Separation and Purification of Epimedin A, B, C, and Icariin from the Medicinal Herb *Epimedium brevicornum* Maxim by Dual-Mode HSCCC

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Abstract

***Epimedium brevicornum* Maxim is a famous medicinal herb which has been widely used for the treatment of impotence, infertility, osteoporosis, cardiovascular diseases, amnesia, and senile functional diseases. A dual-mode high-speed counter-current chromatographic method is developed for the separation and purification of four bioactive flavonoids from the medicinal herb *Epimedium brevicornum* Maxim. The crude flavonoids are obtained by extraction with ethyl acetate and ethanol from the dried aerial parts of *Epimedium brevicornum* Maxim under sonication. High-speed counter-current chromatography with a two-phase solvent system composed of *n*-butanol–ethyl acetate–water (3:7:10, v/v) is performed using a dual-mode method, which yields 3.2 mg of epimedin A at a purity of 98.2%, 5.5 mg of epimedin B at a purity of 92.6%, 12.7 mg of epimedin C at a purity of 90.4%, and 42.9 mg of icariin at a purity of 96.8% based on the HPLC analysis. The recoveries are 95.2%, 89.4%, 91.1%, and 94.8%, respectively. The isolation of these flavonoids in adequate amounts makes them readily available for structure-activity relationship studies and for quality control of the herbal medicine.**

Introduction

Medicinal herbs have been used to treat human diseases in the East for centuries, and people are becoming increasingly interested in these herbs because of their good therapeutic performance and low toxicity. *Epimedium brevicornum* Maxim is a famous medicinal herb which has been widely used for the treatment of impotence, infertility, osteoporosis, cardiovascular diseases, amnesia, and senile functional diseases (1–6). A group of flavonoids (epimedin A, epimedin B, epimedin C, and icariin) are thought to be the main bioactive components of *Epimedium brevicornum* Maxim (1). The chemical structures of these four flavonoids are shown in Figure 1.

In order to further study the biological activities of these flavonoids and to control the quality of the herbal medicine, a large quantity of pure materials is urgently needed. However, the preparative separation and purification of these bioactive components from the herb by conventional methods are very difficult, tedious, and time-consuming (7–10). High-speed counter-current chromatography (HSCCC) is a relatively new chromatographic technique. Because there is no solid support matrix in the HSCCC column, it eliminates irreversible adsorptive loss, denaturation, and contamination of samples onto the solid support matrix used in the conventional chromatographic column. HSCCC has been applied to the separation and purification of a number of natural products (11–23). Because of the liquid nature of the stationary phase of HSCCC, the phase role can be reversed during a run using the dual-mode HSCCC method. That is, the initial mobile phase will become the stationary phase, and vice versa. The separation still progresses after phase reversal (24,25). This method can elute compounds with a wide range of polarities from the column, and remarkably reduce separation time. In this paper, a dual-mode HSCCC method was developed for the preparative separation and purification of four bioactive flavonoids from the medicinal herb *Epimedium brevicornum* Maxim.

Experimental

Apparatus

Preparative HSCCC was carried out with a Model CCC-1000 high-speed counter-current chromatograph (Pharma-Tech Research, Baltimore, MD). The apparatus consisted of three preparative coils, connected in series (inner diameter of tube,

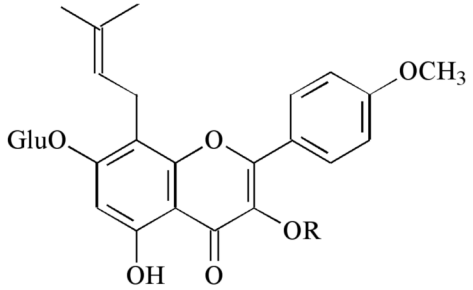

Figure 1. The chemical structures of four flavonoids. Glu = glucose, Rha = rhamnose, Xyl = xylose. For: epimedin A (R = Rha-Glu), epimedin B (R = Rha-Xyl), epimedin A (R = Rha-Rha), icariin (R = Rha).

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1.6 mm; total volume, 320 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 high-performance liquid chromatography (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.

Reagents and materials

All solutions were prepared with analytical grade chemicals. Reverse osmosis water (18 MΩ) from Milli-Q system (Millipore, Billerica, MA) was used for all solutions and dilutions. Ethyl acetate, *n*-butanol, ethanol, and *n*-hexane were obtained from BDH (Poole, UK). The standard icariin was obtained from the Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), and epimedins A, B, and C were purchased from Chromadex (Santa Ana, CA). The dried aerial parts of *Epimedium brevicornum* Maxim were obtained from Beijing Tong-Ren-Tang drug retail outlet in Hong Kong.

