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

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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

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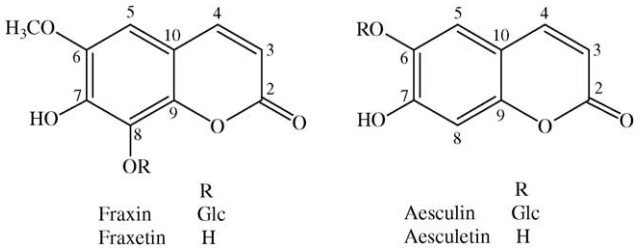

Fig. 1. Chemical structures of coumarin compounds from *Cortex fraxinus*.

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

mobile phase. The flow rate was 1.0 ml min^{-1}, and the effluents were monitored at 254 nm.

Identification of each HSCCC peak fraction was performed by ^{1}H NMR and ^{13}C NMR. The UV spectra were taken from the HPLC three-dimensional spectrum of absorbance versus time and wavelength.

3. Results and discussion

3.1. Optimization of HPLC conditions

Several elution systems such as methanol–H_2O (30:70, v/v), methanol–H_2O (20:80, v/v), methanol–0.1% phosphoric acid (16:84, v/v), acetonitrile–0.1% phosphoric acid (10:90, v/v) were tested in HPLC separation of crude sample. The results indicated that the optimum elution system was methanol–0.1% phosphoric acid (16:84, v/v). The crude sample and the peak fractions separated by HSCCC were analyzed by HPLC. The chromatograms were shown in Fig. 2.

3.2. Selection of HSCCC conditions

Successful separation by HSCCC depends upon the selection of a suitable two-phase solvent system, which provides an ideal range of the partition coefficient (*K*) for the targeted sample. Several two-phase solvent systems were tested and the *K*-values were measured and summarized in Table 1. According to the *K*-values shown in Table 1, it can be seen that the *K*-values of fraxin and aesculin were too small when ethyl acetate–water (5:5) and ethyl acetate–0.5% acetic acid–water (5:1:5) were used as the two-phase solvent system, and the two compounds could not be separated. When *n*-butanol–water (5:5) was used as the two-phase solvent system, the separation time for fraxetin and aesculetin was too long and the peak broadened seriously. So methanol was add to *n*-butanol–water system to reduce the *K*-values of fraxetin and aesculetin. When *n*-butanol–methanol–water (5:0.5:5) was used for HSCCC separation, fraxin, aesculin, and fraxetin could be well separated, but the separation time for aesculetin was still too long. When *n*-butanol–methanol–water (5:1:5) was used for HSCCC separation, the phase separation of the two-phase solvent system was not good and the stationary phase of HSCCC lost seriously. But we found that low concentration of acetic acid could improve the phase separation and the maintenance of the stationary phase. So *n*-butanol–methanol–0.5% acetic acid system was investigated in subsequential study and the results indicated that when *n*-butanol– methanol–0.5% acetic acid (5:1.5:5) was used as the two-phase solvent system, good separation results could be obtained and the separation time was acceptable.

Furthermore, the influence of the flow rate of mobile phase and the column revolution speed were also investigated. The results indicated that reducing the flow rate was propitious to the separation. But at the same time the chromatogram peaks were extended. When the flow rate was increased, the loss of stationary phase became significant. Considering these aspects, the flow rate of the mobile phase was set at 1.5 ml min^{-1}. The rotary speed of the separation coil tube has great influence on the retention percentage of the stationary phase. Expediting the rotary speed can increase the retention of the stationary phase. In this experiment, all separations were performed at 900 rpm.

The crude samples from *C. fraxinus* were separated and purified under the optimum HSCCC conditions. The HSCCC chromatogram is shown in Fig. 3. During the HSCCC separation, when the target compounds were eluted out from the separation column, very small amount of stationary phase was eluted out simultaneously. The baseline of HSCCC chromatogram was high during the compounds were eluted out. Four kinds of coumarins were obtained and yielded 14.3 mg of fraxin (Peak I, collected during 80–92 min), 26.5 mg of aesculin (Peak II, collected during 97–115 min), 5.8 mg of fraxetin(Peak III, collected during 165–190 min) and 32.4 mg of aesculetin (Peak IV, collected during 206–248 min) from 150 mg crude sample. The purity of these compounds was 97.6, 99.5, 97.2 and 98.7%, respectively, as determined by HPLC area normalization method.

3.3. Structure identification

The chemical structure of each peak fraction of HSCCC was identified according to its ^{1}H NMR and ^{13}C NMR data.

