

# Preparative isolation and purification of bioactive constituents from *Aconitum coreanum* by high-speed counter-current chromatography coupled with evaporative light scattering detection

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## Abstract

Preparative high-speed counter-current chromatography (HSCCC) coupled with evaporative light scattering detection (ELSD) was employed for the isolation and purification of alkaloids from the roots of *Aconitum coreanum* (Lèvl.) Rapaics. The two-phase solvent system used in HSCCC was *n*-hexane-ethyl acetate-methanol-0.2 M HCl (1:3.5:2:4.5, v/v/v/v). Six alkaloids were obtained and yielded 10.4 mg of Guanfu base P, 9.2 mg of Guanfu base G, 9.5 mg of Guanfu base F, 8.9 mg of atisine, 11.9 mg of Guanfu base A and 25.7 mg of Guanfu base I from 2 g of crude extracts. The purity of these compounds was 96.9%, 95.7%, 91.5%, 98.9%, 95.8% and 95.5%, respectively, as determined by high-performance liquid chromatography (HPLC). Their chemical structures were identified by MS, <sup>1</sup>H NMR and <sup>13</sup>C NMR.  
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**Keywords:** Preparative chromatography; Counter-current chromatography; *Aconitum coreanum*; Plant materials; Diterpenoid alkaloids

## 1. Introduction

*Aconitum coreanum* (Lèvl.) Rapaics (Guanbaifu in Chinese) is one of the most centuries-old Chinese herbs. It has been used to treat various kinds of disorders such as cardialgia, facial distortion, epilepsy, migraine headache, vertigo, tetanus, infantile convulsion and rheumatic arthralgia [1]. Pharmacological studies and clinical practice demonstrated that its extract has anti-arrhythmia [2], analgesic and anti-inflammatory effects [3]. The bioactive constituents of the herb are diterpenoid alkaloids (chemical structures shown in Fig. 1).

High-speed counter-current chromatography (HSCCC) being a support-free liquid-liquid partition chromatography [4] has been widely used in preparative separation of natural products [5–9]. The separation and purification of alkaloids from the extract of *Aconitum coreanum* using HSCCC has not been reported. In the present paper, an efficient HSCCC–evaporative

light scattering detection (ELSD) method for the preparative separation and purification of alkaloids from the roots of *Aconitum coreanum* was successfully established.

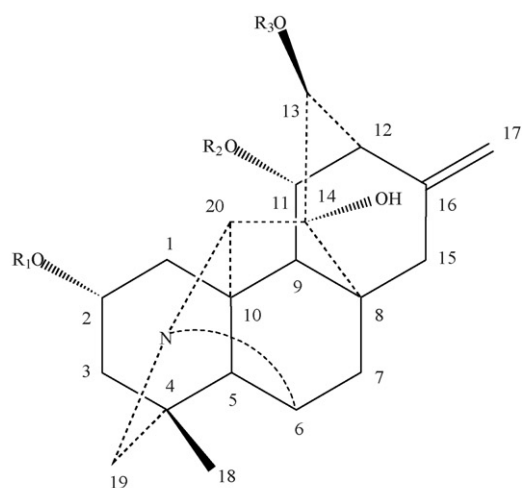
## 2. Experimental

### 2.1. Apparatus

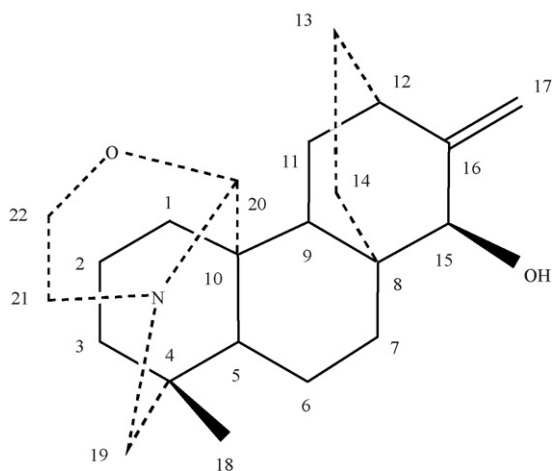
The HSCCC instrument employed in present study is TBE-300A high-speed counter-current chromatography (Tauto Biotechnology, Shanghai, China) with three multi-layer coil separation column connected in series (I.D. of the tubing = 1.6 mm, total volume = 260 ml) and a 20 ml sample loop.

