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Application of analytical and preparative high-speed counter-current chromatography for separation of alkaloids from *Coptis chinensis* Franch

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Abstract

Analytical high-speed counter-current chromatography (HSCCC) was used for the systematic selection and optimization of the two-phase solvent system to separate alkaloids from *Coptis chinensis* Franch. The optimum solvent system thus obtained led to the successful separation of alkaloids from *C. chinensis* Franch by preparative HSCCC. One batch separation yielded four pure alkaloids, including palmatine, berberine, epiberberine and coptisine from the crude alkaloid extract.
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1. Introduction

Analytical-scale counter-current chromatography (CCC) was first carried out by Ito and Bowman [1] by means of the hydrostatic CCC system using a toroidal coil centrifuge in 1970. In 1987 analytical HS CCC utilizing the hydrodynamic CCC system was introduced [2] and it has been applied to the analytical separation of a variety of natural products, including alkaloids [3,4], anthraquinones [5], coumarins [6], flavonoids [7,8], lignans [9–11], macrolides [12,13], and triterpenoids [14]. It has also been effectively used for the determination of octanol–water partition coefficients [15].

The selection of the solvent system is the first and most important step in performing HSCCC. Although a systematic search for the two-phase solvent systems has been introduced for CCC [16,17], the method is focused on hydrophobicity of the solvent system. In order to separate charged analytes such as alkaloids, however, an additional adjustment is required with respect to pH and ionic strength of the solvent system, and at present this can only be possible by a trial and error method. Because of its speedy separation and minimum solvent consumption, analytical HSCCC offers very efficient means of testing the solvent system for providing a proper range of partition coefficients of analytes. Recently, several contributions have already shown that analytical HSCCC is very useful for method development [7,18].

Coptis chinensis Franch is an important traditional

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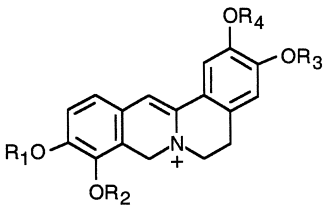

	R_1	R_2	R_3	R_4
palmatine	CH_3	CH_3	CH_3	CH_3
berberine	CH_3	CH_3	CH_2	
epiberberine	CH_2		CH_3	CH_3
coptisine	CH_2		CH_2	
jateorhizine	CH_3	CH_3	H	CH_3
columbamine	CH_3	CH_3	CH_3	H

Fig. 1. Structural illustration of alkaloids from *C. chinensis* Franch.

Chinese medicinal herb which has been effectively used for heat-clearing, damp-drying, relieving fidgetiness and detoxicating, etc. The major active constituents of this herb are alkaloids, including berberine, epiberberine coptisine, palmatine, jateorhizine and columbamine, and their structures are illustrated in Fig. 1. The berberine, present at about 10% in *C. chinensis* Franch, has a strong antibacterial action on shigella dysenteriae, staphylococci and streptococci, and has been used for the treatment of dysentery. All other alkaloids from *C. chinensis* Franch possess a distinct anti-inflammatory action.

In this paper, analytical HSCCC is used for the systematic selection and optimization of the two-phase solvent system for the separation of alkaloids from *C. chinensis* Franch. Using the optimized solvent system the preparative HSCCC separation of the crude alkaloids is performed from the crude extract of *C. chinensis* Franch.

2. Experimental

2.1. Apparatus

The analytical HSCCC instrument employed in the present study is a new Model GS 20 analytical high-speed counter-current chromatograph designed and constructed at Beijing Institute of New Technology Application, Beijing, China. The apparatus holds a pair of column holders symmetrically on the rotary frame at a distance of 5 cm from the central axis of the centrifuge. The multilayer coiled separation column was prepared by winding a 50 m×0.85 mm I.D. PTFE (polytetrafluoroethylene) tube directly onto the holder hub forming multiple coiled layers with a total capacity of 30 ml. The β value varied from 0.4 at the internal terminal to 0.7 at the external terminal ($\beta=r/R$ where r is the distance from the coil to the holder shaft, and R, the revolution radius or the distance between the holder axis and the central axis of the centrifuge). Although the revolution speed of the apparatus could be regulated with a speed controller in a range between 0 to 2000 rpm, an optimum speed of 1500 rpm was used in the present studies.

