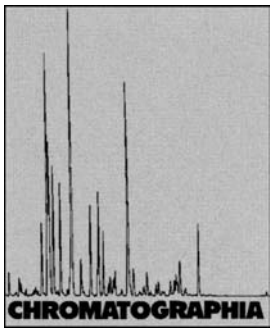
Large-Scale Isolation and Purification of Scoparone from *Herba artemisiae scopariae* by High-Speed Counter-Current Chromatography



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Abstract

A preparative high-speed counter-current chromatography with a two-phase solvent system composed of n-hexane-ethyl acetate-methanol- water (1:1:0.45:1.55, *v/v/v/v*) was successfully performed to isolate scoparone (6,7-dimethoxycoumarin, 6,7-dimethylesculetin, 6,7-DME) from the plant of Herba artemisiae scopariae, a traditional Chinese medicine. 233.5 mg Scoparone with the purity of 96.8% (determined by HPLC) was obtained in one-step elution from 800 mg crude extract. The recovery of scoparone was 91.8%, and the chemical structure of this compound was identified by IR, MS, ^{1}H NMR and ^{13}C NMR spectrum.

Keywords

Column liquid chromatography
Counter-current chromatography
Coumarin compounds
Scoparone
Herba artemisiae scopariae
Artemisia capillaries and Artemisia scoparia

Introduction

Herba artemisiae scopariae (*Yinchen* in Chinese), the dried aerial part of *Artemisia capillaries* Thunb (Compositae) or *Artemisia scoparia* Waldst. et Kit. (Compositae), is a commonly used traditional Chinese medicine listed in the Chinese Pharmacopoeia and used to remove damp-heat and relieve jaundice [1].

Scoparone (Fig. 1) is one of the major coumarins in *Herba artemisiae scopariae*. Modern pharmacological research showed that this compound possesses the choleretic activity through increasing the bile secretion [2, 3], the marked inhibitory activity as a competitive antagonist of norepinephrine on the contractions induced by norepinephrine, 5-hydroxytryptamine, histamine and angiotensin II [4], the potent inhibitory activity on rabbit platelet aggregation induced by four types of agent, ADP, PAF, sodium arachidonate and collagen [5], and the inhibitory activity on the expression of chemokines (IL-8 and MCP-1) in PMA-stimulated U937 cells to treat hepatitis and biliary tract infection in oriental countries [6]. It also has the ability to reduce the proliferative responses of human peripheral mononuclear cells, relax smooth muscle, reduce total cholesterol and triglycerides, retard the characteristic pathomorphological changes in hypercholesterolaemic diabetic rabbits, scavenge reactive oxygen species and inhibit tyrosine kinases and potentiation of prostaglandin generation [7–9]. In view of these benefits, much pure product was required for further pharmacological or clinical research. However, coumarins are often isolated by some conventional methods such as silica gel, polyamide, which require several steps, time consuming and low recoveries of the products. High-speed counter-current chromatography (HSCCC), a support free liquid–liquid partition chromatographic technique, can successfully eliminate irreversible adsorption of the sample onto solid support [10], and was widely used for separation and purification of various natural and synthetic products with an excellent sample recovery. Regrettably, the sample consumption (crude extract) of these experiments was often not above 300 mg [11–14].

The aim of this study was to develop an efficient method for the large-scale isolation and purification of scoparone with high purity from *Herba artemisiae scopariae* by HSCCC. As far as we know, there are no previous scientific literature reports of using HSCCC to isolate and purify scoparone from the plant of *Herba artemisiae scopariae*.

Experimental

Apparatus

Preparative HSCCC was carried out with a model TBE-300A HSCCC (Shenzhen,

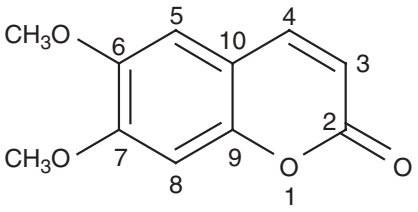

Fig. 1. Structure formula of Scoparone

Tauto Biotech, China). The apparatus was equipped with a polytetrafluoroethylene, three preparative coils (diameter of tube, 2.6 mm, total volume, 300 mL) and a 20 mL sample loop. The revolution radius or the distance between the holder axis and central axis of the centrifuge (R) was 5 cm, and the β value varied from 0.5 at the internal terminal to 0.8 at the external terminal ($\beta = r/R$ where r is the distance from the coil to the holder shaft). The HSCCC system was equipped with a model S constant-flow pump, a model UV-II detector operating at 254 nm, and a model N2010 workstation (Zhejiang University, Hangzhou, China). The experimental temperature was adjusted by HX 1050 constant temperature circulating implement (Beijing Boyikang Lab Implement, Beijing, China).

