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Preparative separation of quaternary ammonium alkaloids from *Coptis chinensis* Franch by pH-zone-refining counter-current chromatography

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Abstract

pH-Zone-refining counter-current chromatography was successfully applied to the preparative separation of five quaternary ammonium alkaloids from the crude extract of *Coptis chinensis* Franch. The separation was performed with a two-phase solvent system composed of chloroform–methanol–water (4:3:3, v/v), where the upper aqueous stationary phase was added with 60 mM of hydrochloric acid and the lower organic mobile phase with 5 mM of triethylamine. From 1.0 g of crude extract, 5.4 mg of columbamine at 96.6% purity, 6.1 mg of jateorhizine at 98.8% purity, 58.3 mg of coptisine at 99.5% purity, 25.6 mg of palmatine at 98.4% purity and 503.9 mg of berberine at 99.5% purity were obtained. The purities of the isolated alkaloids were analyzed by HPLC and the chemical structures were identified by electrospray ionization-mass spectrometry and ¹H-NMR.

Keywords

pH-Zone-refining counter-current chromatography; *Coptis chinensis* Franch; Quaternary ammonium alkaloids

1. Introduction

pH-Zone-refining counter-current chromatography (pH-zone-refining CCC) is a kind of liquid-liquid partition chromatography that enables the separation of organic acids and bases into a succession of highly concentrated rectangular peaks that elute according to their pK_a values and hydrophobicities [1–4]. It extends the capability of standard high-speed counter-current chromatography (HSCCC) by various ways including an over ten-fold increase in

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sample loading capacity, high concentration of fractions, high purity, concentration of minor impurities, etc. In addition, this method enables detection of samples by their pH, which is especially useful if the analyte has low ultraviolet absorbance [2,3,5]. It has been successfully used as a large-scale preparative technique for separating ionizable compounds which include alkaloids [6–8], synthetic colors [9, 10], isomers [11–13], peptide derivatives [14, 15], etc.

Coptis chinensis Franch, as an important source of Traditional Chinese Medicine, mainly distributes in Yunnan and Xizang province of China. This herbal medicine has been officially listed in the Chinese Pharmacopoeia [16] and possesses broad-spectrum antibacterial and antiprotozoal effects. It is effective for clearing away heat and depriving dampness, and has always been used for the treatment of dysentery, hypertension, inflammation, diabetes, cancer, and liver disease in China [17, 18]. The major active constituents of this herb are alkaloids such as columbamine, jateorhizine, coptisine, palmatine and berberine as shown in Fig. 1. It also has been reported that these alkaloids from the root of *Coptis chinensis* Franch have antiprotozoal, antihypertensive, anticholinergic, antiarrhythmic and anticancer biological activities [19–23]. One of the important major component of alkaloid, berberine, has strong anti-inflammatory and antimicrobial activities [24, 25]. Recently, cholesterol-lowering and antidiabetic effects of berberine have been approved [26, 27]. Coptisine, another major component of alkaloid, could inhibit the monoamine oxidase type A, the proliferation of vascular smooth muscle cells and the differentiation of osteoclast. It also has the property of myocardial preservation [28]. Besides, as an important alkaloid, palmatine has the activity of epinephrine and anti-norepinephrine [29]. Considering the aforementioned pharmacological effects, an efficient method for the preparative isolation and purification of alkaloids from *Coptis chinensis* Franch is of great significance. Generally, alkaloids are present as an ionized form in the acidic solution and in as a molecular form in the neutral or basic solution. Therefore, they are good candidates for pH-zone-refining CCC. However, quaternary ammonium alkaloids show high alkalinity and polarity, which may lead to much more difficulties for the separation and purification by pH-zone-refining CCC.

In present study, optimization of the two-phase solvent system and the retainer acid and eluter base of pH-zone-refining CCC was investigated. Five quaternary ammonium alkaloids were successfully separated from the crude extract of *Coptis chinensis* Franch in a single run with the optimized solvent system and their chemical structures were shown in Fig. 1.

2. Materials and methods

2.1 Materials and reagents

All the organic solvents used for the preparation of crude extract and the pH-zone-refining CCC were of analytical grade (Cinc High Purity Solvents (Shanghai) Co., Ltd., Shanghai, China). Acetonitrile used for HPLC analysis was of chromatographic grade (Tedia Company, Inc., Fairfield, USA). The reverse osmosis Milli-Q water (Millipore, USA) was used for all solutions and dilutions.

The material, *Coptis chinensis* Franch, was collected in Sichuan province. The species was identified by Dr. Jia Li (Shandong University of Traditional Chinese Medicine, Jinan, China).