Peak I: ^{1}H NMR (400 MHz, $C^2H_3O^2H$) δ: 7.85 (1H, d, J = 9.5 Hz, H-4), 6.93 (1H, s, H-5), 6.20 (1H, d, J = 9.5 Hz, H-3), 4.96 (1H, d, J = 7.6 Hz, H-1′$_{8\text{-O-Glc}}$), 3.87 (3H, s, 6-OCH_3). ^{13}C NMR (400 MHz, $C^2H_3O^2H$) δ: 163.2 (C-2), 147.4 (C-7), 146.4 (C-4), 145.7 (C-6), 144.2 (C-8), 133.2 (C-9), 113.1 (C-3), 112.2 (C-10), 106.0 (C-5), 105.1 (C-1′$_{8\text{-O-Glc}}$), 78.6 (C-3′), 77.7 (C-5′), 75.5 (C-2′), 70.7 (C-4′), 62.2 (C-6′), 57.0 (C-6-OCH_3). Comparing these data with ref. [3], Peak I was identified as fraxin.

Peak II: ^{1}H NMR (400 MHz, $C^2H_3O^2H$) δ: 7.82 (1H, d, J = 9.5 Hz, H-4), 7.41 (1H, s, H-5), 6.80 (1H, s, H-8), 6.21 (1H, d, J = 9.5 Hz, H-3), 4.83 (1H, d, J = 7.6 Hz, H-1′$_{8\text{-O-Glc}}$). ^{13}C NMR (400 MHz, $C^2H_3O^2H$) δ: 163.2 (C-2), 153.3 (C-7), 152.6 (C-9), 145.9 (C-4), 144.4 (C-6), 116.8 (C-5), 113.2 (C-3), 112.8 (C-10), 104.5 (C-8), 104.3 (C-1′$_{6\text{-O-Glc}}$), 78.5 (C-3′), 77.6 (C-5′), 74.8 (C-2′), 70.5 (C-4′), 62.5 (C-6′). Comparing these data with ref. [3], Peak II was confirmed as aesculin.

Peak III: ^{1}H NMR (400 MHz, $C^2H_3O^2H$) δ: 7.84 (1H, d, J = 9.6 Hz, H-4), 6.72 (1H, s, H-5), 6.23 (1H, d, J = 9.6 Hz, H-3), 3.87 (3H, s, 6-OCH_3). ^{13}C NMR (400 MHz, $C^2H_3O^2H$) δ: 164.0 (C-2), 147.0 (C-7), 146.7 (C-4), 140.8 (C-6), 140.7 (C-8), 134.0 (C-9), 112.6 (C-3), 112.2 (C-10), 101.3 (C-5), 56.8 (C-6-OCH_3). Comparing these data with ref. [2], Peak III was confirmed as fraxetin.

Peak IV: ^{1}H NMR (400 MHz, $C^2H_3O^2H$) δ: 7.82 (1H, d, J = 9.5 Hz, H-4), 6.97 (1H, s, H-8), 6.79 (1H, s, H-5), 6.22 (1H, d, J = 9.5 Hz, H-3). ^{13}C NMR (400 MHz, $C^2H_3O^2H$)

