

PREPARATIVE SEPARATION OF C₁₉-DITERPENOID ALKALOIDS FROM *Aconitum carmichaelii* Debx BY pH-ZONE-REFINING COUNTER-CURRENT CHROMATOGRAPHY

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Recebido em 28/3/13; aceito em 27/6/13; publicado na web em 21/8/13

The technique of pH-zone-refining counter-current chromatography was successfully applied to preparatively separate three C₁₉-diterpenoid alkaloids from the crude extracts of *Aconitum carmichaelii* for the first time using a two-phase solvent system of petroleum ether–ethyl acetate–methanol–water (5:5:1:9, v/v/v/v). Mesaconitine (I), hypaconitine (II), and deoxyaconitine (III) were obtained from 2.5 g of the crude alkaloids in a one-step separation; the yields were 4.16%, 16.96%, and 5.05%, respectively. The purities of compounds I, II, and III were 93.0%, 95%, and 96%, respectively, as determined by HPLC. The chemical structures of the three compounds were identified by electrospray ionization mass spectrometry (ESI-MS) and NMR.

Keywords: *Aconitum carmichaelii* Debx; pH-zone-refining counter-current chromatography; diterpenoid alkaloids.

INTRODUCTION

Aconitum L. (Ranunculaceae) is a large genus with approximately 300 species and is widely distributed in southwestern China. Approximately 76 aconitum species in China have been used for the treatment of various types of pain and rheumatoid arthritis.¹ *Aconitum carmichaelii* Debx is widely distributed in the Yunnan, Guizhou, and Sichuan provinces of China.² The tubers of *Aconitum carmichaelii* are an important ingredient of Chinese medical preparations and have long been used as a cardiotoxic, diuretic, and analgesic.³ The C₁₉-diterpenoid alkaloids are the major bioactive compounds in the tubers of *Aconitum carmichaelii* and have been widely used due to their predominant analgesic, antipyretic, anti-rheumatoid arthritis, and anti-inflammation effects.^{4,5} To further study the biological activities of these alkaloids and to control the quality of this traditional Chinese medicine and its extract, a large quantity of pure compounds are urgently needed. Therefore, the development of an efficient method for the separation and purification of C₁₉-diterpenoid alkaloids from *Aconitum carmichaelii* is required.

Conventional separation methods such as column chromatography and thin-layer chromatography (TLC) are tedious and require multiple chromatographic steps.⁴ In contrast, preparative pH-zone-refining counter-current chromatography (pH-zone-refining CCC), which was first introduced by Ito,⁶ is an efficient and simple liquid-liquid partition chromatography method that has been applied to separate and purify a variety of ingredients, including many natural products.⁷ The method can separate organic acids and bases into a succession of highly concentrated rectangular peaks with minimum overlap, indicating that the mixtures have been separated into highly concentrated fractions of ionized compounds. The method offers various important advantages compared to conventional counter-current chromatography, including a more than 10-fold increase in sample loading capacity, high concentrations of fractions, and low concentrations of minor impurities.⁶ As far as we are aware, no accounts of the use of pH-zone-refining CCC to isolate and purify

C₁₉-diterpenoid alkaloids have been reported in the literature. We herein report an efficient method for the preparative separation and purification of three C₁₉-diterpenoid alkaloids from *Aconitum carmichaelii* by pH-zone-refining CCC. The chemical structures of the three C₁₉-diterpenoid alkaloids are shown in Figure 1.