2.2 Apparatus

pH-Zone-refining CCC instrument involved in this study is an Emilion-300 HSCCC (Beijing Emilion Technology, Co., Ltd., Beijing, China), which has three multilayer coil separation columns connected in series (I.D. of the tubing = 1.6 mm, total volume = 260 ml) and a 20 ml sample loop. The distance between the holder axis and central axis of the centrifuge (R) is 5 cm, and the β values of the multilayer coil range from 0.47 at the internal terminal to 0.73 at the external terminal ($\beta=r/R$, where r is the rotation radius or the distance from the coil to the holder shaft). The revolution speed of this apparatus can be regulated with a speed controller in the range of 0 to 1,000 rpm. In order to control the separation temperature, the HSCCC system is equipped with an HX 1005 constant-temperature circulating implement (Zhengzhou Great Wall Scientific Industrial and Trade Co., Ltd, Zhengzhou, China). Using an Emilion constant-flow pump (Beijing Emilion technology Co., Ltd., Beijing, China), the solvent is pumped into the column. Continuous monitoring of the effluent is performed by a Model 8823A-UV Monitor (Beijing Emilion technology, Co., Ltd., Beijing, China) operating at 254 nm and a Model UB-7 pH meter (Denver Instrument, Beijing, China). A portable recorder (Yokogawa Model 3057, Sichuan Instrument Factory, Chongqing, China) is used for drawing the pH-zone-refining CCC chromatogram.

The analysis of the crude sample and the purified alkaloids is performed by a Waters Millennium 32 system including a Waters 996 Photodiode Array Detection (DAD) system, a Waters 600 Multisolute Delivery System, a Waters 600 System controller, a Waters 600 pump, and a Millennium 32 work-station (Waters, Milford, USA).

2.3 Extraction of crude sample for pH-zone-refining CCC separation from *Coptis chinensis* Franch

Half a kilogram of dried *Coptis chinensis* Franch powder was extracted with 2 L of 70% aqueous ethanol under reflux in water bath (65°C), each for 2 h. The extraction was repeated two additional times and the extract were combined. After filtration, the extract was concentrated on a rotavapor at 55°C. The residues were dissolved in 500 ml of 1.5% H₂SO₄ solution. And the acidic solution was basified to pH 5.0–6.0 with Ca(OH)₂. After two hours' standing, the solution was filtered and concentrated at 55°C under reduced pressure to a volume of 200 ml, in which 1 ml extract equaled to 0.25 g of the dried powder. The extract was acidized to pH 1.0–2.0 with HCl and 5.0% NaCl solution was added to the solution to accelerate the process of crystallization. The container filled with the above solution was stored in the fridge at 4°C overnight. The educts were filtered with Buchner funnel and dried by the vacuum drying apparatus, 150 g of the crude extract was used for further separation [30].

2.4 Preparation of solvent system and sample solution

The two-phase solvent system composed of chloroform (CHCl₃)–methanol (MeOH)–water (H₂O) (4:3:3, v/v) was equilibrated in a separatory funnel, and the two phases were

separated before use. The upper aqueous phase (the stationary phase) was acidified with HCl at the concentration of 60 mM. The lower organic phase (the mobile phase) was rendered basic by adding triethylamine (TEA), resulting in a 5 mM solution.

The sample solution was prepared by dissolving 1.0 g of the crude extract in 10 ml of the aqueous phase with 60 mM HCl and 10 ml of the organic phase without TEA.

2.5 Separation procedure

The separation was initiated by filling the whole column with the upper aqueous phase as the stationary phase at 20 ml/min and then the sample dissolved in a mixture of stationary and mobile phases (in the ratio of 1:1) was injected. After the column was rotated at 850 rpm for a while, the mobile phase was pumped into the column at 2.0 ml/min. The UV absorption of the effluent was continuously monitored by a UV monitor at 254 nm and 10-ml fractions were collected. At the same time, the pH of each fraction was measured with a pH meter. After the separation was completed, the retention of the stationary phase was measured by forcing the column contents into a graduated cylinder with pressurized air.

2.6 Analysis and identification of pH-zone-refining CCC fraction

The purified fractions from the pH-zone-refining CCC as well as the crude sample were analyzed by HPLC with a Shim-pack VP-ODS column (250 mm $\times$ 4.6 mm, i.d., 5 μ m) at 345 nm and column temperature of 25°C. The mobile phase was acetonitrile–0.1% TEA and 2% acetic acid aqueous solution (35:65, v/v). The effluent was monitored by a photodiode array detector and the injection volume was 10 μ L.

