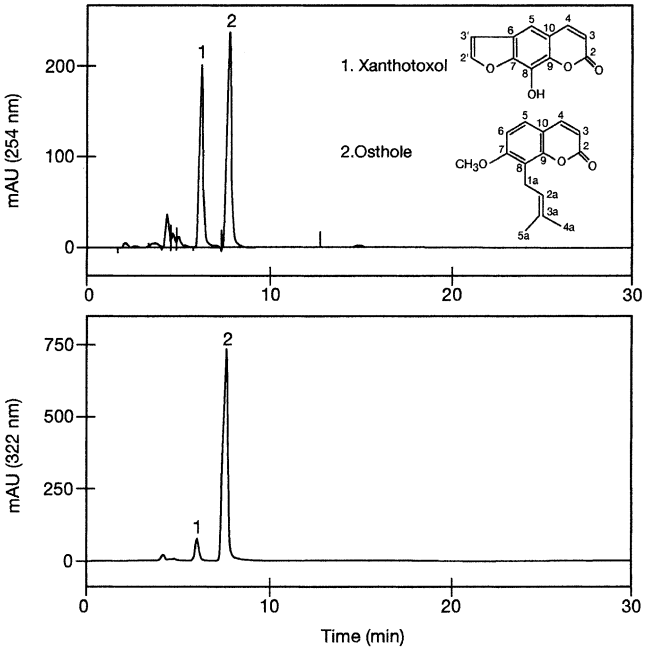

Fig. 1. HPLC analyses of the crude extract of Common Cnidium Fruit. HPLC conditions: a Polaris ODS column (250 mm × 4.6 mm i.d.), column temperature: 35 °C. Mobile phase: methanol–water (75:25, v/v), flow-rate: 1.0 ml/min, monitored at 254 and 322 nm by DAD. Peaks: 1 = xanthotoxol, 2 = osthol.

as stationary phase. Then the lower phase of system 1 was pumped into the head end of the inlet column at a flow-rate of 2 ml/min, while the apparatus was rotated at 800 rpm. After hydrodynamic equilibrium was reached, as indicated by a clear mobile phase eluting at the tail outlet, the sample solution (308 mg in 10 ml of lower phase of solvent system 1) was injected through the injection valve. In the stepwise elution, after certain time of elution using the first solvent system, the mobile phase was switched to the lower phase of the second solvent. 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.

2.5. HPLC analyses and identification of HSCCC peak fractions

The crude extract of Common Cnidium Fruit and HSCCC peak fractions were each analyzed by HPLC. The analyses were performed with a Polaris ODS column (250 mm ×

