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Isolation and purification of baicalein, wogonin and oroxylin A from the medicinal plant *Scutellaria baicalensis* by high-speed counter-current chromatography

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Abstract

The medicinal plant *Scutellaria baicalensis* Georgi has been used widely in traditional Chinese medicine for anti-inflammation, anticancer, antiviral and antibacterial infections, reducing the total cholesterol level and decreasing blood pressures. A high-speed counter-current chromatography (HSCCC) method was developed for the preparative separation and purification of three bioactive flavonoids, namely, baicalein, wogonin and oroxylin A, from *S. baicalensis* Georgi. Preparative HSCCC with a two-phase solvent system composed of *n*-hexane–ethyl acetate–*n*-butanol–water (1:1:8:10, v/v/v/v) was successfully performed by increasing the flow-rate of the mobile phase stepwise from 1.0 to 2.0 ml min^{-1} after 4 h. The components purified and collected were analyzed by high-performance liquid chromatography. The method yielded 144.8 mg of baicalein at 95.7% purity, 50.2 mg of wogonin at 98.5% purity, and 12.4 mg of oroxylin A at 93.2% purity from 500 mg of the crude extract in a one-step separation. The recoveries of baicalein, wogonin and oroxylin A were 92.7%, 91.6% and 92.5%, respectively.

Keywords: *Scutellaria baicalensis*; Counter-current chromatography; Preparative chromatography; Plant materials; Baicalein; Wogonin; Oroxylin A

1. Introduction

Scutellaria baicalensis Georgi is one of the most widely used medicinal plants, and is officially listed in the Chinese Pharmacopoeia. Its roots have been used for anti-inflammation, anticancer, treating bacterial and viral infections, reducing the total cholesterol level and decreasing blood pressures [1–3]. Baicalein, wogonin, oroxylin A and baicalin are the main bioactive components of *S. baicalensis* Georgi [4,5]. Baicalein possesses anti-HIV [6], anti-tumor [7], antioxidant and free radical scavenging effects [8]. Wogonin has anti-respiratory syncytial virus [9], anti-hepatitis B virus [10], anti-tumor [7], antioxidant and free radical scavenging effects [8]. Oroxylin A has anti-respiratory syncytial virus activity [9]. The chemical structures of baicalein, wogonin and oroxylin A are shown in Fig. 1.

High-speed counter-current chromatography (HSCCC) is a unique liquid–liquid partition chromatography with a liquid stationary phase, which has been applied to the separation of a number of natural products [11–20]. Although baicalin has been purified from *S. baicalensis* Georgi by HSCCC [21], preparative separation and purification of baicalein, wogonin and oroxylin A from *S. baicalensis* Georgi, however, has not been explored. The present paper describes successful preparative separation and purification of baicalein, wogonin and oroxylin A from the crude extract of *S. baicalensis* Georgi by HSCCC.

2. Experimental

2.1. Apparatus

Preparative HSCCC was carried out with a Model CCC-1000 high-speed counter-current chromatograph (Pharma-

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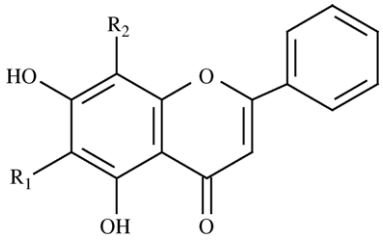

Baicalein (R_1=OH, R_2=H)
Wogonin (R_1=H, R_2=OMe)
Oroxylin A (R_1=OMe, R_2=H)

Fig. 1. Chemical structure of baicalein, wogonin and oroxylin A.

Tech Research, Baltimore, MD, USA). The apparatus consisted of three preparative coils, connected in series (inner diameter of tube, 2.6 mm; total volume, 325 ml). The revolution radius or the distance between the holder axis and central axis of the centrifuge (R) was 7.5 cm, and the β-value varied from 0.47 at the internal terminal to 0.73 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 Series II HPLC pump (Pharma-Tech Research), a Model SPD-10Avp UV–vis detector (Shimadzu, Japan), a Model L 120 E flat-bed recorder (Linseis, Germany), and a sample injection valve with a 10 ml sample loop.