The identification was performed by electrospray ionization tandem mass spectrometry (ESI-MS) with an Agilent 5973N mass selective detector (MSD), and Pentium 4 computer with mass selective detector Productivity Chemstation Software. The mass spectrometer was scanned over the range 29–400 m/z scan 1 s, with an ionizing voltage of 70 eV and an ionization current of 150 mA. The NMR spectrum was recorded with a Varian-600 spectrometer (Varian, Palo Alto, CA, USA) with DMSO as the solvent and tetramethylsilane (TMS) as an internal standard.

3. Results and discussion

3.1 Optimization of the two-phase solvent system for pH-zone-refining CCC

A successful separation by pH-zone-refining CCC requires a suitable two-phase solvent system which could provide ideal K_D values in both acidic ($K_{acid} \ll 1$) and basic ($K_{base} \gg 1$) conditions as well as good solubility of the crude sample [2]. According to the typical separations by pH-zone-refining CCC, two solvent systems composed of MtBE–CH₃CN–water and petroleum ether–ethyl acetate–methanol–water each with different volume ratios were first investigated. Although several volume ratios of these two solvent systems produced suitable K_D values for the main compounds, none of them could be used for the separation of this crude sample because of the poor solubility of lower than 0.2 g in both phases. Considering of the high polarity of the quaternary ammonium alkaloids, we thought that the crude sample might have good solubility in chloroform which is often employed for

the extraction of alkaloids and the solvent system of $\text{CHCl}_3\text{--MeOH--H}_2\text{O}$ was examined. The K_D values of this two-phase solvent system at the volume ratios of 4:2:2 and 4:3:3 were evaluated. The results showed that both of these two ratios provided ideal K_D values, which could be suitable for the separation of these quaternary ammonium alkaloids. And the solubility of the crude sample in the solvent system was greatly improved. After testing the biphasic solvent system at the volume ratio of 4:2:2 with 10 mM HCl in the upper phase and 10 mM TEA in the lower phase on the HSCCC apparatus, the result was not acceptable because the target compounds were eluted with a lot of impurities and the boundaries between two absorbance plateaus were ambiguous. While, when the biphasic solvent system $\text{CHCl}_3\text{--MeOH--H}_2\text{O}$ (4:3:3, v/v) with 10 mM HCl in the upper phase and 10 mM TEA in the lower phase was employed, the resolution of the target quaternary ammonium alkaloids and impurities could be improved. However, the resolution between the target quaternary ammonium alkaloids was also not perfect and the crude sample was only separated into four fractions as shown in Fig. 2. Based on the above experiments, an idea that could enhance the resolution by reducing the concentration of the eluter base and increasing the retainer acid of the biphasic solvent system occurred to us [31]. The concentration of the eluter in the mobile phase and retainer in the stationary phase mainly determined the concentration and retention time of analyte as well as the retention of the stationary phase. In order to achieve efficient resolution of the target compounds, a proper amount of the retainer and eluter should be added to the aqueous stationary phase and organic mobile phase, respectively. Although the target compounds B, C and D did not separate well and compound A was only partly separated with the purity of lower than 95% as determined by HPLC, the resolution was greatly improved and five fractions were clearly observed when the aqueous phase with 40 mM HCl and the organic phase with 10 mM TEA as shown in Fig. 3. However, the rectangular peaks were too narrow, which showed that the alkaloids were eluted too quickly. Therefore, the purification process was not efficient. What to our surprise was that the resolution of the sample was radically improved with 60 mM HCl in the aqueous stationary phase and 5 mM TEA in the organic mobile phase as shown in Fig. 4. Thus, the two-phase solvent system $\text{CHCl}_3\text{--MeOH--H}_2\text{O}$ (4:3:3, v/v) with 60 mM HCl in aqueous stationary phase and 5 mM TEA in organic mobile phase was selected for the separation of the crude alkaloids.

3.3 Preparative separation of alkaloids by pH-zone-refining CCC

Fig. 4 shows a typical chromatogram of pH-zone-refining CCC for the separation of 1.0 g of the crude extract using the two-phase solvent system $\text{CHCl}_3\text{--MeOH--H}_2\text{O}$ (4:3:3, v/v) with 5 mM TEA in organic mobile phase and 60 mM HCl in aqueous stationary phase. The total separation time was about 10 h. These quaternary ammonium alkaloids were eluted as five irregular rectangular peaks or (plateaus A, B, C, D and E in Fig. 4). The measurement of pH values of collected fractions also revealed five flat pH-zones, indicating the successful separation of the five target quaternary ammonium alkaloids. This separation yielded 5.4 mg of compound A, 6.1 mg of compound B, 58.3 mg of compound C, 25.6 mg of compound D, and 503.9 mg of compound E from 1.0 g of crude sample with a purity of over 96.6% as determined by HPLC (in Fig. 4).