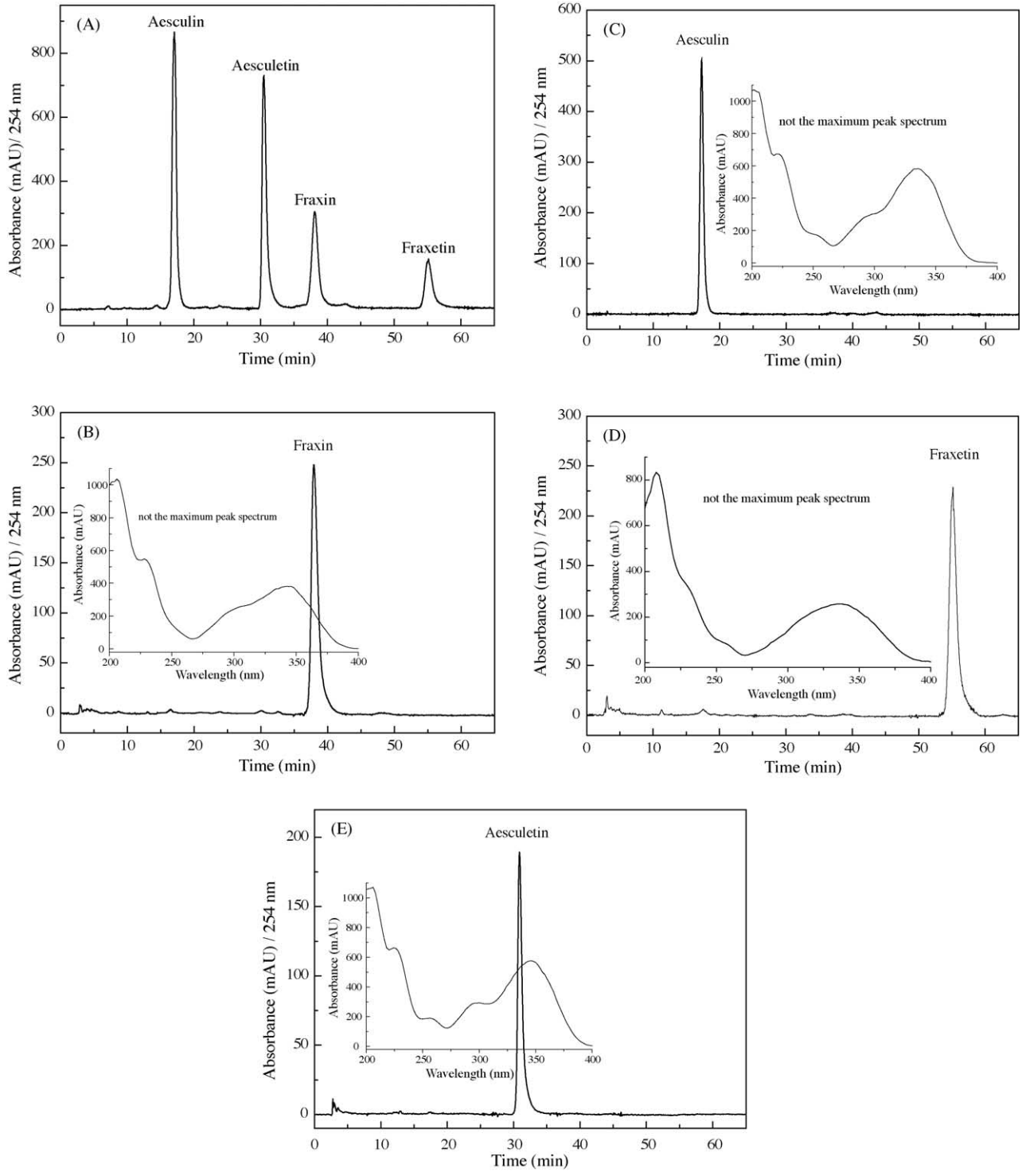

Fig. 2. HPLC chromatogram of crude extract from *Cortex fraxinus* and HSCCC peak fractions. Conditions: column: SPHERIGEL ODS-C_{18} column (250 mm × 4.6 mm i.d., 5 μm); mobile phase: methanol–0.1% phosphoric acid (16:84, v/v); flow-rate: 1.0 ml min^{-1}; detection wavelength: 254 nm. crude extract from *Cortex fraxinus*; (B)–(E) HSCCC peak fraction I–IV in Fig. 3.

δ: 164.6 (C-2), 152.1 (C-7), 150.5 (C-9), 146.2 (C-4), 144.6 (C-6), 113.1 (C-5), 112.8 (C-10), 112.5 (C-3), 103.7 (C-8). Comparing the data with ref. [2], Peak IV was identified as aesculetin.

The result of our studies described above clearly demonstrated that HSCCC is very successful in the separation and purification of fraxin, aesculin, fraxetin and aesculetin from *C. fraxinus*.

Table 1
K-values as the same as in paragraph 1 of Section 3.2 of HSCCC conditions

Solvent system (v/v)	*K*			
	Fraxin	Aesculin	Fraxetin	Aesculetin
Ethyl acetate–water (5:5)	0.05	0.02	1.36	3.48
Ethyl acetate–0.5% acetic acid–water (5:1:5)	0.06	0.09	1.47	8.87
n-Butanol–water (5:5)	0.51	1.47	6.54	10.39
n-Butanol–methanol–water (5:0.5:5)	0.49	1.25	2.48	7.70
n-Butanol–methanol–water (5:1:5)	Phase separation was not satisfactory			
n-Butanol–0.5% acetic acid (5:5)	0.55	1.17	4.53	10.28
n-Butanol– methanol–0.5% acetic acid (5:1:5)	0.72	1.20	3.35	5.26
n-Butanol–methanol–0.5% acetic acid (5:1.5:5)	0.83	1.23	2.65	3.58

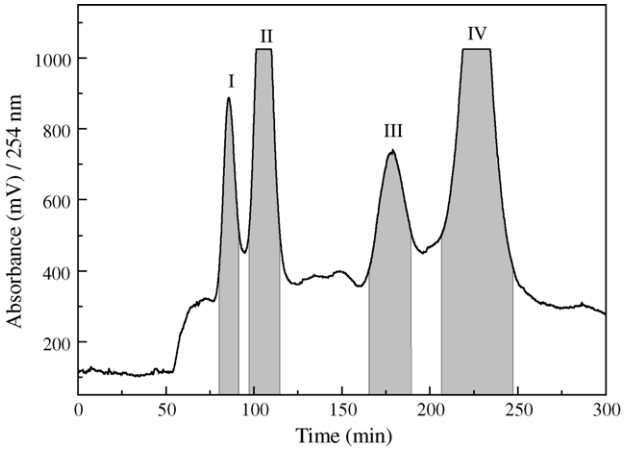

Fig. 3. HSCCC chromatogram of crude extract from *Cortex fraxinus*. Two-phase solvent system: *n*-butanol–methanol–0.5% acetic acid (5:1.5:5); mobile phase: the lower phase; flow-rate: 1.5 ml min^{-1}; revolution speed: 900 rpm; detection wavelength: 254 nm; sample size: 150 mg of crude sample dissolved in 5 ml of the upper phase; separation temperature: 25 °C; retention percentage of the stationary phase: 30%.

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