Preparation of crude flavonoids from *Epimedium brevicornum* Maxim

Preparation of crude flavonoids was carried out according to the literature (7,26) with slight modification. In brief, the dried aerial parts of *Epimedium brevicornum* Maxim were ground to powder. The powder (100 g) was extracted with 500 mL of ethyl acetate under sonication for 30 min. The mixture was filtered with a 0.22-µm filter membrane of Type GV (Millipore), and the residue was then extracted twice with 70% ethanol under sonication for 30 min (500 mL each time). The filtrate from ethyl acetate and 70% ethanol extraction was combined, and the extract was evaporated to dryness by rotary vaporization. The dried extract was partitioned between 200 mL of water and 100 mL of *n*-hexane. The resulting water portion was then extracted with 100 mL of ethyl acetate. It was followed by two more successive extractions of the water portion with *n*-butanol (100 mL each time). Afterwards, the extract from ethyl acetate and *n*-butanol was combined and dried by rotary vaporization. The dried crude flavonoids (4.518 g) were stored in a refrigerator for the subsequent HSCCC separation.

Preparation of two-phase solvent system and sample solution

In the present study, we selected a two-phase solvent system composed of *n*-butanol–ethyl acetate–water (3:7:10, v/v). The solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature, and the two phases were separated shortly before use. Because the sample was not easily dissolved in either phase alone, the sample solution was prepared by dissolving the crude flavonoids in a solvent mixture of both the lower phase and the upper phase (1:1, v/v) of the solvent system used for HSCCC separation.

HSCCC separation procedure

Preparative separation was performed using a dual-mode HSCCC method. In each run, 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. After the mobile phase front emerged and hydrodynamic equilibrium was established in the column, ~ 10 mL of the sample solution containing 300 mg of the crude extract was injected into the head of the column through the injection valve. The flow-rate was held at 1.0 mL/min for the first 3 h. Afterwards, the flow-rate was increased to 2.0 mL/min, and was held for 8 h of operation. After 8 h, the inlet and outlet of the column were switched, and the upper phase, which was originally used as the stationary phase, was eluted in the tail-to-head direction through the column, while the direction of the column rotation was held unaltered. Meanwhile, the flow-rate was shifted back to 1.0 mL/min and was held for the rest of run. The effluent of the column was continuously monitored with a UV–vis detector at 270 nm. Peak fractions were collected according to the elution profile.

HPLC analysis

The crude extract and each peak fraction obtained by HSCCC were analyzed by HPLC according to the literature (26,27) with slight modification. The HPLC system used throughout this study consisted of two Waters 510 pumps (Waters, Milford, MA), a sample injector (Rheodyne, Cotati, CA) 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 × 4.6 mm i.d., 5 µm, Beckman, Fullerton, CA). The mobile phase was acetonitrile (solvent A)–water (solvent B) in the gradient mode as follows: 0–16 min, 26% A; 16–24 min, 26–80% A; 24–25 min, 80–26% A; 25–35 min, 26% A. The flow-rate was 1.0 mL/min, and the effluent was monitored at 270 nm. Routine sample calculation was made by comparison of the peak area with that of the standard.

Results and Discussion

In the extraction procedure, several resolvents were sequentially used to improve extraction efficiencies because the target compounds have a broad range of polarities. Furthermore, the partitioning steps were adopted to concentrate the target compounds (7,26). The crude extracts obtained from *Epimedium brevicornum* Maxim were analyzed by HPLC (Figure 2). The contents of epimedin A, B, C, and icariin were 1.1%, 1.9%, 4.2%, and 14.6%, respectively. As shown in Figure 2, the crude extract contained a high number of different compounds with a broad range of polarities. In addition, the chemical structures of epimedin A, B, and C were very similar (Figure 1), which hinted that it would be very difficult to separate and purify these compounds from the crude extract.