In the past, alkaloids from *Coptis chinensis* Franch have been separated by the standard HSCCC method and the sample-loading capacity was 0.2 g [32]. The present system using pH-zone-refining CCC could separate a much larger amount of target compounds at higher purity in a single run, indicating that pH-zone-refining CCC had many advantages over the standard HSCCC. It had about 10-fold increase in sample-loading capacity with higher purity, higher yield, higher recovery and higher concentration of the separated fractions.

3.4 HPLC analysis of the crude sample and pH-zone-refining CCC fractions

Analytical HPLC, as one of the most rapid and exact analytical method, was employed to analyze the crude sample and the purified alkaloids. The target compounds could get baseline separation when acetonitrile–0.1% TEA and 2% acetic acid aqueous solution (35:65, v/v) was used as mobile phase and the flow-rate was set at 0.7 ml/min [33]. The HPLC chromatogram of crude extract is shown in Fig. 2. It mainly contained five peaks (peak A, B, C, D, and E), which correspond to columbamine, jateorhizine, coptisine, palmatine and berberine in sequence, based on the peak area normalization method at the optimized detective wavelength of 345 nm.

Fig. 2 shows HPLC chromatogram of crude sample in which peak A represented 0.78% of the total sample and peak B, C, D, and E was 1.04%, 10.65%, 3.96%, and 81.28% of the total sample, respectively. Fig. 4 displays the HPLC chromatograms of fractions separated by pH-zone-refining CCC. The results showed that these quaternary ammonium alkaloids were obtained with the purities of all over 96.6%.

3.5 Structural identification

Identification of the alkaloids purified by pH-zone-refining CCC was carried out by UV, ESI-MS and ¹H-NMR. The results for each alkaloid were as follows:

Compound A (peak A in Fig. 2): light yellow spiculas in chloroform, UV (λ MeOH max) absorption: 263 and 345nm. Positive ESI-MS, m/z 338 $[M+H]^+$. ¹H-NMR (600 MHz, DMSO): 7.57 (1H, s, H-1), 3.90 (3H, s, 3-OCH₃), 7.06 (1H, s, H-4), 3.19 (2H, t, J=6.0 Hz, H-5), 4.93 (2H, t, J=6.0 Hz, H-6), 9.88 (1H, s, H-8), 4.07 (3H, s, 9-OCH₃), 4.09 (3H, s, 10-OCH₃), 8.20 (1H, d, J=9.0 Hz, H-11), 8.06 (1H, d, J=9.0 Hz, H-12), 8.82 (1H, s, H-13). Compared with the data given in reference [33], compound A was identified as columbamine.

Compound B (peak B in Fig. 2): light red spiculas in chloroform, UV (λ MeOH max) absorption: 264 and 345nm. Positive ESI-MS, m/z 338 $[M+H]^+$. ¹H-NMR (600 MHz, DMSO): 7.69 (1H, s, H-1), 3.94 (3H, s, 2-OCH₃), 6.86 (1H, s, H-4), 3.14 (2H, t, J=6.0 Hz, H-5), 4.91 (2H, t, J=6.0 Hz, H-6), 9.86 (1H, s, H-8), 4.07 (3H, s, 9-OCH₃), 4.09 (3H, s, 10-OCH₃), 8.19 (1H, d, J=9.0 Hz, H-11), 8.01 (1H, d, J=9.0 Hz, H-12), 8.98 (1H, s, H-13). Compared with the data given in reference [34], compound B was identified as jateorhizine.

Compound C (peak C in Fig. 2): reddish-brown spiculas in chloroform, UV (λ MeOH max) absorption: 264 and 358nm. Positive ESI-MS, m/z 320 $[M+H]^+$. ¹H-NMR (600 MHz, DMSO): 7.79 (1H, s, H-1), 6.18 (2H, s, 2, 3-OCH₂O), 7.09 (1H, s, H-4), 3.20 (2H, t, J=6.0 Hz, H-5), 4.89 (2H, t, J=6.0 Hz, H-6), 9.97 (1H, s, H-8), 6.54 (2H, s, 9, 10-OCH₂O), 8.04

(1H, d, J=9.0 Hz, H-11), 7.83 (1H, d, J=9.0 Hz, H-12), 8.98 (1H, s, H-13). Compared with the data given in reference [35], compound C was identified as coptisine.