Reagents

n-Hexane, ethyl acetate, methanol were analytical grade and purchased from WuLian Chemical Factory, Shanghai, China. Acetonitrile was HPLC grade (Merck, Germany). Reverse osmosis Milli-Q water (18 MΩ) (Millipore, USA) was used for all solutions and dilutions.

Herba artemisiae scopariae was purchased from Shanghai Yanghetang Traditional Chinese Medicine Co., LTD. Shanghai, P. R. China, in July 2005 and identified by Professor C.G. Huang of Shanghai Institute of Materia Medica, Chinese Acadamy of Sciences (CAS). Their voucher specimens (YC-050714) were deposited at the Herbarium of Shanghai Institute of Materia Medica, CAS. D101 macroporous resin (outer appearance: cream white opaque ball pellet resin; grain length: (0.3–1.25 mm) ≥90%; moisture: 65–75%; moisture looking density: 0.65–0.75 g/ml; comparing specific surface area: 500–550 m^2/g; even hole diameter: 90–100 Å; polar nature: non-polarity) was purchased from Shanghai Institute of Pharmaceutical Industry (Shanghai, China).

Preparation of the Crude Extract

300 g of the *Herba artemisiae scopariae* was added to a bottle and extracted by reflux with 1500 mL volume of 95% aqueous ethanol for 2 h. The mixture was filtered, and the filtrate was collected. The extract was then concentrated to dryness by rotary vaporization at 60 °C under reduced pressure to give 110 g residue which was divided into three subfraction A1-A3 by D101 macroporous resin (200 g, 40 × 320 mm) eluted with ethanol-water (10:90, 30:70, 100:0, each 2500 mL). Subfraction A2 (4.2 g) was subjected to HSCCC separation.

Determination of Partition Coefficient (*K*) value

The composition of the two-phase solvent system was selected according to the partition coefficient (K) of the target compound in the crude example. The partition coefficients were determined by HPLC as described before [15]. Briefly, approximately 2 mg of the crude extract was weighed in a 10 mL test tube to which 4.0 mL of each phase of the equilibrated two-phase solvent system was added. The tube was shaken vigorously for 2 min to equilibrate the sample thoroughly with the two phases. Then the two-phases were separated and evaporated to dryness under reduced pressure. The residue was diluted with the mobile phase used in the HPLC analysis and then analyzed by HPLC. The partition coefficient (K) value was expressed as the peak area of target component in the upper phase divided by that in the lower phase.

Preparation of Two-Phase Solvent System and Sample Solution

Two-phase solvent systems were used in the present study. *n*-Hexane - ethyl acetate–methanol–water (1:1:0.45:1.55, *v*/*v*/*v*/*v*) was prepared. The solvent mixture was thoroughly equilibrated in a separated funnel at room temperature and the two phases were separated shortly before use. The sample solution was prepared by dissolving the sample (800 mg) in the equal volume of the two phases (total 20 mL) of solvent system for isolation and purification.

HSCCC Separation Procedure

In the crude sample isolation and separation, the coil column was first entirely filled with the upper phase of the solvent system. Then the apparatus was rotated at 800 rpm, while the lower phase was pumped into the column at a flow rate of 1.2 mL min^{-1}. After the mobile phase front emerged and hydrodynamic equilibrium was established in the column, 20 mL sample solution containing 800 mg of the crude extract was injected through the injection value. The effluent of the column was continuously monitored with a UV–vis detector at 254 nm. Peak fractions were collected according to the elution profile.

HPLC Analysis and Identification of Scoparone

The analytical HPLC system used throughout this study consisted of LC–10AT pump (Shimadzu, Japan), a SPD–10A UV–vis detector (Shimadzu, Japan), and a model N2000 workstation (Zhejiang University, Hangzhou, China). The crude sample and the peak fraction obtained by HSCCC were analyzed by HPLC with a 20 μL sample loop. The column used was a Diamonsil C_{18} (4.6 mm × 200 mm i.d. 5 ìm) (Dikma Technologies) with a precolumn equipped with the same stationary phase. The mobile phase was acetonitrile–water (25:75, *v*/*v*). The flow rate was 1.0 mL min^{-1}, the effluent was monitored at 254 nm and the column temperature was set at 30 °C.