The solvent was pumped into the column with a Model NS-1007 constant-flow pump (Beijing Institute of New Technology Application, Beijing, China). Continuous monitoring of the effluent was performed with a Model 8823A-UV Monitor (Beijing Institute of New Technology Application) at 254 nm. A manual sample injection valve with a 1.0-ml loop (Tianjin High-New Science and Technology Company, Tianjin, China) was used to introduce the sample into the column. A portable recorder (Yokogawa Model 3057, Sichuan Instrument Factory, Chongqin, China) was used to draw the chromatogram.

The details of the preparative HSCCC system employed have been described earlier [19]. A Model RE-90 rotary evaporator and a Model FC-95 autofraction collector (both from Beijing Institute of New Technology Application) were also used.

2.2. Reagents

All organic solvents and hydrochloric acid used for preparation of the two-phase solvent system are of analytical grade and purchased from Beijing Chemical Factory (Beijing, China).

2.3. Extraction of crude alkaloids

Raw roots of *C. chinensis* Franch (ca. 1.0 kg) were extracted with methanol three times at room tem-

perature. The extracts were combined and evaporated to dryness under reduced pressure. The above crude extracts containing alkaloids as major components was directly subjected to HSCCC.

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

The present study utilized a series of two-phase solvent systems composed of chloroform, methanol and dilute hydrochloric acid. Each solvent system was prepared by mixing the solvents at the specified volume ratios and thoroughly equilibrating in a separatory funnel at room temperature. The two phases were separated shortly before use.

The sample solutions were prepared by dissolving the crude alkaloid extract of *C. chinensis* Franch in the lower organic phase at suitable concentrations for analytical and preparative purposes.

2.5. Separation procedure

The analytical HSCCC was performed with a Model GS 20 HSCCC instrument as follows: the multilayer coiled column was first entirely filled with the upper aqueous stationary phase. The lower organic mobile phase was then pumped into the head end of the column at a flow-rate of 1.0 ml/min, while the apparatus was run at a revolution speed of 1500 rpm. (Head–tail relationship of the coil is defined by an Archimedean Screw force where all objects different in density move toward the head of the coil). After hydrodynamic equilibrium was reached, as indicated by a clear mobile phase eluting at the tail outlet, the sample solution (2.5 mg in 1 ml mobile phase) was injected through the sample port. The effluent from the tail end of the column was continuously monitored with a UV detector at 254 nm. After the separation was completed, the apparatus was stopped and the column contents were collected into a graduated cylinder by connecting the inlet of the column to a nitrogen line under about 80 p.s.i. (1 p.s.i.=6894.76 Pa). The retention of the stationary phase relative to the total column capacity was computed from the volume of the stationary phase collected from the column.

The preparative HSCCC was performed with a Model GS10A2 HSCCC centrifuge equipped with a multilayer coil column of 1.6 mm I.D. and 230 ml in total volume and a 20-ml sample injection loop. The mobile phase was eluted at a flow-rate of 2.0 ml/min at a revolution speed of 800 rpm. The peak fractions were collected with test tubes according to the chromatogram.

2.6. Analyses and structural identification of CCC peak fractions

All peak fractions were analyzed using silica gel G thin-layer chromatography (TLC) plates by developing with a solvent mixture composed of C_6H_6–EtOAc–CH_3OH–$(CH_3)_2CHOH$–aqueous NH_3 (12:6:3:3:1, v/v) and staining with a Dragendorff reagent to detect alkaloids. Alternatively, the spots on the TLC plates were observed under ultraviolet lamp. The alkaloids purified by HSCCC were identified by mass spectrometry (MS), nuclear magnetic resonance (NMR), IR and UV detection.