Compound D (peak D in Fig. 2): yellowish-brown pillars in chloroform, UV (λ MeOH max) absorption: 272 and 345nm. Positive ESI-MS, m/z 352 $[M+H]^+$. 1H -NMR (600 MHz, DMSO): 7.73 (1H, s, H-1), 3.94 (3H, s, 2-OCH₃), 3.88 (3H, s, 3-OCH₃), 7.10 (1H, s, H-4), 3.23 (2H, t, J=6.0 Hz, H-5), 4.96 (2H, t, J=6.0 Hz, H-6), 9.90 (1H, s, H-8), 4.08 (3H, s, 9-OCH₃), 4.10 (3H, s, 10-OCH₃), 8.20 (1H, d, J=9.0 Hz, H-11), 8.04 (1H, d, J=9.0 Hz, H-12), 9.08 (1H, s, H-13). Compared with the data given in reference [35], compound D was identified as palmatine.

Compound E (peak A in Fig. 2): yellow pillars in chloroform, UV (λ MeOH max) absorption: 263 and 346nm. Positive ESI-MS, m/z 336 $[M+H]^+$. 1H -NMR (600 MHz, DMSO): 7.80 (1H, s, H-1), 6.18 (2H, s, 2, 3-OCH₂O), 7.09 (1H, s, H-4), 3.21 (2H, t, J=6.0 Hz, H-5), 4.94 (2H, t, J=6.0 Hz, H-6), 9.90 (1H, s, H-8), 4.07 (3H, s, 9-OCH₃), 4.10 (3H, s, 10-OCH₃), 8.21 (1H, d, J=9.0 Hz, H-11), 8.01 (1H, d, J=9.0 Hz, H-12), 8.96 (1H, s, H-13). Compared with the data given in reference [35], compound E was identified as berberine.

4. Conclusions

The efficient pH-zone-refining CCC method for the separation and purification of five quaternary ammonium alkaloids from the crude extract of *Coptis chinensis* Franch was developed using CHCl₃-MeOH-H₂O (4:3:3, v/v) as the two-phase solvent system. After the two-phase solvent system had been equilibrated, 5 mM TEA was added to the organic mobile phase as an eluter and 60 mM HCl was added to the aqueous stationary phase as a retainer. From 1.0 g of the crude sample columbamine (5.4 mg) in 96.6% purity, jateorhizine (6.1 mg) in 98.8% purity, coptisine (58.3 mg) in 99.5% purity, palmatine (25.6 mg) in 98.4% purity, and berberine (503.9 mg) in 99.5% purity were obtained in a single run. The results of our studies could demonstrate the following three points: (1) the alkaloid sample could be pre-purified by the acid-base partitioning method, which is very important for an efficient separation; (2) for pH-zone-refining CCC a good separation is obtained by optimizing the concentration of the retainer in the stationary phase and the eluter in the mobile phases; (3) the pH-zone-refining CCC is a rapid and efficient method for the separation and purification of highly polar compounds such as these quaternary ammonium alkaloids.

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	R ₁	R ₂	R ₃	R ₄
columbamine	CH ₃	CH ₃	CH ₃	H
jatrorrhizine	CH ₃	CH ₃	H	CH ₃
coptisine
palmatine	CH ₃	CH ₃	CH ₃	CH ₃
berberine	CH ₃	CH ₃

Fig. 1.
Chemical structures of alkaloids from *Coptis chinensis* Franch.

Fig. 2. pH-Zone-refining CCC and HPLC control for the separation of the crude extract from *Coptis chinensis* Franch. Experimental conditions: Solvent system: CHCl_3 -MeOH- H_2O (4:3:3, v/v); 10 mM HCL in upper aqueous phase and 10 mM TEA in lower organic phase; revolution speed: 850 rpm; flow-rate: 2 ml/min; sample size: 1.0 g; UV detection wavelength: 254 nm.

Fig. 3. pH-Zone-refining CCC and HPLC control for the separation of the crude extract from *Coptis chinensis* Franch. Experimental conditions: Solvent system: CHCl_3 -MeOH- H_2O (4:3:3, v/v); 40 mM HCL in upper aqueous phase and 10 mM TEA in lower organic phase; revolution speed: 850 rpm; flow-rate: 2 ml/min; sample size: 1.0 g; UV detection wavelength: 254 nm.

Fig. 4.

pH-Zone-refining CCC and HPLC control for the separation of the crude extract from *Coptis chinensis* Franch. Experimental conditions: Solvent system: CHCl_3 -MeOH- H_2O (4:3:3, v/v); 60 mM HCL in upper aqueous phase and 5 mM TEA in lower organic phase; revolution speed: 850 rpm; flow-rate: 2 ml/min; sample size: 1.0 g; UV detection wavelength: 254 nm.