Separation and Purification Technology 72 (2010) 406–409

Contents lists available at ScienceDirect

Separation and Purification Technology

journal homepage: www.elsevier.com/locate/seppur

Short communication

High-speed counter-current chromatography preparative separation and purification of phloretin from apple tree bark

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ARTICLE INFO

Article history:
Received 20 November 2009
Received in revised form 19 February 2010
Accepted 19 February 2010

Keywords:
Phloretin
High-speed counter-current chromatography
Apple tree bark
Separation

ABSTRACT

Phloretin is one of the main polyphenols found in apple tree bark. The separation and purification of phloretin from the crude phloretin extract of apple tree bark was achieved by high-speed counter-current chromatography (HSCCC) with a two-phase solvent system composed of *n*-hexane–ethyl acetate–ethanol–water (2:2:1:2, v/v) on a preparative scale. The lower phase was used as the mobile phase in the head-to-tail elution mode. Double separations were performed with the same two-phase solvent system. 39.2 mg of phloretin were obtained from 767.3 mg of the crude phloretin extract, which contained 5.72% of phloretin. The purity of phloretin was 98.2%, and the recovery was 88.7%. All HSCCC fractions were analyzed by high-performance liquid chromatography and the chemical structure of target compound was identified by UV, IR, ESI-MS, ^{1}H NMR and ^{13}C NMR analysis. The optimum extracting conditions of phloretin from apple tree bark were also investigated by orthogonal test.

1. Introduction

Phloretin (shown in Fig. 1), which is the aglucone of phlorizin, belongs to falconoid. It is one of the naturally occurring non-steroid estrogens, and is abundantly present in apple tree bark which is tonic and febrifuge [1]. Phloretin has been known mostly as cosmeceutical ingredient [2]. Besides its potential antioxidant property, phloretin is known to be served as anti-inflammatory, immunosuppressive effect, antitumor, antimutagenic and anticarcinogenic agents [3–6]. Therefore, an effective method for the isolation and purification of phloretin from natural sources is warranted.

The conventional methods of preparative separating and purifying phloretin require multiple chromatographic steps using silica gel, polyamide and Sephadex LH-20 column, which are easy to enlarge the scale of separation. However, these methods are tedious, time consuming and have low recovery due to irreversibly absorptive effect of the solid matrix. High-speed counter-current chromatography (HSCCC) is a unique liquid–liquid distribution chromatography technique that no solid support matrix is needed. It eliminates the irreversible adsorptive loss of samples onto the solid support matrices used in conventional methods. The separation process is entirely based on the composition of the two-phase solvent system, which provides an ideal partition coefficient of the target compound between the mobile and stationary phases. It has the unique features of high recovery, high efficiency and ease to scale-up [7]. This method has been successfully used for the preparative separation of various natural products [8–13]. However, no report has been published on the isolation and purification of phloretin by HSCCC. The present paper describes successful preparative separation and purification of phloretin from apple tree bark by HSCCC. The best isolation conditions were optimized after investigating the effects of two-phase solvent system, flow rate, temperature and rotation speed. The chemical structure of target compound was elucidated by UV, IR, ESI-MS, ^{1}H NMR and ^{13}C NMR. Otherwise, the optimum extracting conditions of phloretin from apple tree bark were also investigated by orthogonal test L_{16} (4^5).

2. Experimental

2.1. Reagents and materials

Phloretin was purchased from Sigma (USA). *n*-Hexane, ethyl acetate (AcOEt), ethanol and methanol were of HPLC grade and purchased from Institute of Jinan Chemical Industry (China). Fuji apple tree bark was collected from apple tree trunk after apple has matured in apple farm (China). Water used was purified by Cascada Lab Water Systems (18.2 MΩ cm) (Pall, USA).

2.2. Apparatus

The counter-current chromatography apparatus used in the present study is a TBE-300A multilayer coil planet centrifuge for performing standard HSCCC (Shanghai Tauto Biotechnique Company, China), equipped with a polytetrafluoroethylene three

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doi:10.1016/j.seppur.2010.02.020

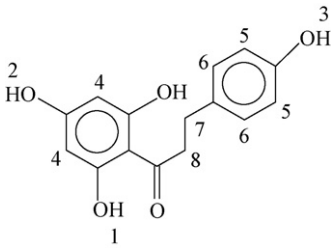

Fig. 1. Chemical structure of phloretin

preparative multilayer coils (total volume, 260 mL, diameter of tube, 1.6 mm). The β value of the preparative column varied from 0.6 at the internal layer to 0.8 at the external layer ($\beta = r/R$, where r is the distance from the coil to the holder shaft, and R is the revolution radius or the distance between the holder axis and central axis of the centrifuge). The rotation speed is adjustable from 0 rpm to 1000 rpm, and 850 rpm was used in the present study. The separation column was installed in a vessel that was maintained at 10 °C by a MultiTemp III constant-temperature circulating implement (ThermoHeake GmbH, Germany). The two-phase solvent system was pumped into the column by an AKTA purifier pump (Amersham, USA) at a flow rate of up to 10 mL min^{-1} under an optimum flow rate of 2.0 mL min^{-1} (maximum column pressure: 25 MPa). The continuous effluent was monitored at 280 nm. The data were collected with a PrimeView^{TM} workstation (Amersham, USA).