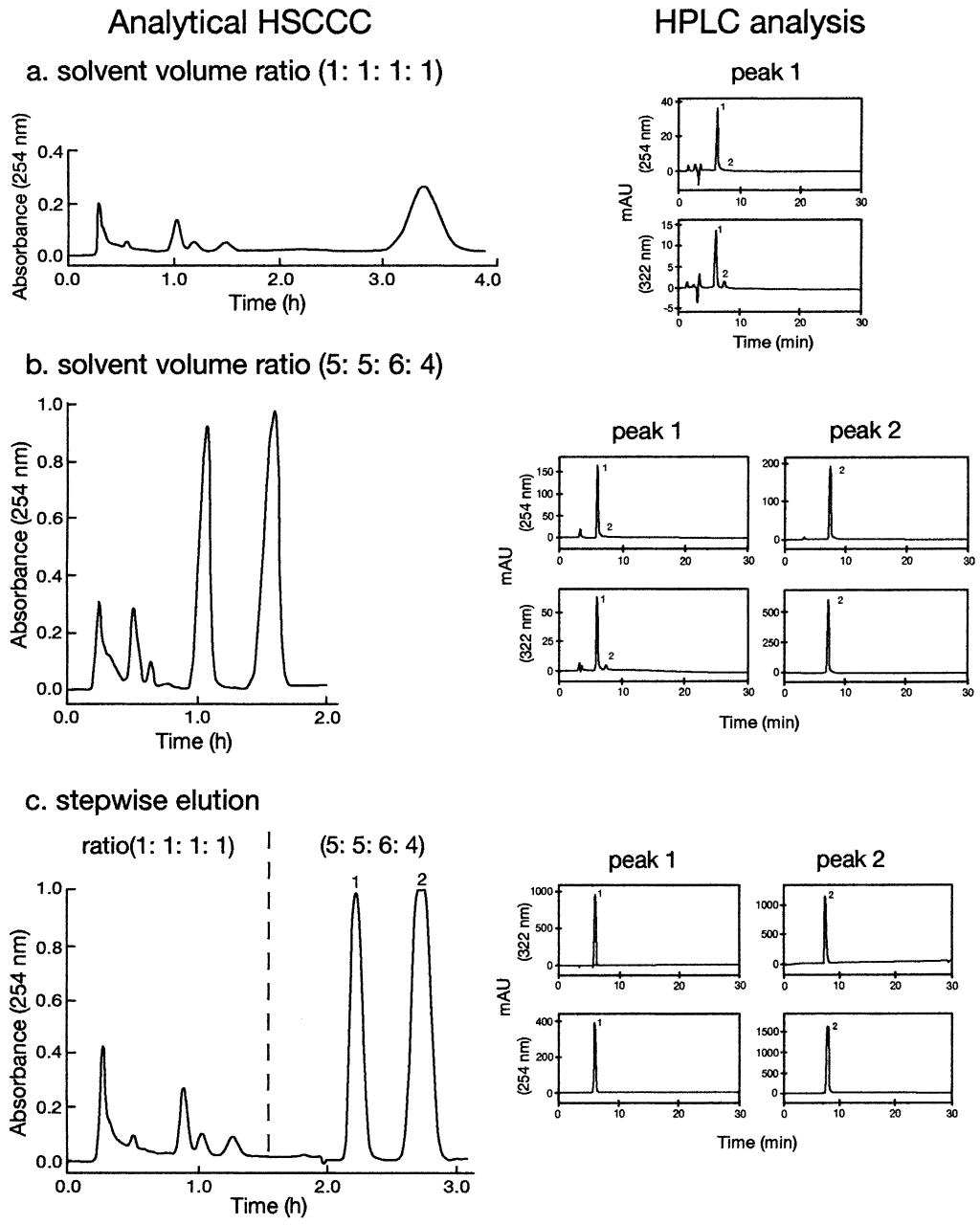

Fig. 2. Optimization of stepwise elution mode using analytical HSCCC for separation of the crude extract of Common Cnidium Fruit. Solvent system: *n*-hexane–ethyl acetate–methanol–water (1:1:1:1, v/v) (a), (5:5:6:4, v/v) (b), and both systems in stepwise elution (c); stationary phase: organic phase; mobile phase: lower aqueous phase; flow-rate: 1.0 ml/min; revolution speed: 1800 rpm; sample: 8 mg dissolved in 1.0 ml lower phase. HPLC conditions: a Polaris ODS column (250 mm × 4.6 mm i.d.), column temperature: 35°C. Mobile phase: methanol–water (75:25, v/v), flow-rate: 1.0 ml/min, monitored at 254 and 322 nm by DAD. Peaks: 1 = xanthotoxol, peak 2 = osthol.

4.6 mm i.d.) at column temperature of 35 °C. The mobile phase composed of methanol–water (75:25, v/v) was isocratically eluted at a flow-rate of 1.0 ml/min and the effluent monitored at 254 and 322 nm by DAD.

Identification of the target compounds (osthol and xanthotoxol) was based on MS, ^{1}H NMR; and ^{13}C NMR spectra.

3. Results and discussion

As shown in Fig. 1, the HPLC analysis of the crude extract of Common Cnidium Fruit shows several compounds where the purity of osthol in crude extract is 45% based on HPLC peak area percentage.

In order to achieve an efficient resolution of target compounds, a two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water was examined using analytical HSCCC by varying the mutual volume ratio, since this solvent system has been successfully applied to various samples with a moderate degree of polarity. The results are illustrated in Fig. 2a–c.