The identification of the target compound scoparone was carried out as follows: Melting points were determined using a SGW X-4 melting point apparatus (Shanghai Precision & Scientific Instrument Co., LTD) and uncorrected. IR spectra were obtained on a Perkin Elmer 577 spectrometer (KBr disc). Mass spectra were attained on Finngen-LCQ^{DECA} mass spectrometer (ESIMS). UV spectra were measured on a Shimadzu UV-3000 spectrophotometer. NMR spectra were recorded on Bruker DRX-400 spectrometers with TMS as internal standard.

Results and Discussion

Optimization of HPLC Method

The crude extract and the fractions obtained by HSCCC were determined by

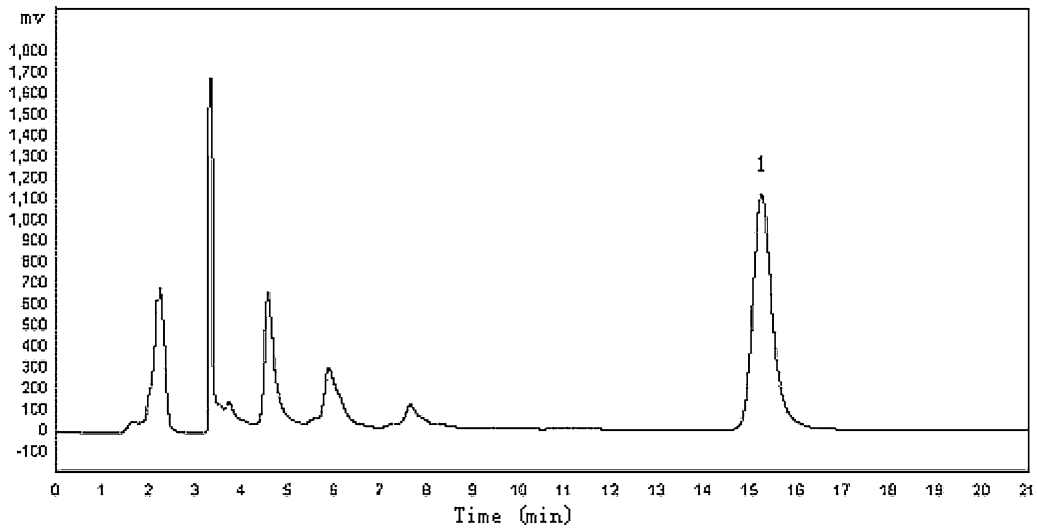

Fig. 2. HPLC chromatography of the crude extract from *Herba artemisiae scopariae*. HPLC analysis conditions: column: reversed-phase Diamonsil C_{18} (4.6 mm × 200 mm i.d. 5 μm); mobile phase: acetonitrile–water (25:75, *v*/*v*); flow rate: 1.0 mL min^{-1}; UV wavelength: 254 nm; injection solvent: 20 μL; peak 1 corresponds to scoparone

HPLC. Different mobile phase (methanol–water, acetonitrile–water), flow rate, column temperature and detection wavelength were all tested. The result indicated that acetonitrile–water at a ratio of 25:75 (*v*/*v*), a flow rate of 1.0 mL min^{-1}, the column temperature of 25 °C and detection wavelength of 254 nm was the best operated condition. No complex gradient of mobile phase and no buffer were necessary. The HPLC chromatogram of the crude extract is given in Fig. 2. Peak 1 corresponds to scoparone, which presented at the content of 31.8%.

Selection of Two-Phase Solvent System and Other Conditions of HSCCC

In HSCCC separation, a suitable two-phase solvent system was critical for a successful isolation and purification. In our experiment, several kinds of solvent systems were evaluated and the partition coefficients were measured and listed in Table 1. According to the *K* values, the result indicated that the solvent systems composed of ethyl acetate-water, ethyl acetate-methanol-water, hexane-ethyl acetate-methanol-water at the ratios of 1:1(*v*/*v*), 8:3:8 (*v*/*v*/*v*) and 1:1:0.30:1.70 (*v*/*v*/*v*/*v*), respectively, had large *K* values and more time will be required to elute the target compound, while hexane-ethyl acetate-methanol-water at the ratio of 1:1:0.85:1.15 (*v*/*v*/*v*/*v*) had a small *K* values which attributed to poor resolution. The other three solvent systems hexane-ethyl acetate-methanol-water at the ratios of 1:1:0.60:1.40 (*v*/*v*/*v*/*v*), 1:1:0.525:1.475 (*v*/*v*/*v*/*v*) and 1:1:0.45:1.55 (*v*/*v*/*v*/*v*) had suitable *K* values.