2.2. Reagents

All solutions were prepared with analytical grade compounds. Reverse osmosis Milli-Q water (18 MΩ) (Millipore, USA) was used for all solutions and dilutions. Ethyl acetate, *n*-hexane, *n*-butanol, absolute ethanol, methanol and acetic acid were obtained from BDH (Poole, UK).

The dried root of *S. baicalensis* Georgi was obtained from Beijing Tong-Ren-Tang drug retail outlet in Hong Kong.

2.3. Preparation of crude extracts from S. baicalensis Georgi

Preparation of crude extracts was carried out according to the literature [4,22]. In brief, the dried roots of *S. baicalensis* Georgi were ground to powder. The powder (100 g) was extracted with 400 ml *n*-hexane under sonication for 30 min. The mixture was filtered, and then the residue was extracted using ethyl acetate and ethanol for 30 min, respectively (400 ml each time). The filtrate was combined, and the extract was evaporated to dryness by rotary vaporization. The residue (12.35 g) was stored in a refrigerator for the subsequent HSCCC separation.

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

In the present study, we selected a two-phase solvent system composed of *n*-hexane–ethyl acetate–*n*-butanol–water at various volume ratios. Each solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and the two phases were separated shortly before use.

The sample solution was prepared by dissolving the crude sample in the solvent mixture of lower phase and upper phase (1:1, v/v) of the solvent system used for separation because the sample was not easily dissolved in either phase.

2.5. HSCCC separation procedure

In each separation, the coiled column was first entirely filled with the upper phase (stationary phase), and then the apparatus was rotated at 1000 rpm, while the lower phase (mobile phase) was pumped into the column in the head-to-tail elution mode at a flow-rate of 1.0 ml min^{-1}. After the mobile phase front emerged and hydrodynamic equilibrium was established in the column, approximately 10 ml of the sample solution containing 500 mg of the crude extract was injected into the head of the column through the injection valve. After 4 h, the flow-rate of the mobile phase was increased to 2.0 ml min^{-1}. The effluent of the column was continuously monitored with a UV–vis detector at 280 nm. Peak fractions were collected according to the elution profile.

2.6. HPLC analysis

The crude sample and each peak fraction obtained by HSCCC were analyzed by HPLC according to the literature [23]. The HPLC system used throughout this study consisted of two Waters 510 pumps (Waters, Milford, MA, USA), a sample injector (Rheodyne, Cotati, CA, USA) with a 20 μl loop, and a Waters 996 photodiode array detector. Evaluation and quantification were made on a Millennium chromatography data system (Waters). The column used was a reversed-phase Ultrasphere C_{18} column (250 mm × 4.6 mm i.d., 5 μm, Beckman, Fullerton, CA, USA). The mobile phase was methanol containing 1% acetic acid (solvent A)–water containing 1% acetic acid (solvent B) in the gradient mode as follows: 0–15 min, 25–55% A; 15–18 min, 55–70% A; 18–26 min, 70% A; 26–28 min, 70–25% A. The flow-rate was 1.0 ml min^{-1}, and the effluent was monitored at 280 nm. The peak identification was based on the retention time and the UV spectrum against the standard. Routine sample calculation was made by comparison of the peak area with that of the standard.

3. Results and discussion

The crude extract obtained from *S. baicalensis* Georgi was analyzed by HPLC, and the chromatogram is shown in Fig. 2. The contents of baicalein, wogonin and oroxylin A were 29.9%, 10.8% and 2.5%, respectively.

In HSCCC, the selection of the two-phase solvent system is the most important for successful separation, and is also the most difficult step; it is estimated that about 90% of the entire

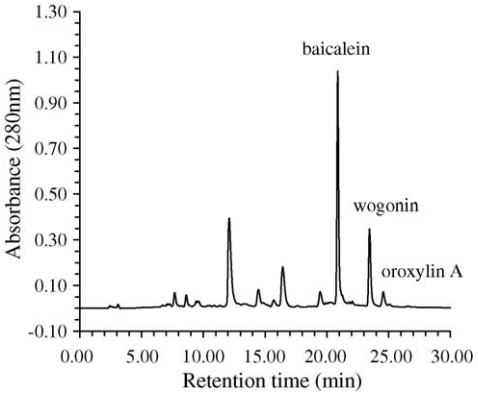

Fig. 2. Chromatogram of the crude extract from *Scutellaria baicalensis* Georgi by HPLC analysis. Conditions: column, reversed-phase Ultrasphere C_{18} column (250 mm × 4.6 mm i.d., 5 μm); mobile phase, methanol containing 1% acetic acid (solvent A)–water containing 1% acetic acid (solvent B) in the gradient mode as follows: 0–15 min, 25–55% A; 15–18 min, 55–70% A; 18–26 min, 70% A; 26–28 min, 70–25% A; flow-rate, 1.0 ml min^{-1}; detection at 280 nm.