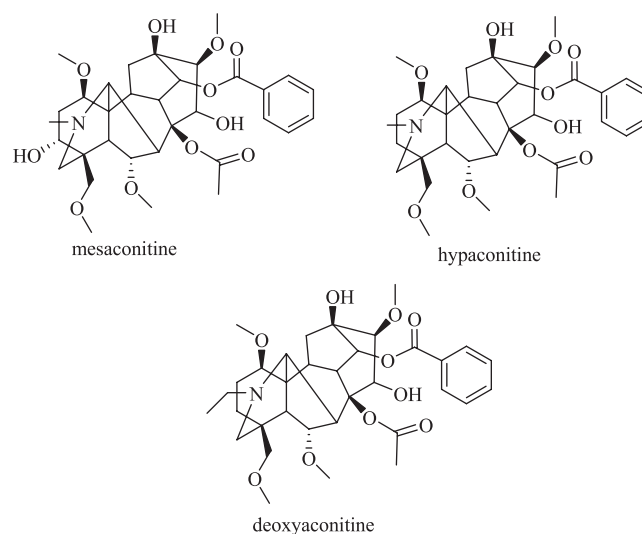


Figure 1. Chemical structures of compounds from *Aconitum carmichaelii* Debx

EXPERIMENTAL

Apparatus

The pH-zone-refining CCC method was performed using a model TBE-300A ordinary CCC system (Shanghai, Tauto Biotech, China) equipped with three PTFE preparative coils (internal diameter of tube: 1.6 mm; total volume: 300 mL) and a 20-mL sample loop. The β -values of this preparative column ranged from 0.47 at the internal to 0.73 at the external ($\beta = r/R$, where r is the rotation radius or the distance from the coil to the holder shaft, and R ($R = 7.5$ cm)

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is the revolution radius or the distance between the holder axis and the central axis of the centrifuge). The solvent was pumped into the column by a model NS-1007 constant-flow pump (Beijing Emilion Science & Technology, Beijing, China). The effluent was continuously monitored with a model 8823A UV detector (Beijing Emilion Science & Technology) at 254 nm and a model UB-7 pH meter (Denver Instruments, Beijing, China). A model 3057 portable recorder (Yokogawa, Sichuan Instrument Factory, Chongqing, China) was used to record the chromatogram.

The HPLC system was equipped with a Waters 600 pump, a Waters 600 controller and a Waters 996 photodiode array detector (Waters, USA). Evaluation and quantification were performed on an Empower pro data handling system (Waters, USA).

Reagents and materials

All organic solvents used for the preparation of extracts and for CCC separation were of analytical grade (Juye Chemical Factory, Jinan, China). Methanol used for HPLC was chromatographic grade (Yucheng Chemical Factory, Yucheng, China). The water used throughout the study was purified by a Milli-Q water purification system (Millipore, USA).

The root of *Aconitum carmichaelii* were collected from Yunnan province and were identified by Dr. Jia Li (Shandong University of Traditional Chinese Medicine, China).

Preparation of crude extract

Five kilograms of dried root of *Aconitum carmichaelii* was ground into powder and extracted three times using an ultrasonic circulation extraction apparatus (Hong Xianglong Company, Binjing, China) with 10 L of an 80% ethanol solution that contained 10 mL of HCl. After filtration, the extract was combined and evaporated to dryness by rotary vaporization under reduced pressure. The residue was then dissolved in 2 L of 1% HCl. The acidic extracts were basified to pH 9.5 with NH₃-water after being extracted with petroleum ether. The basified extracts were then extracted with chloroform and evaporated to dryness. Sixteen grams of crude alkaloids were obtained and were used for further isolation and separation; the yield of crude alkaloids was 0.32%.

Determination of the partition coefficient (*K*)

The two-phase solvent system was selected based on the partition coefficient (*K*) of the target components according to the rules introduced by Ito.⁸ The *K* values were determined using HPLC analysis as follows. Volumes (0.5 mL) of the upper and lower phases were placed into 1.5-mL tubes. A suitable amount of sample was added, as was 5 μL of HCl (*K*_{acid}) or NH₃-water (*K*_{base}). The solution was then shaken. Volumes (200 μL) of the upper phase were placed into another tube and were dried with nitrogen. The residues were diluted to 200 μL with methanol and analyzed by HPLC. Volumes (200 μL) of the lower phase were placed into another tube and were dried with nitrogen. Residues were diluted to 200 μL with methanol and were analyzed by HPLC. The *K* value was defined as the peak area of the target compound in the upper phase divided by the peak area of the lower phase.⁶

Preparation of solvent systems and sample

Two liters of solvent systems composed of Pet-EtAc-MeOH-H₂O were equilibrated in a separatory funnel, and the two phases were subsequently separated. Then, HCl was added to the mobile lower phase to obtain a final concentration of 10 mM, which served as an

eluter, whereas triethylamine was added to the stationary upper phase to obtain a final concentration of 10 mM, which served as a retainer.

