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

Received 22 November 2004; received in revised form 18 February 2005; accepted 3 March 2005

Available online 21 March 2005

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 1H NMR and 13C NMR.

© 2005 Elsevier B.V. All rights reserved.

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 Biotechne 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...
2.3. Preparation of sample

Hundred gram of *C. fraxinus* was purchased from a local drug store and 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 under reduced pressure. The residual 9.3 g was red-brown tracta and 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 multi-layer coil simultaneously by using an AKTA prime system, according to the volume ratio of 70:30. 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-C18 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.
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 ¹H NMR and ¹³C 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–H₂O (30:70, v/v), methanol–H₂O (20:80, v/v), methanol–0.1% phosphoric acid (16:84, v/v), acetone–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 gradient 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. The results indicated that 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 fraxin and aesculin was too long and the peak broadened seriously. So methanol was added to n-butanol–water system to reduce the K-values of fraxin and aesculin. Table 1 shows the K-values of fraxin (Peak I) and aesculin (Peak II) when different two-phase solvent systems were used.

3.3. Structure identification

The chemical structure of each peak fraction of HSCCC was identified according to its ¹H NMR and ¹³C NMR data. The ¹H NMR spectra of Peak I, II, III, and IV are shown in Table 1. The rotary speed of the separation coil tube has a 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, 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 aesculetin (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.
Fig. 2. HPLC chromatogram of crude extract from Cortex fraxinus and HSCCC peak fractions. Conditions: column: Spherigel ODS-C$_{18}$ column (250 mm x 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

<table>
<thead>
<tr>
<th>Solvent system (v/v)</th>
<th>Fraxin</th>
<th>Aesculin</th>
<th>Fraxetin</th>
<th>Aesculetin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethyl acetate–water (5:5)</td>
<td>0.05</td>
<td>0.02</td>
<td>1.36</td>
<td>3.48</td>
</tr>
<tr>
<td>Ethyl acetate–0.5% acetic acid–water (5:1.5)</td>
<td>0.06</td>
<td>0.09</td>
<td>1.47</td>
<td>8.87</td>
</tr>
<tr>
<td>n-Butanol–water (5:5)</td>
<td>0.51</td>
<td>1.47</td>
<td>6.54</td>
<td>10.39</td>
</tr>
<tr>
<td>n-Butanol–methanol–water (5:0.5:5)</td>
<td>0.49</td>
<td>1.25</td>
<td>2.48</td>
<td>7.70</td>
</tr>
<tr>
<td>n-Butanol–methanol–water (5:1:5)</td>
<td>Phase separation was not satisfactory</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n-Butanol–0.5% acetic acid (5:5)</td>
<td>0.53</td>
<td>1.17</td>
<td>4.53</td>
<td>10.28</td>
</tr>
<tr>
<td>n-Butanol–methanol–0.5% acetic acid (5:1.5)</td>
<td>0.72</td>
<td>1.20</td>
<td>3.35</td>
<td>5.26</td>
</tr>
<tr>
<td>n-Butanol–methanol–0.5% acetic acid (5:1.5:5)</td>
<td>0.83</td>
<td>1.23</td>
<td>2.65</td>
<td>3.58</td>
</tr>
</tbody>
</table>

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%.

References