The versatility of HSCCC makes it an ideal choice for the isolation of bioactive natural products. Because HSCCC is an all-liquid chromatographic technique and there is no solid support matrix in its column, any change of the mobile phase composition is likely to change the stationary phase composition or volume. Therefore, isocratic elution is most widely used to

develop a HSCCC method. In the literature (22), chloroform–methanol–water (4:3.5:2) was used as the two-phase solvent system for the isolation and purification of epimedokoreanoside I, icariin, and icariside II from *Epimedium koreamum* Nakai. In this study, preliminary HSCCC studies were carried out with a two-phase solvent system composed of *n*-hexane–ethanol–water, which was more advantageous than the two-phase solvent system containing organic chloride (such as tetrachloromethane and chloroform), when health and environmental questions were considered. Firstly, *n*-hexane–ethanol–water at a volume ratio of 10:5:5 was tested, and the flavonoids could not be separated from other components in the crude extract because their retention times were too short. In order to increase the polarity of the mobile phase, *n*-hexane–ethanol–water at a volume ratio of 10:4:6 was attempted. Although the retention times were increased, they could not be separated from other components yet. Then, *n*-hexane–ethanol–water at a volume ratio of 10:3:7 was tested because the mobile phases had stronger polarity. The flavonoids could not be separated from each other. Thus, the two-phase solvent system composed of *n*-hexane–ethanol–water at the different volume ratios (10:5:5, 10:4:6, and 10:3:7) was not suitable for the separation of these flavonoids from the plant extract.

In another study (17), *n*-hexane–*n*-butanol–methanol–water (1:4:2:6) was used as the two-phase solvent system for the purification of icariin from *Epimedium segittatum*. In present studies, a two-phase solvent system composed of *n*-hexane–ethyl acetate–ethanol–water was evaluated in terms of peak resolution. When the two-phase solvent system at a volume ratio of 5:5:5:5 was tested, the flavonoids could not be separated from other compounds in the crude extract because their retention times in the column were short. Then, the two-phase solvent system at a volume ratio of 5:5:4:6 was attempted because the mobile phases had higher polarity. The flavonoids could not be separated from each other. The two-phase solvent system at a volume ratio of 5:5:3:7 was also tested. Epimedin A, B, and C could not be separated from each other, although icariin could be separated from the other compounds. Therefore, the two-phase solvent system composed of *n*-hexane–ethyl acetate–ethanol–water at the different volume ratios (5:5:5:5, 5:5:4:6, and 5:5:3:7) was not suitable for the separation of these flavonoids from the crude extract.

Several compounds contained all 2 or 3 saccharide groups (Figure 1). This indicates that these compounds all have strong polarities. For these kinds of compounds, maybe, the main organic solvent used in the two-phase solvent system should be of relative polarity. In the subsequent studies, a two-phase solvent system composed of *n*-butanol–ethyl acetate–water at different volume ratios was attempted. Firstly, *n*-butanol–ethyl acetate–water at a volume ratio of 1:4:5 was tested. Epimedin A and B could be separated from other components in the crude extract; however, it was very difficult to elute epimedin C and icariin from the column. In order to increase the eluting ability of the mobile phase, *n*-butanol–ethyl acetate–water at a volume ratio of 2:3:5 was attempted. Epimedin A could be separated from other compounds in the crude extract. Although epimedin C could be eluted from the column, the overlap of chromatographic peaks of epimedin B and epimedin C remained a question. In addition, it was very difficult to elute icariin from the column. Then, *n*-butanol–ethyl acetate–water at a volume ratio of 10:3:7 was tested. Epimedin A, B, and C could be separated from each other, but it was still very difficult to elute icariin from the column. Because of the liquid nature of the stationary phase of HSCCC, the phase role could be reversed during a run to elute all the injected compounds from the column in a short separation time. Thus, the dual-mode HSCCC method was attempted. That is, the initial mobile phase become the stationary phase after epimedin A, B, and C were eluted from the column. In addition, the mobile phase (the original stationary phase) was pumped into the column in the tail-to-head elution mode instead of original head-to-tail elution mode.