The sample solution was prepared by dissolving 2.5 g of crude alkaloids in 20 mL of the solvent consisting of approximately 10 mL of the upper phase with triethylamine and 10 mL of the lower phase without HCl.

The CCC separation procedure

In this separation process, the upper phase was pumped into the multilayer-coiled column as stationary phase, and the sample solution was subsequently loaded. The apparatus was rotated at 850 rpm in head-to-tail mode while the lower phase, as the mobile phase, was pumped through the column at flow rate of 2 mL/min. The absorbance of the effluent was continuously monitored at 254 nm, and 10-mL fractions were collected. The pH of each eluted fraction was measured with a pH meter. After the separation was completed, we measured the retention of the stationary phase by collecting the column contents into a graduated cylinder by forcing them out of the column with pressurized nitrogen gas. The fractions collected were brought to dryness using rotary vaporization under reduced pressure and were analyzed by HPLC. The whole process of separation was conducted at room temperature.

Analysis and identification of peak fractions

The crude sample and each purified fraction were analyzed by HPLC using an Inertsil ODS-SP column (4.6 mm × 250 mm, I.D., 5 μm). The mobile phase was an 80:20, v/v solution of methanol and water that contained 0.2% triethylamine. Other parameters were as follows: flow rate, 1.0 mL/min; wavelength, 230 or 254 nm; column temperature, 25 °C. The purity of the target compounds were determined using the peak-area method, where the peak area of the target compound was divided by the peak area of the total area of all peaks.

The identification of CCC peak fractions was performed by electrospray ionization mass spectrometry (ESI-MS) on an Agilent 1100/MS-G1946 (Agilent Technologies, USA) equipped with an electrospray ionizer. The spray voltage was set at (+)4000 V or (-)4000 V. Nitrogen was used as the nebulizer gas, and the nebulizer pressure was set at 35 psi. Desolvation gas (nitrogen) was heated to 300 °C and was delivered at a flow rate of 10 L/min. The NMR spectra were recorded with a Varian 600 spectrometer (Varian, Palo Alto, CA, USA) using tetramethylsilane (TMS) as an internal standard.

RESULTS AND DISCUSSION

Selection of two-phase solvent system

The selection of a suitable solvent system is the critical step in pH-zone-refining CCC separations. A successful two-phase solvent system should have suitable partition coefficient (*K*) values in both acidic (*K*_{acid} << 1) and alkaline (*K*_{base} >> 1) conditions as well as the ability to dissolve the sample.⁸ According to the physical and chemical properties of the C₁₉-diterpenoid alkaloids, three two-phase Pet-EtAc-MeOH-H₂O solvent systems (5:5:5:5, 5:5:3:7, 5:5:1:9, v/v/v/v) (10 mM triethylamine in the upper phase and 10 mM hydrochloric acid in the lower phase) were selected for testing. As shown in Table 1, the *K* values of these systems were suitable for the separation.

The results for the three tested two-phase solvent systems are shown in Figure 2. When the two-phase solvent system of Pet-EtAc-MeOH-H₂O (5:5:5:5, v/v/v/v) (10 mM TEA in the upper phase and 10 mM HCl in the lower phase) was used, the target compounds were eluted too quickly, resulting in a bad separation (Figure 2(A)).

Table 1. The partition coefficient (K) values of different systems under both acidic and alkaline conditions

Solvent system (Pet–EtAc–MeOH–H ₂ O) (v/v/v/v)	K_{I}	K_{II}	K_{III}	
5:5:5:5	K_{acid}	0	0	0
	K_{base}	5.6	5.9	6.4
5:5:3:7	K_{acid}	0	0	0
	K_{base}	8.4	9.1	9.7
5:5:1:9	K_{acid}	0.07	0.05	0.11
	K_{base}	11.2	13.4	16

Compound I was only partly separated, and compounds I, II, and III were mixed.