The revolution radius was 5 cm, and the  $\beta$ -values of the multi-layer coil varied from 0.5 at internal terminal to 0.8 at the external terminal. The revolution speed of the apparatus can be regulated with a speed controller in the range between 0 and 1000 rpm. An HX 1050 constant-temperature circulating implement (Beijing Boyikang Lab Instrument, Beijing, China) was used to control the separation temperature. Shimadzu LC-8A

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1. GFP R<sub>1</sub>=isobutyryl R<sub>2</sub>=Ac R<sub>3</sub>=Ac
2. GFG R<sub>1</sub>=Ac R<sub>2</sub>=Ac R<sub>3</sub>=Ac
3. GFF R<sub>1</sub>=isobutyryl R<sub>2</sub>=H R<sub>3</sub>=Ac
5. GFA R<sub>1</sub>=Ac R<sub>2</sub>=H R<sub>3</sub>=Ac
6. GFI R<sub>1</sub>=Ac R<sub>2</sub>=H R<sub>3</sub>=H



4. Atisine

Fig. 1. Chemical structures of diterpenoid alkaloids.

(Shimadzu, Japan) was used to pump the two-phase solvent system. Flow-splitter was used to connect to the evaporative light scattering detection. An Alltech 500 ELSD instrument was used to monitor the effluent (Alltech Associates, USA). The data were collected with a Sepu 3000 chromatography workstation (Hangzhou Puhui Science Apparatus, Hangzhou, China).

The HPLC equipment used was an Agilent 1100 system including a G1311A QuatPump, a G1315B UV-vis photodiode array detector, a G1313A Auto-sampler, a G1332A degasser and Agilent HPLC workstation.

Mass spectra were recorded on an Agilent 1100 matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer.

The nuclear magnetic resonance (NMR) spectrometer used was a Bruker AV300 instrument (Bruker, Germany).

## 2.2. Reagents and materials

All organic solvents used for preparation of crude extract and HSCCC separation were of analytical grade (Nanjing Reagent Factory, Nanjing, China). Acetonitrile used for HPLC was of chromatographic grade (Merck), and water was distilled water. C<sup>2</sup>HCl<sub>3</sub> was used as the solvent for NMR determination.

*Aconitum coreanum* was provided by Mayinglong Pharmaceutical Factory (Baicheng, Jilin Province, China) and identified by Professor Weichun Wu (Shenyang Pharmaceutical University, Shenyang, China).

## 2.3. Preparation of crude extract

*Aconitum coreanum* was ground to powder (about 30 mesh) by a disintegrator. The powder (23 kg) was extracted three times with 95% ethanol. The extract was combined and evaporated to dryness under reduced pressure, which yielded 2.6 kg of dry powder. Then, the dry powder was dissolved with 1.5 l water. After filtration, the aqueous solution was extracted three times with 1.5 l of water-saturated light petroleum (b.p. 60–90 °C, ethyl acetate and *n*-butanol successively which yielded 260 g of light petroleum extract, 110 g of ethyl acetate extract and 80 g of *n*-butanol extract after being combined and evaporated to dryness under reduced pressure. The ethyl acetate dried powder was stored in a refrigerator (–2 °C) for the subsequent HSCCC separation.

## 2.4. Selection of two-phase solvent system

The two-phase solvent system was selected according to the partition coefficient (*K*) of each target component. The *K* values were determined by HPLC as follows: 10 mg of crude extract was added to a test tube, to which 5 ml of each phase of the two-phase solvent system was added. The test tube was shaken violently for several minutes. Then the upper and lower phases were analyzed by HPLC. The partition coefficients (*K*) of all components in sample were obtained by peak area obtained from the upper phase to that of the lower phase.