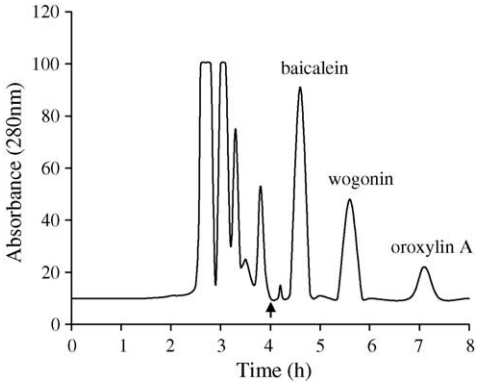

Fig. 3. Chromatogram of the crude extract from *Scutellaria baicalensis* Georgi by HSCCC separation. Conditions: column, multilayer coil of 2.6 mm i.d. PTFE tube with a total capacity of 325 ml; rotary speed, 1000 rpm; solvent system, *n*-hexane–ethyl acetate–*n*-butanol–water (1:1:8:10, v/v/v/v); mobile phase, lower phase (water phase); flow-rate, 0–4 h, 1.0 ml min^{-1} and 4–8 h, 2.0 ml min^{-1}; detection at 280 nm; sample size, 500 mg; retention of the stationary phase, 51%. The arrow indicates the flow-rate of the mobile phase was increased stepwise from 1.0 to 2.0 ml min^{-1} after 4 h.

work in HSCCC is spent on that. If only one component needs to be separated from the others, the standard HSCCC method, which uses a constant flow-rate of the mobile phase, could be used. In order to separate more different compounds, stepwise elution or stepwise increasing the flow-rate of the mobile phase might be adopted [24–26]. Preliminary HSCCC experiments were carried out with the two-phase solvent system composed of *n*-butanol–ethyl acetate–water at a volume ratio of 2:3:5. Although baicalein could be separated from other

