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Short communication

Preparative isolation and purification of two new isomeric diterpenoid alkaloids from *Aconitum coreanum* by high-speed counter-current chromatography

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ABSTRACT

Preparative high-speed counter-current chromatography (HSCCC) coupled with evaporative light scattering detection (ELSD) was used to isolate and separate bioactive constituents from the roots of *Aconitum coreanum*. Two new diterpenoid alkaloid isomers were successfully separated for the first time by HSCCC with an optimized two-phase solvent system composed of ethyl acetate–*n*-butanol–methanol–2% acetic acid (3.5:1.5:2:4.5, v/v/v/v), 25.4 mg of GFT (**1**) and 18.3 mg of GFU (**2**) were isolated form 1 g crude extract in one step HSCCC experiment. The purities of the two new compounds were all over 95% as analyzed by HPLC and their structures were identified by ESI–MS, ^{1}H NMR, ^{13}C NMR, and 2D NMR analysis.

1. Introduction

The roots of *Aconitum coreanum* (Lèvl.) Rapaics (Guanbaifu in Chinese) has been extensively used to treat various kinds of disorders such as cardialgia, facial distortion, epilepsia, migraine headache, vertigo, tetanus, infantile convulsion, and rheumatic arthralgia [1]. The pharmacological research and clinical practice shows that its extract has anti-arrhythmia [2], analgesic, and anti-inflammatory effects [3]. Major active components isolated from this herb are diterpenoid alkaloids and many of them are homologous compounds (chemical structures shown in Fig. 1), thus separation and purification of them are a tough job but indispensable to study the structure and activity relationship of these compounds.

High-speed counter-current chromatography 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 diterpenoid alkaloids from the extract of *A. coreanum* using HSCCC has been reported previously [10–11]. This is the first report of two new isomeric diterpenoid alkaloids successfully separated by high-speed counter-current chromatography, an efficient HSCCC coupled with ELSD method was successfully established by optimizing the two-phase solvent system to separate and purify the two diterpenoid alkaloid isomers.

2. Experimental

2.1. Apparatus

The HSCCC instrument employed in present study is TBE-300A high-speed counter-current chromatography (Tauto Biotechnique, 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 β-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 (Shimadzu Corporation, Kyoto, Japan) was used to pump the two-phase solvent system. Flow-splitter (split ratio = 3:1; 3 for collector and 1 for ELSD) was used to connect to the evaporative light scattering detection. An

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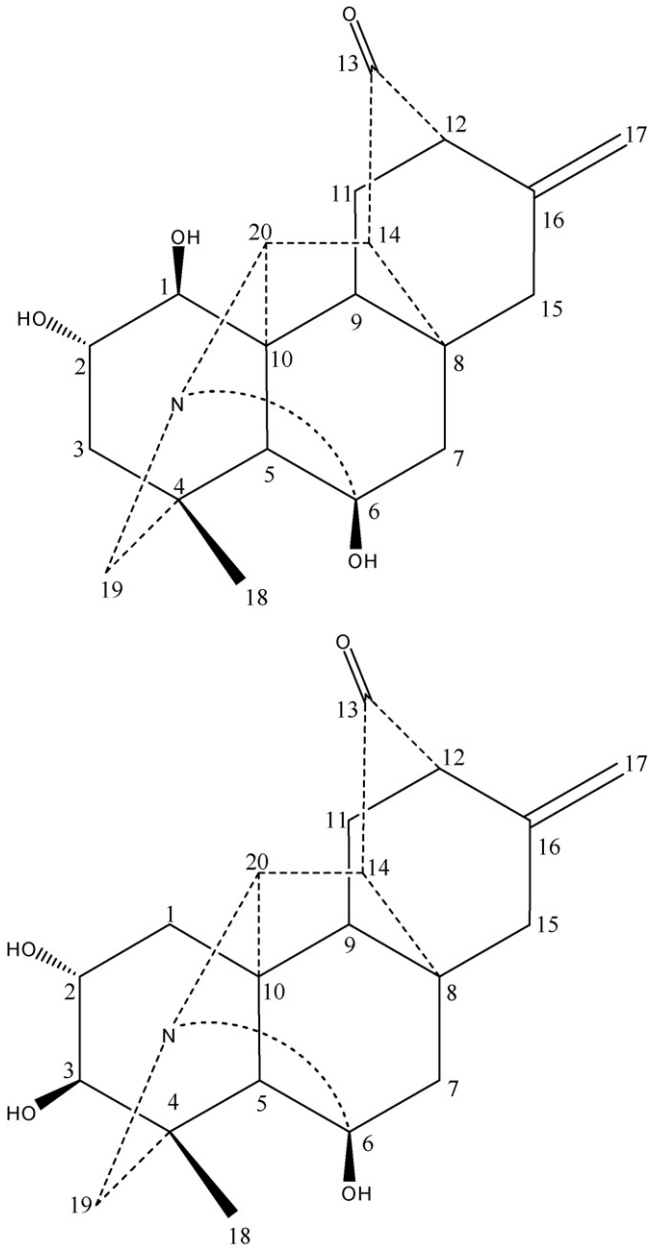