## 2.5. Preparation of two-phase solvent system and sample solution

In the present study, the two-phase solvent system composed of *n*-hexane-ethyl acetate-methanol–0.2 M HCl (1:3.5:2:4.5, v/v/v/v) was used for HSCCC separation. The solvents were added to a separatory funnel according to the volume ratios and thoroughly equilibrated after shaking violently. Then the upper phase and the lower phase were separated and degassed by supersonic bath for 30 min shortly before use.

The sample solution for HSCCC separation was prepared by dissolving 2 g of the dried powder of ethyl acetate extracts in the 10 ml of the upper phase of the two-phase solvent system.

## 2.6. HSCCC separation procedure

HSCCC was performed as follows: The multilayer-coiled column was first entirely filled with the upper phase as stationary

phase. The lower aqueous mobile phase was then pumped into the head end of the column inlet at a flow-rate of 4.0 ml/min, while the apparatus was run at a revolution speed of 950 rpm. After hydrodynamic equilibrium was reached (about half an hour), as indicated by a clear mobile phase eluting at the tail outlet, 10 ml of the sample solution containing 2 g of the ethyl acetate extracts were introduced into the column through the injection valve. All through the experiment, the separation temperature was controlled at 25 °C. The effluent from the tail end of the column was introduced into the ELSD system through the flow-splitter. The detector was preheated for 30 min; nitrogen gas was the result of an arbitrary flow setting of 3.0 l min<sup>-1</sup> on the detector, and the chromatogram was recorded immediately after the sample injection. Each peak fraction was collected manually according to the obtained chromatogram and each collection was evaporated under reduced pressure and dissolved by 0.2 M HCl for subsequent purity analysis by HPLC.

### 2.7. HPLC analysis and identification of HSCCC peak fractions

The HPLC analysis was performed with a reversed-phase Diamonsil C18 column (250 mm × 4.6 mm I.D., 5 μm) at room temperature. The mobile phase was (solvent A) 2 mg/ml sodium 1-heptanesulfonic (including 0.2% triethylamine and the pH was adjusted to 3.0 with phosphoric acid) and (solvent B) acetonitrile in gradient mode as follows: 0–20 min, 17–30% acetonitrile; 20–40 min, 30–35% acetonitrile. The effluent was monitored at 205 nm and the flow-rate was at 1.0 ml min<sup>-1</sup> constantly.

The structural identification of each HSCCC peak fractions were carried out by <sup>1</sup>H NMR and <sup>13</sup>C NMR.

## 3. Results and discussion

### 3.1. Optimization of HSCCC conditions

The ethyl acetate extracts of *Aconitum coreanum* were analyzed by HPLC and the chromatogram was given in Fig. 2. The selection of the two-phase solvent system is the most important step in performing HSCCC. Preliminary HSCCC experiments were carried out with the two-phase solvent system composed of ethyl acetate–methanol–water in a volume ratio of 3:1:3 or 3:2:4. The results showed that it could not be separated. In the subsequent studies, 0.2 M HCl instead of water, and the two-phase solvent system was tested again. Although two diterpenoid alkaloids could be separated from other compounds, it was poor to separate the main bioactive constituents. Finally the two-phase solvent system composed of *n*-hexane-