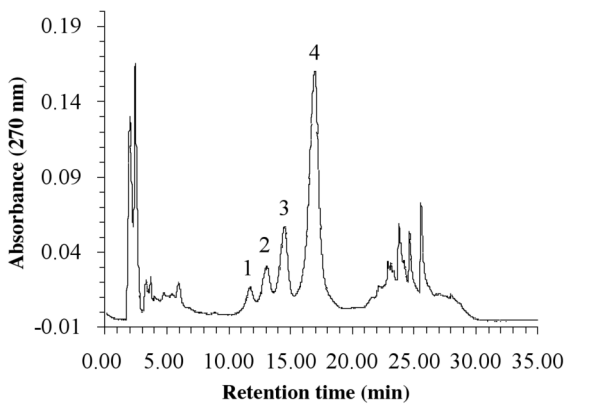

Figure 2. Chromatogram of the crude extract from *Epimedium brevicornum* Maxim by HPLC analysis: where peak 1 is epimedin A; peak 2 is epimedin B; peak 3 is epimedin C; and peak 4 is icariin. Experimental conditions: a reversed-phase Ultrasphere C18 column (250 × 4.6 mm i.d., 5 µm); acetonitrile (solvent A)–water (solvent B) as the mobile phase in the gradient mode as follows: 0–16 min, 26% A; 16–24 min, 26–80% A; 24–25 min, 80–26% A; 25–35 min, 26%; flow-rate: 1.0 mL/min; detection at 270 nm.

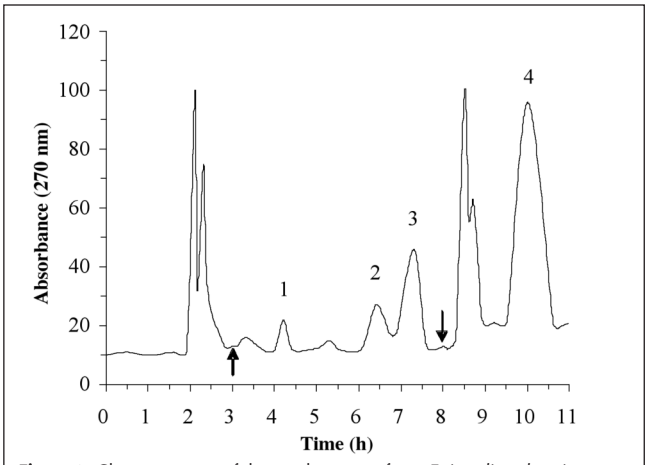

Figure 3. Chromatogram of the crude extract from *Epimedium brevicornum* Maxim by HSCCC separation: where peak 1 is epimedin A; peak 2 is epimedin B; peak 3 is epimedin C; and peak 4 is icariin. Experimental conditions: multilayer coil of 1.6 mm PTFE tube with a total capacity of 320 mL; rotary speed, 1000 rpm; solvent system, *n*-butanol–ethyl acetate–water (3:7:10, v/v); mobile phase, 0–8 h (the lower phase), and 8–12 h (the upper phase); flow-rate, 0–3 h (1 mL/min), 3–8 h (2 mL/min), and 8–11 h (1 mL/min); detection at 270 nm; sample size, 300 mg. The up arrow indicates that the flow-rate of the mobile phase was increased from 1 to 2 mL/min at 3 h, and the down arrow indicates that the flow-rate was decreased from 2 to 1 mL/min at 8 h, and the phase role was reversed.

When the two-phase solvent system composed of *n*-butanol–ethyl acetate–water at a volume ratio of 10:3:7 was used in dual-mode HSCCC method, all flavonoids could be eluted out in 11 h, and the peak resolution was satisfactory. Figure 3 shows the preparative HSCCC separation of 300 mg of the crude extract using a dual-mode method. The relative standard deviations of the retention times in the HSCCC separation were 2.1–4.6% (n = 8). HPLC analysis of each peak fraction of this preparative HSCCC revealed that four relatively pure flavonoids could be obtained from the crude extract in a one-step separation. The peaks were identified by comparison with the retention times and UV spectra of the standard flavonoids because they are the known structure compounds typically present in Epimedium brevicornum Maxim, and are also the main bioactive components of the plant (1). The 3.2 mg of epimedin A, 5.5 mg of epimedin B, 12.7 mg of epimedin C, and 42.9 mg of icariin were obtained, respectively, from 300 mg of the crude extract in a single run, which was the mean value of eight separations. Their purities were 98.2%, 92.6%, 90.4%, and 96.8%, and recoveries were 95.2%, 89.4%, 91.1%, and 94.8%, respectively.

Conclusions

An efficient method for the separation and purification of the bioactive components from the medicinal herb *Epimedium brevicornum* Maxim was developed by dual-mode HSCCC. The four relatively pure flavonoids epimedin A, B, C, and icariin were obtained from 300 mg of the crude extract in a one-step separation. Their purities were 90.4–98.2%, and the recoveries were 89.4–95.2%. The present study indicates that HSCCC is a powerful tool for the separation and purification of bioactive components from natural sources.

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