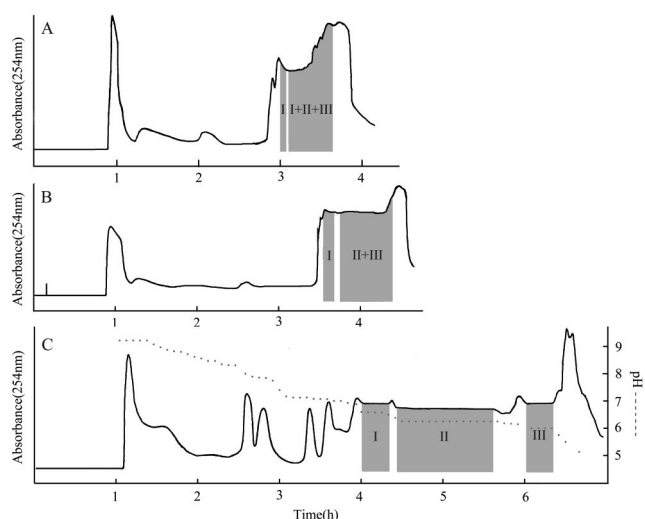


Figure 2. pH-zone-refining CCC chromatograms of the preparative separation of alkaloids compounds from *Aconitum carmichaelii* Debx. Conditions: (A) Two-phase solvent system: Pet–EtAc–MeOH–H₂O (5:5:5:5, v/v/v/v), with 10 mM TEA in the upper phase and 10 mM HCl in the lower phase; flow rate: 2 mL/min; detection wavelength: 254 nm; revolution speed: 850 rpm; sample size: 2.0 g. Retention of the stationary phase: 53%. (B) Two-phase solvent system: Pet–EtAc–MeOH–H₂O (5:5:3:7, v/v/v/v), with 10 mM TEA in the upper phase and 10 mM HCl in lower phase; flow rate: 2 mL/min; detection wavelength: 254 nm; revolution speed: 850 rpm; sample size: 2.1 g. Retention of the stationary phase: 55%. (C) Two-phase solvent system: Pet–EtAc–MeOH–H₂O (5:5:1:9, v/v/v/v), with 10 mM TEA in the upper phase and 10 mM HCl in the lower phase; flow rate: 2 mL/min; detection wavelength: 254 nm; revolution speed: 850 rpm; sample size: 2.5 g. Retention of the stationary phase: 62%

According to the rules for the selection of the solvent system,⁸ a reduction of the ratio of the bridge solvent (methanol) should reduce the elution speed of the target alkaloids and thereby enhance the resolution. Therefore, Pet–EtAc–MeOH–H₂O (5:5:3:7, v/v/v/v) (10 mM triethylamine in the upper phase and 10 mM hydrochloric acid in the lower phase) was selected for the purification. As shown in Figure 2(B), typical rectangular peaks were obtained. Although compound I was successfully separated, compounds II and III were eluted simultaneously. Different separation conditions, such as different flow rates and sample sizes, were also tested; however, the separation was not satisfactory. To separate compounds II and III, the eluting speed should be reduced.

Therefore, we again reduced the ratio of methanol and tested Pet–EtAc–MeOH–H₂O (5:5:1:9, v/v/v/v) (10 mM triethylamine in

the upper phase and 10 mM hydrochloric acid in the lower phase). As shown in Figure 2(C), a typical pH-zone-refining CCC chromatogram was obtained. The alkaloids were eluted as irregular rectangular peaks, whereas impurities or minor components were highly concentrated at the front and rear boundaries. The pH measurement of the collected fractions also revealed a flat pH zone, which corresponded to the previously discussed absorbance plateaus; these results suggested that the components were successfully separated. Based on the HPLC analysis and the elution curve of the pH-zone-refining CCC, the corresponding fractions were combined and evaporated to dryness under reduced pressure at 45°C. The yields of mesaconitine (I), hyaconitine (II), and deoxyaconitine (III) were 4.16%, 16.96%, and 5.05%, and their purities were 93.0%, 95%, and 96%, respectively. The HPLC chromatograms are shown in Figure 3.

