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Isolation and purification of coumarin compounds from *Cortex fraxinus* by high-speed counter-current chromatography

Renmin Liu *, Qinghua Sun, Ailing Sun, Jichun Cui

College of Chemistry and Chemical Engineering, Liaocheng University, Liaocheng 252059, China

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Abstract

High-speed counter-current chromatography (HSCCC) was successfully used for the isolation and purification of coumarin compounds from *Cortex fraxinus*, the Chinese herbal drug. *n*-Butanol–methanol–0.5% acetic acid (5:1.5:5, v/v) was used as the two-phase solvent system. 14.3 mg of fraxin, 26.5 mg of aesculin, 5.8 mg of fraxetin and 32.4 mg of aesculetin with the purity of 97.6, 99.5, 97.2 and 98.7%, respectively were obtained from 150 mg of crude extracts of *C. fraxinus* in a single run. The structures of the isolated compounds were identified by ^{1}H NMR and ^{13}C NMR.

Keywords: Counter-current chromatography; *Cortex fraxinus*; Fraxin; Aesculin; Fraxetin; Aesculetin

1. Introduction

Cortex fraxinus, a kind of commonly used Chinese herbal drug, is officially listed in the Chinese Pharmacopoeia [1]. People usually use it to clear away pathogenic heat and remove the toxin, eliminate pathogenic heat from the blood to treat dysentery, remove excessive heat from liver to improve visual acuity [2]. *C. Fraxinus* could inhibit the growth of dysentery bacillus. Furthermore, it also has been shown to possess expectorant, antitussive and antiasthmatic effects [3]. The main active components of *C. fraxinus* are coumarin compounds including fraxin, aesculin, fraxetin and aesculetin. The chemical structures of these compounds are shown in Fig. 1.

In view of the beneficial effects of the active components of *C. fraxinus*, an efficient method for the separation and purification of these compounds from natural sources is warranted. The conventional methods, such as crystallization, column chromatography, are tedious and usually require multiple steps [3,4]. High-speed counter-current chromatography (HSCCC) uses no solid support, so the adsorbing effects on stationary phase material and artifact formation can be eliminated [5]. Many natural products have been efficiently separated by high-speed counter-current chromatography [6–12].

The Present paper describes the successful isolation and purification of four coumarin compounds from *C. fraxinus* by high-speed counter-current chromatography. *n*-Butanol–methanol–0.5% acetic acid (5:1.5:5, v/v) was used as the two-phase solvent system of HSCCC. Four kinds of major coumarins including fraxin, aesculin, fraxetin and aesculetin could be obtained in a single run.

2. Experimental

2.1. Apparatus

The HSCCC instrument employed in the present study is TBE-300A high-speed counter-current chromatography (Tauto Biotechnique Company, Shanghai, China) with three multilayer coil separation column connected in series (i.d. of the tubing = 1.6 mm, total volume = 260 ml) and a 20 ml sample loop. The revolution radius was 5 cm, and the β values of

* Corresponding author. Tel.: +86 6358239840.
E-mail address: renminliu@lctu.edu.cn (R. Liu).

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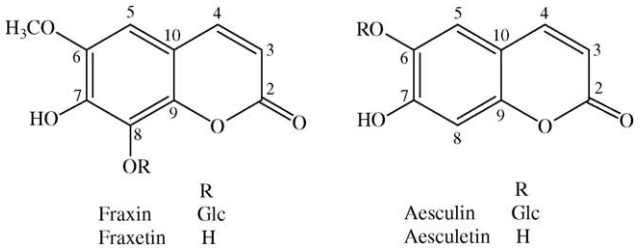

Fig. 1. Chemical structures of coumarin compounds from *Cortex fraxinus*.

the multilayer coil varied from 0.5 at internal terminal to 0.8 at the external terminal. The revolution speed of the apparatus can be regulated with a speed controller in the range between 0 and 1000 rpm. An HX 1050 constant-temperature circulating implement (Beijing Boyikang Lab Instrument Company, Beijing, China) was used to control the separation temperature. An ÄKTA prime (Amersham Pharmacia Biotechnique Group, Sweden) was used to pump the two-phase solvent system and perform the UV absorbance measurement. It contains a switch valve and a mixer, which were used for gradient formation. The data were collected with Sepu 3000 chromatography workstation (Hangzhou Puhui Science Apparatus company, Hangzhou, China).