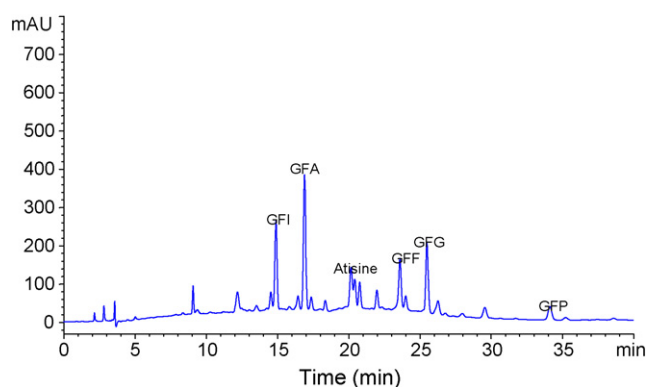


Fig. 2. HPLC analyses of the ethyl acetate fraction. Column: Diamonsil C18 column (250 mm × 4.6 mm I.D., 5 μm); the mobile phase was (solvent A) 2 mg/ml sodium 1-heptanesulfonic (including 0.2% triethylamine and the pH was adjusted to 3.0 with phosphoric acid) and (solvent B) acetonitrile in gradient mode as follows: 0–20 min, 17–30% acetonitrile; 20–40 min, 30–35% acetonitrile; detection: 205 nm; flow-rate: 1.0 ml min<sup>-1</sup>.

ethyl acetate–methanol–0.2 M HCl was tested. Although the two-phase solvent systems at different volume ratios were tested, the two-phase solvent system was selected according to the *K* values of each target components. In order to achieve efficient resolution of target compounds, the *K* values of the target components in different solvent systems were determined by HPLC and the *K* values were summarized in Table 1. The two-phase solvent systems listed in Table 1 were also tested in HSCCC separation. The purity of the compounds obtained by HSCCC was determined by HPLC. The results indicated that when *n*-hexane-ethyl acetate–methanol–0.2 M HCl (1:3.5:2.5:3.5, v/v/v/v) was used as the two-phase solvent system [10], the six compounds could not be separated. When *n*-hexane-ethyl acetate–methanol–0.2 M HCl (1:3.5:1.5:4.5, v/v/v/v) was used, the six compounds can be well separated, but the separation time was long and the peak broadened seriously. When *n*-hexane-ethyl acetate–methanol–0.2 M HCl (1:3.5:2:4.5, v/v/v/v) was used, six peaks were well separated and the separation time was also acceptable. When *n*-hexane-ethyl acetate–methanol–0.2 M HCl (1:3.5:2.5:4.5, v/v/v/v) was used, the six compounds could not be separated well and the purity of them became poor. So *n*-hexane-ethyl acetate–methanol–0.2 M HCl (1:3.5:2:4.5, v/v/v/v) was used as the two-phase solvent system of HSCCC. The influence of revolution speed, flow-rate of the mobile phase, and temperature on HSCCC peak resolution was also investigated [11]. The results indicated that when the flow-rate was 4.0 ml min<sup>-1</sup>, revolution speed was 950 rpm, and separation temperature was 25 °C, retention percentage of the stationary phase was 70%, good separation results can be obtained.

Table 1  
The *K* values of the target components in different solvent systems

Solvent system: <i>n</i> -hexane-ethyl acetate–methanol–0.2 M HCl	<i>K</i>					
	GFI	GFA	Atisine	GFF	GFG	GFP
1:3.5:2.5:4.5, v/v/v/v	32.62	24.03	18.71	11.84	8.13	5.76
1:3.5:2:4.5, v/v/v/v	11.10	9.76	7.95	5.23	3.22	2.17
1:3.5:1.5:4.5, v/v/v/v	5.47	5.22	4.81	4.03	3.52	3.13