Fig. 1. Chemical structures of diterpenoid alkaloids.

Alltech 500 ELSD (Alltech Associates, Deerfield, IL, USA) was used to monitor the effluent. The data were collected with Sepu 3000 chromatography workstation (Hangzhou Puhui Science Apparatus, Hangzhou, China).

The HPLC equipment used was an Agilent 1100 system (Agilent Technologies, Palo Alto, CA, USA) 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 Aglient 1100 electrospray ionization-time of fright (ESI-TOF) mass spectrometer.

Nuclear magnetic resonance (NMR) spectrometer used here was Bruker AVANCE 500 (Bruker, Switzerland).

2.2. Reagents and materials

All organic solvents used for preparation of crude extract and HSCCC separation were of analytical grade (Nanjing Chemical Reagent Corporation, Nanjing, China). Acetonitrile used for HPLC was of chromatographic grade (Merck, Darmstadt, Germany), and water was distilled water. DMSO-d6 (Cambridge Isotope Laboratories, Andover, MA, USA) was used as the solvent for NMR determination.

A. 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

A. coreanum was ground to powder (about 30 meshes) by a disintegrator. The powder (10 kg) was extracted three times with 95% ethanol. After concentration under vacuum, the residue (120 g) was purified by a D101 macroporous resin column, eluted with distilled water, 30% ethanol, 60% ethanol, and 95% ethanol. The 60% ethanol fraction (10 g), which contained target compounds was evaporated under reduced pressure and dried to powder, the 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 vigorously 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, two-phase solvent system composed of ethyl acetate–*n*-butanol–methanol–2% acetic acid (3.5:1.5:2:4.5, v/v/v/v) was used for HSCCC separation. Each solvent was added to a separatory funnel and thoroughly equilibrated at room temperature. 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 1 g of the dried powder of the 60% ethanol fraction 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 multi-layer 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 2.0 ml/min, while the apparatus was run at a revolution speed of 850 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 1 g of crude 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 evaporative light scattering detection (ELSD) through the flow-splitter. The detector was preheated for 30 min; nitrogen gas was introduced to the detector at 3.0 L min^{-1} 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, Dikma Technologies, Shanghai, China) at room temperature. The mobile phase was (A) 2 mg/ml sodium 1-heptansulfonate (including 0.2% triethylamine and the pH was adjusted to 3.0 with phosphoric acid) and (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 flow rate was 1.0 ml min^{-1}. The structural identification of each HSCCC peak fractions was carried out by ^{1}H NMR, ^{13}C NMR and 2D NMR data.