The HPLC equipment used was Agilent 1100 HPLC system including a G1311A QuatPump, a G1315B DAD, a Rheodyne 7725i injection valve with a 20 μl loop, a G1332A degasser and Agilent HPLC workstation.

Nuclear magnetic resonance (NMR) spectrometer used here was Mercury Plus 400 NMR (Varian Inc., USA).

2.2. Reagents and materials

All organic solvents used for preparation of crude sample and HSCCC separation were of analytical grade (Jinan Reagent Factory, Jinan, China). Methanol used for HPLC was Chromatographic grade (Yucheng Chemical plant, Yucheng, China), and water used was distilled water.

C. fraxinus was purchased from a local drug store and identified by Professor Yongqing Zhang (Shandong University of Traditional Chinese Medicine, Jinan, China).

2.3. Preparation of sample

Hundred gram of *C. fraxinus* was dried at 50 °C for 4 h under vacuum and then pulverized to about 30 mesh. The powder was extracted with 500 ml of 95% ethanol for 1 h under reflux. The extraction procedure was repeated three times. The extracts were combined together and evaporated by a rotary evaporator at 45 °C under reduced pressure. Finally, 9.3 g of red–brown tracta was obtained. It was stored in a refrigerator (4 °C) for further purification by HSCCC.

2.4. Selection of the two-phase solvent system

The composition of the two-phase solvent system was selected according to the partition coefficient (*K*) of the target compounds. The *K*-values were determined by HPLC as follows: approximately 0.2 mg of crude sample was added to a test tube to which 3.0 ml of the lower phase of the pre-equilibrated two-phase solvent system was added. After the crude sample thoroughly dissolved, equal volume of the upper phase of the pre-equilibrated two-phase solvent system was added and shaken violently for several minutes. Finally, the upper and lower phase were analyzed by HPLC. The *K*-values of all components in sample were calculated according to the ratio of the peak areas. $K = A_U/A_L$, where A_U is the peak area of the upper phase, and A_L, the peak area of the lower phase.

2.5. Preparation of two-phase solvent system and sample solution

The selected solvent system, *n*-butanol–methanol–0.5% acetic acid (5:1.5:5, v/v), was prepared by adding all the solvents to a separation funnel according to the volume ratios and thoroughly equilibrated by shaking repeatedly. After thoroughly equilibrated, the upper phase and lower phase were separated and degassed by sonication for 30 min prior to use.

The sample solution was prepared by dissolving the crude sample (150 mg) in 5 ml of the mixture of equal volume of lower phase and upper phase of the solvent system used for HSCCC separation.

2.6. Separation procedure

The whole procedure was carried out as follows: The upper phase and the lower phase of *n*-butanol–methanol–0.5% acetic acid (5:1.5:5, v/v) were pumped into the multiplayer-coiled column simultaneously by using ÄKTA prime system, according to the volume ratio of 30:70. When the column was totally filled with the two phases, only the lower phase was pumped through the column at a flow-rate of 1.5 ml/min while the column was rotated at 900 rpm. After the hydrodynamic equilibrium was reached, about 30 min later, 150 mg of crude sample in 5 ml of the mixture of equal volume of lower phase and upper phase was injected into the separation column. The separation temperature was controlled at 25 °C. The effluent from the outlet of the column was continuously monitored at 254 nm 80 min after the sample injection. Each peak fraction was manually collected according to the chromatogram and evaporated under reduced pressure. The residuals were dissolved in methanol for subsequent HPLC analysis.

2.7. HPLC analysis and identification of HSCCC peak fractions

The crude sample and HSCCC peak fractions were analyzed by HPLC. HPLC analysis was performed using an Agilent 1100 HPLC-DAD system with a SPHERIGEL ODS-C_{18} column (250 mm × 4.6 mm i.d., 5 μm) at room temperature. Methanol–0.1% phosphoric acid (16:84, v/v) was used as the