As seen in Fig. 2a, the separation using solvent ratio (1:1:1:1) purified xanthotoxol (peak 1) from polar impurities while osthol is long retained in the column. On the other hand, the use of the solvent ratio (5:5:6:4) completely separated two target compounds in a short elution time, while xanthotoxol (peak 1) was contaminated with unknown impurities as shown in Fig. 2b. These results suggest that

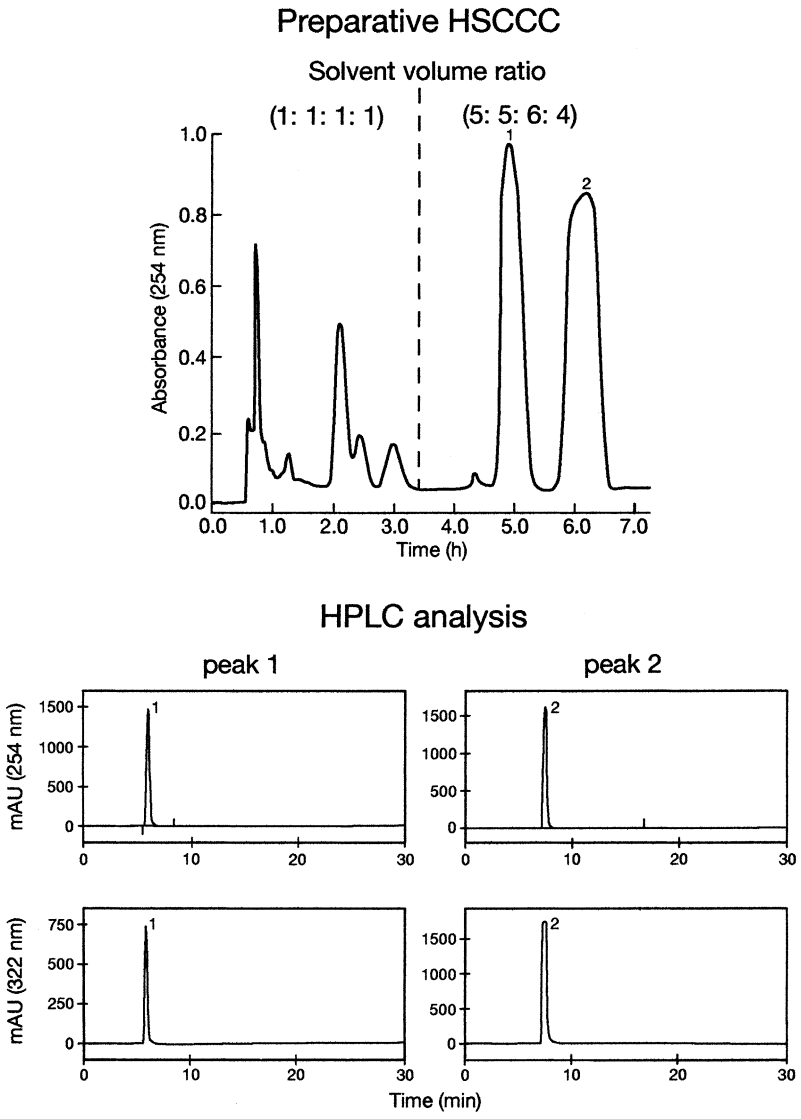

Fig. 3. Preparative HSCCC separation of the crude extract of Common Cnidium Fruit using stepwise elution with solvent systems 1 and 2. Solvent system 1: *n*-hexane–ethyl acetate–methanol–water (1:1:1:1, v/v), solvent system 2: *n*-hexane–ethyl acetate–methanol–water (5:5:6:4, v/v); stationary phase: upper organic phase of solvent system 1; mobile phase: 400 ml lower aqueous phase of solvent system 1 and 420 ml lower aqueous phase of solvent system 2; flow-rate: 1.0 ml/min; revolution speed: 800 rpm; sample: 308 mg dissolved in 10 ml lower phase of solvent system 1. HPLC conditions: a Polaris ODS column (250 mm × 4.6 mm i.d.), column temperature: 35 °C. Mobile phase: methanol–water (75:25, v/v), flow-rate: 1.0 ml/min, monitored at 254 and 322 nm by DAD. Peaks: 1 = xanthotoxol, 2 = osthol.

the combined use of these two solvents in stepwise elution would provide an excellent purification of these two target compounds. This strategy was successfully demonstrated in Fig. 2c where the crude extract was first eluted with the solvent system with a volume ratio at 1:1:1:1 until the polar impurities were eluted out (see dotted line in Fig. 2c) followed by the elution with the second solvent system with a volume ratio at 5:5:6:4. In this way these two components were purified within 3 h as shown in Fig. 2c.