3. Results and discussion

During the selection of two-phase solvent system, a solvent system composed of $CHCl_3$–MeOH–water (4:3:2, v/v) was first applied for the analytical separation of the crude alkaloids from *C. chinensis* Franch. In this separation, there were no alkaloids eluted in 2.5 h. In the following separation with a solvent system composed of $CHCl_3$–MeOH–0.05 *M* HCl (4:3:2, v/v), all major alkaloid components were eluted within 2 h but with poor resolution. Further studies were carried out using the above solvent system composed of chloroform, methanol and dilute HCl by systematically modifying the relative volumes of methanol (4:3:2–4:1.5:2) and the concentration of HCl (0.3–0.1 *M*).

Fig. 2 shows the results of separations of the crude alkaloids from *C. chinensis* Franch by analytical HSCCC using a set of the above mentioned solvent systems. In this figure, nine chromatograms are arranged in such a way that the effects of methanol and HCl concentrations on the separation of alkaloids are each clearly visualized. As seen from the top to the bottom rows, decreasing the relative volumes of methanol in the solvent system from 4:3:2 to 4:1.5:2 increases the retention time of alkaloids. A similar effect is also produced by

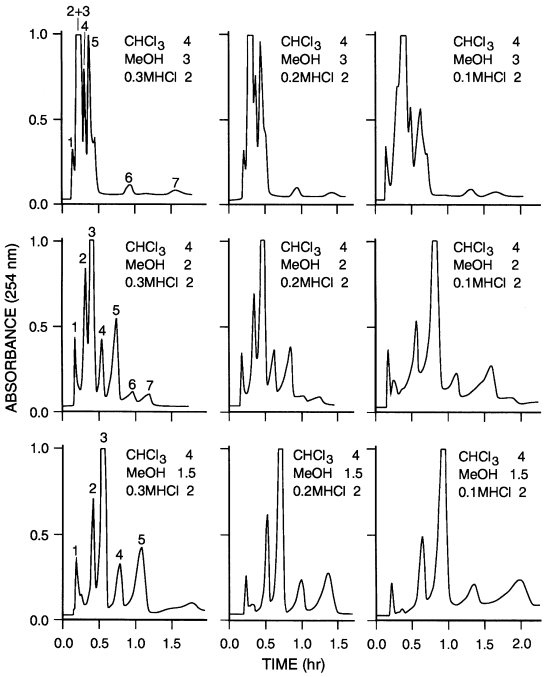

Fig. 2. Chromatograms of the crude alkaloids from *C. chinensis* Franch by analytical HSCCC. Nine chromatograms are arranged in such a way that the effects of methanol and HCl concentrations on the alkaloid separation are each clearly visualized. Experimental conditions: apparatus: analytical HSCCC instrument equipped with a multilayer coil of 0.85 mm I.D. and 30 ml capacity; sample: 2.5 mg of crude alkaloid extract of *C. chinensis* Franch; solvent system: shown above each chromatogram; mobile phase: lower organic phase; flow-rate: 1 ml/min; revolution: 1500 rpm. Retention of the stationary phase was as follows: $CHCl_3$–MeOH–(0.1–0.3 *M* HCl) (4:3:2), 77%; $CHCl_3$–MeOH–(0.1–0.3 *M* HCl) (4:2:2), 80%; and $CHCl_3$–MeOH–(0.1–0.3 *M* HCl) (4:1.5:2), 77%.

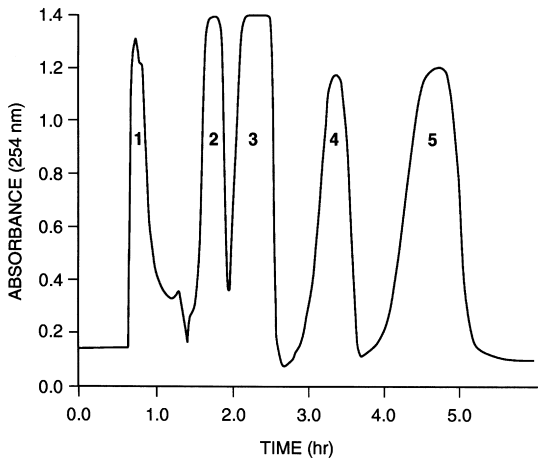

Fig. 3. Chromatogram of the crude alkaloids from *C. chinensis* Franch by preparative HSCCC with $CHCl_3$–MeOH–0.2 *M* HCl (4:1.5:2, v/v). Mobile phase: lower phase; flow-rate: 2.0 ml/min; sample size: 200 mg dissolved in 10 ml mobile phase. 1: unknown compound; 2: palmatine; 3: berberine; 4: epiberberine; 5: coptisine. Retention of the stationary phase was 73.3%.