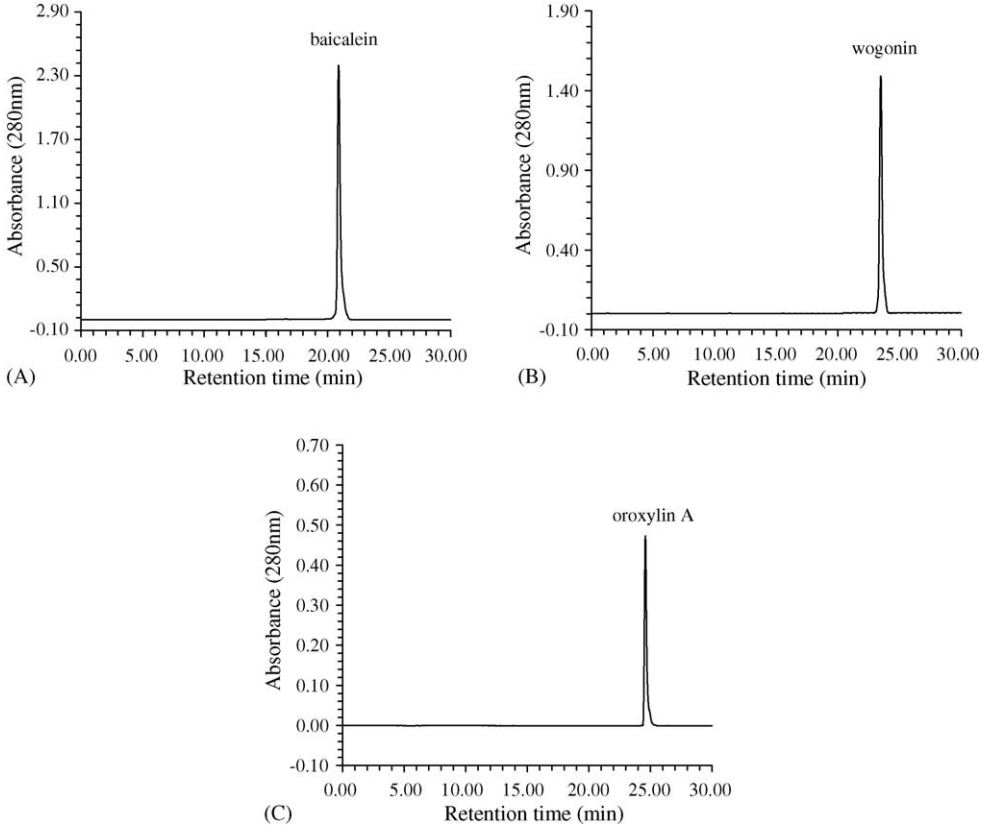

Fig. 4. HPLC chromatograms of baicalein, wogonin and oroxylin A purified from *Scutellaria baicalensis* Georgi by HSCCC. Conditions: column, reversed-phase Ultrasphere C_{18} column (250 mm × 4.6 mm i.d., 5 μm); mobile phase, methanol containing 1% acetic acid (solvent A)–water containing 1% acetic acid (solvent B) in the gradient mode as follows: 0–15 min, 25–55% A; 15–18 min, 55–70% A; 18–26 min, 70% A; 26–28 min, 70–25% A; flow-rate, 1.0 ml min^{-1}; detection at 280 nm.

compounds, it was difficult to purify wogonin and oroxylin A. In the subsequent studies, another two-phase solvent system was investigated.

A two-phase solvent system composed of *n*-hexane–*n*-butanol–ethanol–water at a volume ratio of 3:7:1:9 was first evaluated. It was, however, very difficult to purify target compounds from the crude extract, because the time they were retained in the column was short. Subsequently, a two-phase solvent system composed of *n*-hexane–ethyl acetate–*n*-butanol–ethanol –water at a volume ratio of 1:1:8:1:9 was tested. Although the peak resolution was improved, and baicalein could be separated from other components, it was difficult to purify wogonin and oroxylin A. With the two-phase solvent system composed of *n*-hexane–ethyl acetate–*n*-butanol–water at a volume ratio of 1:1:8:10, the peak resolution was improved, but oroxylin A was retained in the column for a long period of time (10 h). Finally, the method with stepwise increasing the flow-rate of the mobile phase was attempted with this two-phase solvent system. That is, the flow-rate of the mobile phase was kept at 1.0 ml min^{-1} before 4 h, and subsequently increased to 2.0 ml min^{-1} after 4 h. The separation of the target compounds was achieved with good peak resolution, and the retention of the stationary phase was satisfactory (about 51%). After baicalein, wogonin and oroxylin A were eluted out, in order to save solvents and time, the remaining compounds in the column were removed by forcing out the stationary phase with pressurized nitrogen gas instead of eluting them with the mobile phase because the stationary phase was not to be reused. Fig. 3 shows the preparative HSCCC separation of 500 mg of crude extract using the solvent system composed of *n*-hexane–ethyl acetate–*n*-butanol–water at a volume ratio of 1:1:8:10 by increasing the flow-rate of the mobile phase stepwise from 1.0 to 2.0 ml min^{-1} after 4 h. This separation yielded 144.8 mg of baicalein at 95.7% purity, 50.2 mg of wogonin at 98.5% purity, and 12.4 mg of oroxylin A at 93.2% purity according to HPLC analysis. The recoveries of baicalein, wogonin and oroxylin A were 92.7%, 91.6% and 92.5%, respectively. The HPLC chromatograms of baicalein, wogonin and oroxylin A as purified from the preparative HSCCC are shown in Fig. 4.

In conclusion, an HSCCC method for the preparative separation and purification of bioactive compounds from the medicinal plant *S. baicalensis* Georgi was developed by stepwise increasing the flow-rate of the mobile phase. Baicalein, wogonin and oroxylin A with high purities could be obtained from the crude extract in a one-step separation, and their recoveries were also high. The present study indicates that HSCCC is a very powerful tool for the preparative isolation and purification of bioactive components from medicinal plants.

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