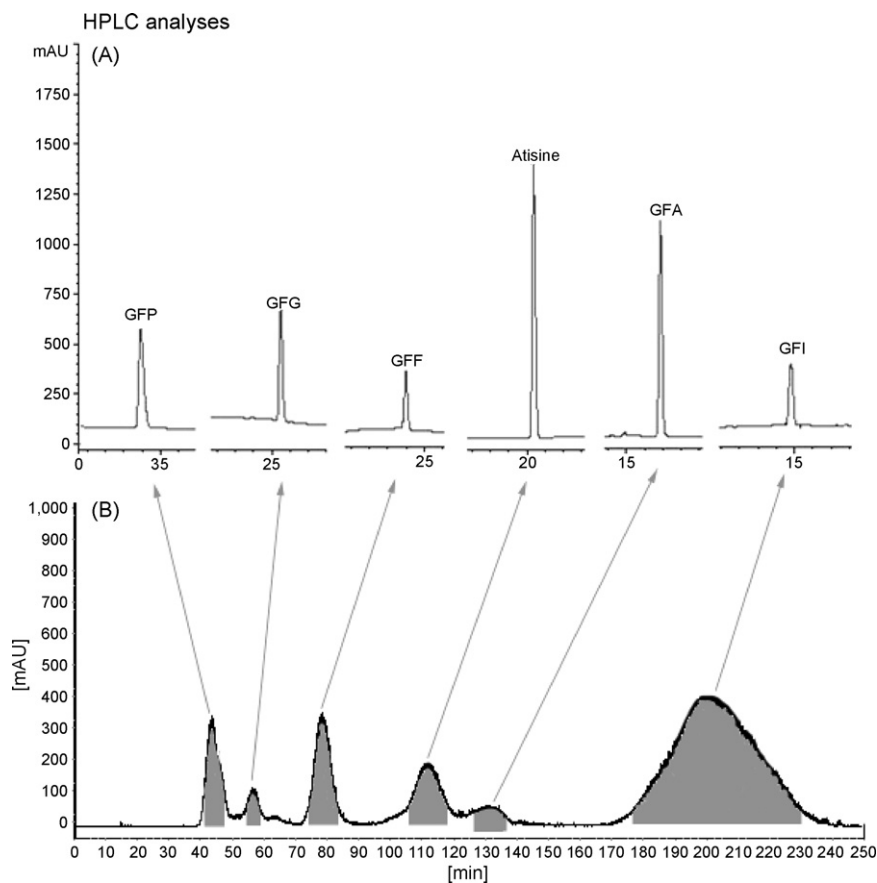


Fig. 3. (A) HPLC chromatograms of the six alkaloids from *Aconitum coreanum* (Lévl.) Rapaics and (B) HSCCC peak fractions. Conditions: HPLC conditions same as in Fig. 2. HSCCC solvent system: *n*-hexane-ethyl acetate–methanol–0.2 M HCl (1:3.5:2:4.5, v/v/v/v); mobile phase: the lower phase; elution mode: head to tail; flow-rate: 4.0 ml/min; sample size: 2.0 g; injection volume: 10 ml; revolution speed: 950 rpm.

### 3.2. Optimization of ELSD conditions

Because of the low UV absorption of these diterpenoid alkaloids and the HSCCC solvent system contained UV restricted agents such as ethyl acetate, HSCCC-ELSD is developed to detect this kind of compounds in our separation procedure. The result showed that this unique hyphenated technology was a rapid and convenient way to detect the non-volatile compounds in the volatile UV restricted solvents utilized in the HSCCC. In our experience, the ELSD conditions can be mainly optimized by changing drift tube temperature and gas flow rate. So the influence of drift tube temperature and gas flow rate on detect efficiency was studied. Signal-to-noise ratios were well obtained when the drift tube temperature and nitrogen gas flow rate were set at 110 °C and 3.01 min<sup>-1</sup>.

### 3.3. Structural identification

The chemical structures of the peaks in Fig. 3 were identified according to their MS, <sup>1</sup>H and <sup>13</sup>C NMR data.

Peak 1: Positive electrospray ionization (ESI) MS, *m/z* 500 [*M*+H]. <sup>1</sup>H NMR (C<sup>2</sup>HCl<sub>3</sub>, 300 MHz) δ1.05 (3H, s, 18-H), δ1.10, 1.15, 2.36 (3H, d, 3H, d, 1H, m, 2-isobutyryl), δ2.01 (3H, s, 11-Ac), δ2.03 (3H, s, 13-Ac), δ4.81 (1H, br. s, 17-H),

δ4.84 (1H, br. s, 17-H), δ4.95 (1H, m, 13-H), δ5.01 (1H, d, 11-H), δ5.13 (1H, m, 2-H). Comparing the above data with [12], the obtained product was identified as GFP.