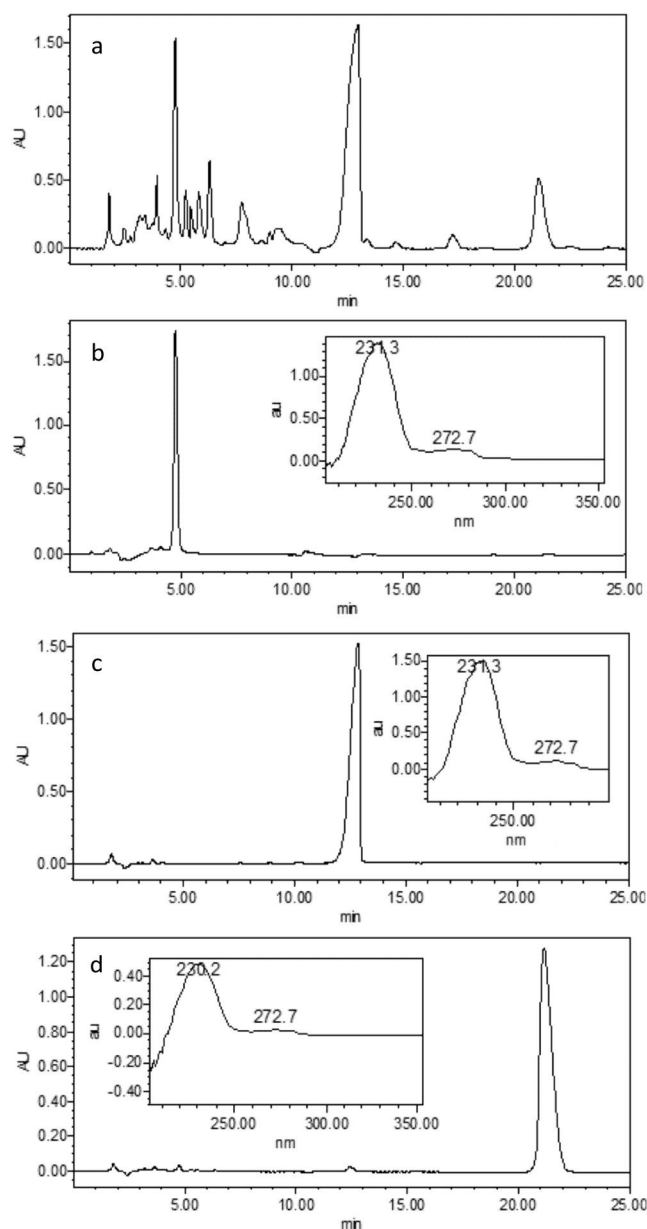


Figure 3. HPLC chromatograms of (a) the crude extracts from *Aconitum carmichaelii* Debx; (b) mesaconitine; (c) hyaconitine; and (d) deoxyaconitine. Column: Inertsil ODS-SP (4.6 mm × 250 mm, I.D., 5 μm). The mobile phase was an 80:20 v/v solution of methanol and water that contained 0.2% triethylamine. Other parameters were as follows: flow rate, 1.0 mL/min; wavelength, 230 nm; column temperature, 25 °C

Identification of the isolated compounds

The chemical structures of the target compounds were identified according to their ESI-MS and NMR data. Based on the data reported by Ishimi *et al.*⁴ and Jiang *et al.*,⁹ the alkaloids corresponded to mesaconitine (I), hypaconitine (II), and deoxyaconitine (III).

CONCLUSION

We developed an efficient pH-zone-refining CCC method for the separation and purification of three C₁₉-diterpenoid alkaloids from *Aconitum carmichaelii*. Diterpenoid alkaloids with high purity were obtained from the crude extract in a one-step separation. Three major alkaloids were obtained from the crude sample in a single run. The results clearly demonstrated that pH-zone-refining CCC produced efficient separation of three alkaloids from crude extracts of *Aconitum carmichaelii*. The present method could be applied to purification of various other alkaloids from natural products.

SUPPLEMENTARY MATERIAL

Supplementary material containing information related to the ESI-MS and NMR data is available at <http://quimicanova.sbg.org.br>, in a pdf file with free access.

ACKNOWLEDGEMENTS

Financial support from the Natural Science Foundation of China (20872083, 21202094), the Natural Science Foundation of Shandong Province (ZR2012HQ020), and the Key Science

and Technology Program of Shandong Province are gratefully acknowledged.

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