decreasing the concentration of HCl from 0.3 *M* to 0.1 *M* in the solvent system (from left to right in each row). Among those, the solvent system composed of $CHCl_3$–MeOH–0.2 *M* HCl (4:1.5:2, v/v) (bottom row, center) produced the best separation of all four major components of alkaloids (peaks 2–5) within 1.5 h while minor components (peaks 6 and 7) were still retained in the column. On the other hand, the solvent systems containing a large volume of methanol (top row) produced a good separation for peaks 6 and 7 within 2 h.

In order to separate a large amount of alkaloids from *C. chinensis* Franch by preparative HSCCC, the above solvent system composed of $CHCl_3$–MeOH–0.2 *M* HCl (4:1.5:2, v/v) was chosen, and 200 mg of the crude alkaloids sample dissolved in 10 ml mobile phase was injected into the column. Fig. 3 shows the results of this separation. Four main alkaloids, berberine, epiberberine, coptisine and palmatine, were well resolved within 5 h.

Fig. 4 shows the result of the TLC analyses of various samples including the crude alkaloid extract of *C. chinensis* Franch and HSCCC fractions corresponding to peaks 2, 3, 4 and 5 (Fig. 3). Silica gel G TLC plates were developed with a solvent mixture composed of C_6H_6–EtOAc–CH_3OH–$(CH_3)_2CHOH$–aqueous NH_3 (12:6:3:3:1, v/v) and stained with a Dragendorff reagent to detect the alkaloids. Alternatively, spots on the TLC plates were observed under a UV lamp. The result indicated that HSCCC fractions corresponding to peaks 2, 3, 4 and 5 each contained a single alkaloid species which was identified as palmatine, berberine, epiberberine and coptisine, respectively, by MS, NMR, IR and UV.

The results of our studies clearly demonstrated the advantage of HSCCC in both analytical and preparative separations of alkaloids from a crude extract of *C. chinensis* Franch. Especially analytical HSCCC with its speedy separation and minimum solvent

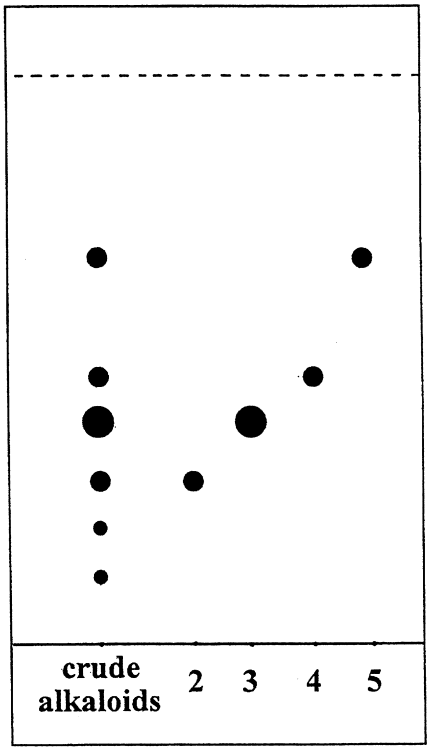

Fig. 4. The result of TLC analyses of the crude alkaloid extract of *C. chinensis* Franch and HSCCC fractions corresponding to peaks 2, 3, 4 and 5 made by a silica gel G TLC plate developed with a solvent system composed of C_6H_6–EtOAc–CH_3OH–$(CH_3)_2CHOH$–aqueous NH_3 (12:6:3:3:1, v/v).

consumption offers a very efficient means to carry out optimization of solvent systems for separation and purification of natural products by preparative HSCCC.

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