The HPLC equipment used was an Agilent 1100 system (Agilent, USA) including a G1311A QuatPump, a sample injector with 20 μL-injection loop, an Agilent temperature control module, and a G1314A UV–vis detector. An Agilent Eclipse XDB-C_{18} column (4.6 mm × 150 mm i.d., 5 μm) was used to separate phloretin. Evaluation and quantification were made on Agilent HPLC workstation (USA).

2.3. Preparation of crude phloretin extract

Apple tree bark was dried to constant weight and then pulverized by medicine grinder (China). Ten grams of the pulverized sample were put into a 250 mL flask, to which 150 mL of 90% aqueous ethanol and 3 mL of concentrated hydrochloric acid were added. After extracting at boiling water bath for 2 h by reflux extraction [14], the mixture was filtered. The extraction procedure was repeated twice. All the filtrates were combined and concentrated to dryness by rotary evaporation at reduced pressure. The residue was extracted with 50 mL of AcOEt five times. All the AcOEt extracts were combined and concentrated to dryness by rotary vaporization. 767.3 mg of crude phloretin extract containing 5.76% of phloretin were yielded. The crude phloretin extract was stored in a refrigerator for subsequent HSCCC separation.

The sample solution was prepared by dissolving the crude phloretin extract in the mixture solution of the upper and lower phases (1:1, v/v) of the two-phase solvent system used for HSCCC separation.

2.4. HSCCC solvent system

In order to achieve efficient separation of phloretin from the crude phloretin extract, three kinds of solvent systems at different volume ratios were tested: (1) chloroform–methanol–water, (2) *n*-butanol–ethyl acetate–water, (3) *n*-hexane–ethyl acetate–ethanol–water. The partition coefficient (K) values were determined according to literature [7]. Briefly, approximately 3 mg of the crude phloretin extract were put into a 10 mL test tube, to which 3.0 mL of each phase of the pre-equilibrated two-phase solvent system were added. The solution was shaken vigorously for 5 min to equilibrate the sample thoroughly with the two phases. After centrifugation at 3000 × g for 10 min, the same volume of each phase was evaporated to dryness under water bath. The residue was diluted with the same volume of HPLC mobile phase and then analyzed by HPLC. The partition coefficient (K) value was expressed as the peak area of phloretin in the upper phase divided by that in the lower phase.

After thorough equilibration of the selected solvent system in a separation funnel by repeating vigorous shaking, the two immiscible phases were separated and degassed by sonication before use. The head-to-tail elution mode with the upper phase as the stationary phase was adopted in the HSCCC experiments, while the lower phase was used as the mobile phase.

2.5. HSCCC separation

The multilayer coiled column was first entirely filled with the upper phase. Then, the apparatus was rotated at 850 rpm in the head-to-tail elution mode, while the lower phase was pumped into the column at a flow rate of 2 mL min^{-1}. The separation temperature was 10 °C. After the mobile phase front emerged and equilibrium was established in the column, 20 mL of the sample solution containing 767.3 mg of the crude phloretin extract was injected. The effluent was continuously monitored at 280 nm and the chromatogram was recorded. Each peak fraction was collected according to the chromatogram and analyzed by HPLC. After the separation was completed, the retention of the stationary phase relative to the total column capacity was computed by the volume of the stationary phase collected from the column.

2.6. HPLC analysis and identification of target compound

The crude phloretin extract, phloretin (standard) and each fraction corresponding to various portions of the major peaks in HSCCC were analyzed by HPLC. The HPLC analysis was performed with a reversed-phase Agilent Eclipse XDB-C_{18} column (150 mm × 4.6 mm, i.d., 5 μm) at a column temperature of 30 °C. The separation was performed with an isocratic elution using methanol–water–acetic acid (40:59:1, v/v) at a flow rate of 1.0 mL min^{-1} and the effluent was monitored at 286 nm. All the components were confirmed from their retention times. Although there was much interference present in the crude phloretin extract, they did not affect the separation and determination of phloretin (Fig. 3(a)). Routine sample calculations were made by comparison of the peak area with that of the standard. The analytical curve was $c\,(\mu g\,L^{-1}) = 1.78 \times 10^{-5} \times A - 0.20$ where c is the concentration of phloretin determined, and A is the peak area of phloretin. The linearity of the calibration curve was observed in the concentration range of 18 μg L^{-1} to 14 mg L^{-1} with a correlation coefficient of 0.9997.

The identification of the target compound was carried out by IR, electrospray ionization (ESI)-MS, ^{1}H NMR and ^{13}C NMR spectra as well as comparing its HPLC retention time and UV spectra with that of the standard. IR spectra were recorded on a Nicolet IR 200 spectrometer (USA). ESI-MS spectra were performed on a Mariner API-TOF (ABI, USA). ^{1}H NMR and ^{13}C NMR spectra were measured in DMSO-*d*6 on a JNM ECP-600 spectrometer (JEOL, Japan) with tetramethylsilane (TMS) as internal standard. All were run at room temperature.

3. Results and discussion

3.1. Optimization of sample extraction

Using orthogonal test $L_{16}(4^5)$ [15], phloretin was extracted from apple tree bark by reflux extraction with ethanol solvent, while