Table 1. The partition coefficients (*K*) of scoparone in different solvent systems

Solvent system	Partition coefficients (*K*)
Ethyl acetate-water 1:1	4.23
Ethyl acetate-methanol-water 8:3:8	3.37
n-Hexane-ethyl acetate-methanol-water 1:1:0.85:1.15	0.60
n-Hexane-ethyl acetate-methanol-water 1:1:0.60:1.40	1.51
n-Hexane-ethyl acetate-methanol-water 1:1:0.525:1.475	1.65
n-Hexane-ethyl acetate-methanol-water 1:1:0.45:1.55	1.89
n-Hexane-ethyl acetate-methanol-water 1:1:0.30:1.70	3.25

The influence of flow rate of mobile phase, the separation temperature and the revolution speed were also investigated. The result indicated that slow flow speed can produce a good separation, but more time and more mobile phase will be needed. Considering the large-scale sample consumption, the flow rate was selected 1.2 mL min^{-1} in the present study. The temperature has significant effect on *K* values, the retention of stationary phase and the mutual solvency of the two phases. After tested at 15, 20, 25, 30, 35 and 40 °C, it can be seen that good result can be obtained when the separation temperature was controlled at 25 °C. The revolution speed has a great influence to the retention of stationary phase. High rotary speed can increase the retention of the stationary phase. In our experiment, the revolution speed was set at 800 rpm.

Under above conditions, three solvent systems with suitable *K* values were evaluated by large-scale HSCCC separation in terms of peak resolution. When hexane-ethyl acetate-methanol-water (1:1:0.60:1.40 or 1:1:0.525:1.475, *v*/*v*/*v*/*v*) was used, scoparone could not be well separated from the extract and their purities were 87.5% and 92.1%, respectively, indicating it was not suitable for the high-purity separation. At last, the two-phase solvent system composed of hexane-ethyl acetate-methanol-water (1:1:0.45:1.55, *v*/*v*/*v*/*v*) was found to be satisfactory for the separation of scoparone with a purity of 96.8% determined by HPLC (Fig. 3) from the partially purified extract within 511 min (800 mg sample consumption). Fig. 4 showed the separation by HSCCC using this solvent system (peak I corresponds to scoparone). The recovery of scoparone was 91.8%.

The Structural Identification

Scoparone: pale yellow needles (CH_3OH); mp 146 ~ 147 °C (lit. [16] 147 °C); ESIMS (positive mode) m/z: 207 $[M + H]^+$; IRν_{max} cm^{-1}: 1717, 1711, 1644, 1571, 1460, 1411, 1279, 825; UV (CH_3OH) λ_{max} nm (logε) 345 (3.39), 295