Peak 2: Positive ESI-MS, *m/z* 472 [*M*+H]. <sup>1</sup>H NMR (C<sup>2</sup>HCl<sub>3</sub>, 300 MHz) δ1.01 (3H, s, 18-H), δ2.03 (3H, s, 2-Ac), δ2.06 (3H, s, 11-Ac), δ2.09 (3H, s, 13-Ac), δ4.82 (1H, s, 17-H), δ5.01 (1H, s, 17-H), δ5.03 (1H, m, 11-H), δ5.11 (1H, d, 13-H), δ5.16 (1H, m, 2-H). Comparing the above data with [12], the obtained product was identified as GFG.

Peak 3: Positive ESI-MS, *m/z* 458 [*M*+H]. <sup>1</sup>H NMR (C<sup>2</sup>HCl<sub>3</sub>, 300 MHz) δ1.04 (3H, s, 18-H), δ1.09, 1.12, 2.39 (3H, d, 3H, d, 1H, m, 2-isobutyryl), δ2.03 (3H, s, 13-Ac), δ4.82 (1H, br. s, 17-H), δ4.71 (1H, br. s, 17-H), δ5.03 (1H, m, 11-H), δ5.11 (1H, d, 9.0Hz, 13-H), δ5.16 (1H, m, 2-H). Comparing the above data with [13], the obtained product was identified as GFF.

Peak 4: Positive ESI-MS, *m/z* 344 [*M*+H]. <sup>1</sup>H NMR (C<sup>2</sup>HCl<sub>3</sub>, 300 MHz) δ1.12 (3H, s, 18-H), δ4.09 (2H, s, 19-H), δ4.82 (2H, m, 21-H), δ4.71 (2H, br. m, 22-H), δ5.07 (1H, br. s, 17-H), δ5.11 (1H, br. s, 17-H), δ8.16 (1H, s, 15-OH), <sup>13</sup>C NMR (CDCl<sub>3</sub>, 75 MHz) δ154.5 (16-C), δ110.7 (17-C). Comparing the above data with [14], the obtained product was identified as atisine.

Peak 5: Positive ESI-MS, *m/z* 430 [*M*+H]. <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>, 300 MHz) δ1.10 (3H, s, 18-H), δ1.98 (3H, s, 13-Ac), δ2.08

(3H, s, 2-Ac),  $\delta$ 4.74 (1H, br. s, 17-H),  $\delta$ 4.89 (1H, br. s, 17-H),  $\delta$ 5.02 (1H, d, 13-H),  $\delta$ 5.10 (1H, m, 2-H). Comparing the above data with [15], the obtained product was identified as GFA.

Peak 6: Positive ESI-MS,  $m/z$  388 [ $M+H$ ].  $^1\text{H}$  NMR ( $\text{C}^2\text{HCl}_3$ , 300 MHz)  $\delta$ 1.03 (3H, s, 18-H),  $\delta$ 2.03 (3H, s, 2-Ac),  $\delta$ 4.70 (1H, br. s, 17-H),  $\delta$ 4.89 (1H, br. s, 17-H),  $\delta$ 5.13 (1H, m, 2-H). Comparing the above data with [15], the obtained product was identified as GFI.

In conclusion, an efficient HSCCC method was developed for the purification of diterpenoid alkaloids and their separation from the *Aconitum coreanum*. High purity diterpenoid alkaloids could be obtained from the crude extract in a one-step separation; six major components were obtained and yielded 10.4 mg of GFP (peak 1 collected during 42–48 min), 9.2 mg of GFG (peak 2 collected during 54–58 min), 9.5 mg of GFF (peak 3 collected during 76–84 min), 8.9 mg of atisine (peak 4 collected during 107–118 min), 11.9 mg of GFA (peak 5 collected during 127–135 min) and 25.7 mg of GFI (peak 6 collected during 178–230 min) from 2 g of crude extracts. Each HSCCC peak fraction was analyzed by HPLC. The purity of these peak fractions was 96.9%, 95.7%, 91.5%, 98.9%, 95.8% and 95.5%. The present study indicates that HSCCC is a very powerful technique for the separation and purification of bioactive substances from Chinese herbs.

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