This stepwise elution was applied for the preparative separation of 308 mg of the crude extract of Common Cnidium Fruit. As shown in Fig. 3, the separation was started with the solvent system 1 (1:1:1:1) and, after most of the polar impurities were eluted (3 h and 20 min at the dotted line in Fig. 3), the mobile phase was switched to the lower phase of solvent system 2 (5:5:6:4). Then, xanthotoxol (peak 1) and osthol (peak 2) were well resolved and eluted in less than 7 h. This separation yielded 88.3 mg of osthol and 19.4 mg of xanthotoxol at a high purity of over 98% based on HPLC analysis.

The structural identification of osthol and xanthotoxol was carried out by MS, ^{1}H NMR and ^{13}C NMR spectra as follows: the electron impact (EI) MS: *m*/*z* 244, 229, 213, 201, 189, 175, 159, 139, 131, 115, 103, 79, 63, 51. It showed the molecular ion at *m*/*z* 244, which is in agreement with the molecular formula $C_{15}H_8O_6$ of osthol. The EI-MS: *m*/*z* 202, 174, 157, 146, 118, 101, 89, 63, 51, 45. It showed the molecular ion at *m*/*z* 202, which is in agreement with the molecular formula $C_{11}H_6O_4$ of xanthotoxol [8].

Osthol: ^{1}H NMR (500 MHz, $C^{2}HCl_3$) δ ppm: 6.162 (3C–H), 7.565 (4C–H), 7.240 (5C–H), 6.790 (6C–H), 3.476 (1aC–H), 5.197 (2aC–H), 1.793 (CH_3–H), 3.865 (OCH_3–H) [9].

Osthol: ^{13}C NMR (500 MHz, $C^{2}HCl_3$) δ ppm: 160.148 (2C), 112.706 (3C), 143.797 (4C), 126.250 (5C), 117.709 (6C), 161.322 (7C), 107.338 (8C), 152.685 (9C), 112.869 (10C), 21.837 (1aC), 121.123 (2aC), 132.444 (3aC), 17.836 (4aC), 25.693 (5aC), 55.962 (OCH_3–C) [10].

Xanthotoxol: ^{1}H NMR [500 MHz, dimethyl sulfoxide (DMSO)] δ ppm: 6.405 (3C–H), 8.087 (4C–H), 7.072 (5C–H), 7.077 (2′C–H), 6.425 (3′C–H).

Xanthotoxol: ^{13}C NMR (500 MHz, DMSO) δ ppm: 159.825 (2C), 114.203 (3C), 145.345 (4C), 125.723 (5C), 114.127 (6C), 147.832 (7C), 130.560 (8C), 143.209 (9C), 116.385 (10C), 146.433 (2′C), 107.093 (3′C). The results were similar to those in refs. [10,11].

Here it is worthwhile to discuss the advantage of the above stepwise elution applied in the present studies. As shown in Fig. 2a, one of target peaks with a much greater partition coefficient will be retained in the column for a long period of time. In this case the chromatographic condition should be changed to facilitate elution of the retained peaks without loss of peak resolution. In fact, the stepwise elution in counter-current chromatography is an efficient way to achieve this goal. However, this method needs a careful choice of the second mobile phase so that the volume of the stationary phase in the column should not be significantly altered. This can be easily examined by mixing the stationary phase of the first solvent system with the mobile phase of the second solvent system in a small graduated cylinder.

The results of our studies demonstrated that stepwise counter-current chromatography is a useful method for the preparative separation of osthol and xanthotoxol from a crude extract of Common Cnidium Fruit.

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