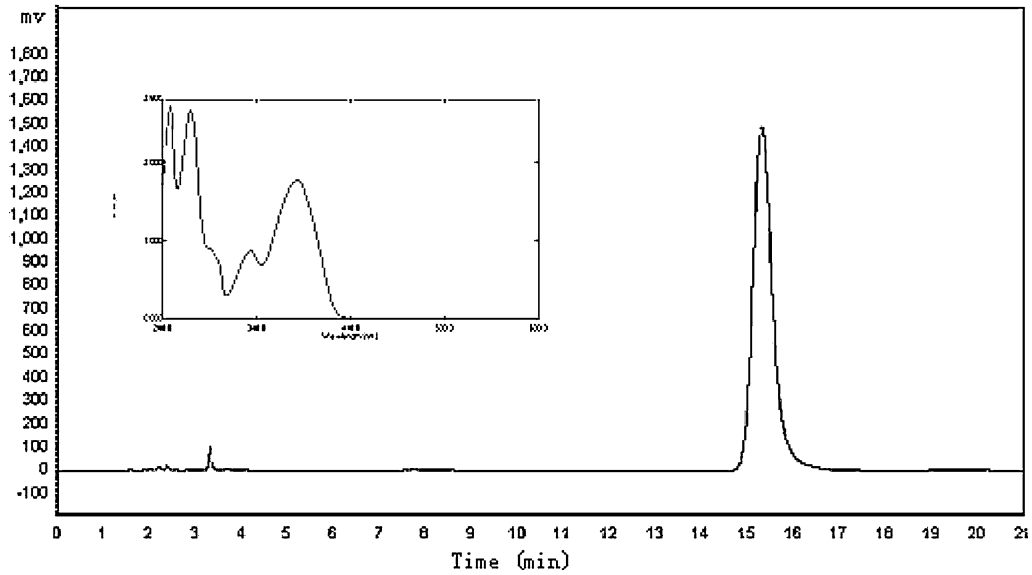

Fig. 3. HPLC chromatography of the fraction I obtained by HSCCC. HPLC analysis conditions and the peaks are the same as Fig. 2

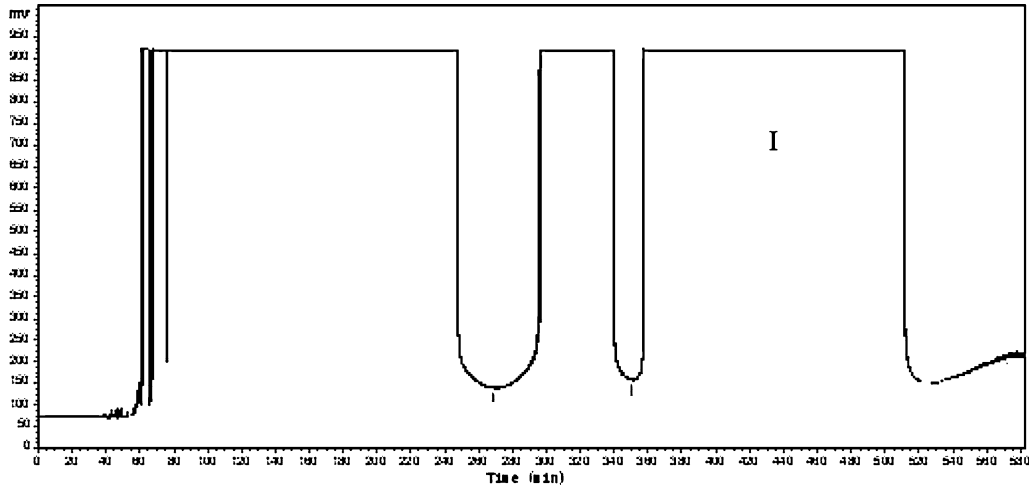

Fig. 4. Chromatogram of the crude extract by preparative HSCCC. Conditions: hexane-ethyl acetate–mathanol–water (1:1:0.45:1.55, *v*/*v*/*v*/*v*); stationary phase: upper phase; mobile phase: lower phase; revolution speed: 800 rpm; sample size: 800 mg; sample loop: 20 mL; detection wavelength: 254 nm. Flow rate: 1.2 mL min^{-1}, retention of stationary phase: 66.7%

(3.61), 235 (4.09); ^{1}H-NMR ($CDCl_3$,400 MHz): δ = 7.65 (1H, d, J = 9.6 Hz, H-4), 6.88 (1H, s, H-5), 6.83 (1H, s, H-8), 6.28 (1H, d, J = 9.6 Hz, H-3), 3.96, 3.94 (each 3H, s, CH_3O-); ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 161.7 (C-2), 153.0 (C-7), 150.2 (C-6), 146.6 (C-9), 143.6 (C-4), 113.7 (C-10), 111.7 (C-3), 108.2 (C-5), 100.2 (C-8), 56.6 (CH_3O-), 56.6 (CH_3O-); The UV and NMR data of this compound were in agreement with those reported [16,17].

Conclusion

Our study demonstrates that HSCCC is a powerful method in separating and isolating of scoparone at high purity from *Herba artemisiae scopariae* with a two-phase solvent system composed of hexane-ethyl acetate–mathanol–water (1:1:0.45:1.55, *v*/*v*/*v*/*v*). 233.5 mg scoparone of high purity was obtained from 800 mg crude extract in a one-step isolation. The method is simple, fast and without complex solvent system or gradient elution.

Acknowledgement

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