3. Results and discussion

3.1. Optimization of HSCCC conditions

The 60% ethanol fraction of *A. coreanum* was analyzed by HPLC (Fig. 2), the HPLC mobile phase containing sodium 1-heptansulfonate, triethylamine, and phosphoric acid, which can offer good separation, was not suitable for subsequent purifications, so we prefer to use HSCCC to separate compounds **1** and **2**. Preliminary HSCCC experiments were carried out with the two-phase solvent system composed of ethyl acetate–*n*-butanol–methanol–0.2 M HCl (3.5:1:1:3.5, v/v/v/v) according to our early research [11], results showed that this two-phase solvent system was suitable for separation of alkaloids from *A. coreanum* except 1 and 2. In the subsequent studies, the volume ratio of organic solvent was modified to find the suitable *K* values of compounds **1** and **2** for HSCCC separation, but the *K* values of compounds **1** and **2** were very similar and changed little (Table 1). Finally, instead of HCl, acetic acid was tested in the composition of the two-phase solvent system, and the *K* values of target components were discriminated (Table 1). The *K* values of the target components in different solvent systems were detected by HPLC and were summarized in Table 1, it showed that when the volume ratio of acetic acid was increased, the difference between *K* values of compounds **1** and **2** was enlarged subsequently. The addition of acetic acid instead of HCl made the two-phase solvent system good selectivity for separating the two target compounds. When ethyl acetate–*n*-butanol–methanol–2% acetic acid (3.5:1.5:2:4.5, v/v/v/v) was used as the two-phase solvent system [12], the two compounds could separate properly and the total experiment time was also acceptable (Fig. 3). The influence of revolution speed, flow-rate of the mobile phase, and temperature on HSCCC peak resolution was also investigated [13], it indicated that when the flow-rate was 2.0 ml min^{-1}, revolution speed was 850 rpm, and separation temperature was 25 °C, retention percentage of the stationary phase was 60%, separation results were satisfactory.

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

Solvent systems	K	
	13-dehydro-1β,2α,6β-trihydroxyhetisine	13-dehydro-2α,3β,6β-trihydroxyhetisine
Ethyl acetate–*n*-butanol–methanol–0.2 M HCl		
3.5:1:1:3.5 (v/v/v/v)	2.54	2.36
4:1:1:3 (v/v/v/v)	2.87	2.75
4.5:1:2:3.5 (v/v/v/v)	2.43	2.31
3:1.5:2:4.5 (v/v/v/v)	2.79	2.67
Ethyl acetate–*n*-butanol–methanol–2% acetic acid		
3.5:1:1:3.5 (v/v/v/v)	3.09	2.57
3.5:1:2:4 (v/v/v/v)	3.19	2.21
3.5:1.5:2:4.5 (v/v/v/v)	3.28	1.73

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 hyphenated technology was a convenient way to detect the

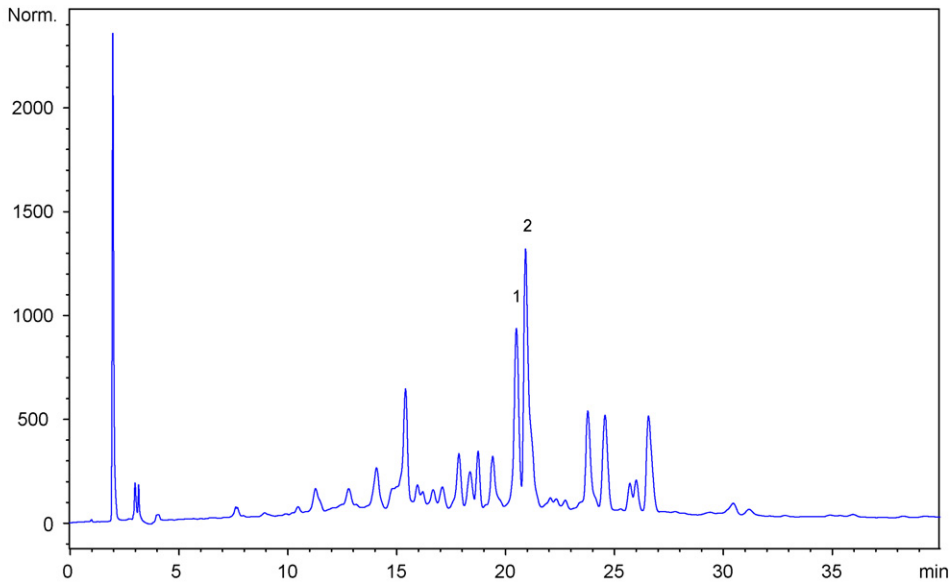

Fig. 2. HPLC analyses of the 60% ethanol fraction. Column: diamonsil C_{18} column (250 mm × 4.6 mm I.D., 5 μm); the mobile phase was (A) 2 mg/ml sodium 1-heptansulfonate (including 0.2% triethylamine and the pH was adjusted to 3.0 with phosphoric acid) and (B) acetonitrile in gradient mode as follows: 0–20 min, 17–30% acetonitrile; 20–40 min, 30–35% acetonitrile; detection: 205 nm; flow-rate: 1.0 ml min^{-1}.

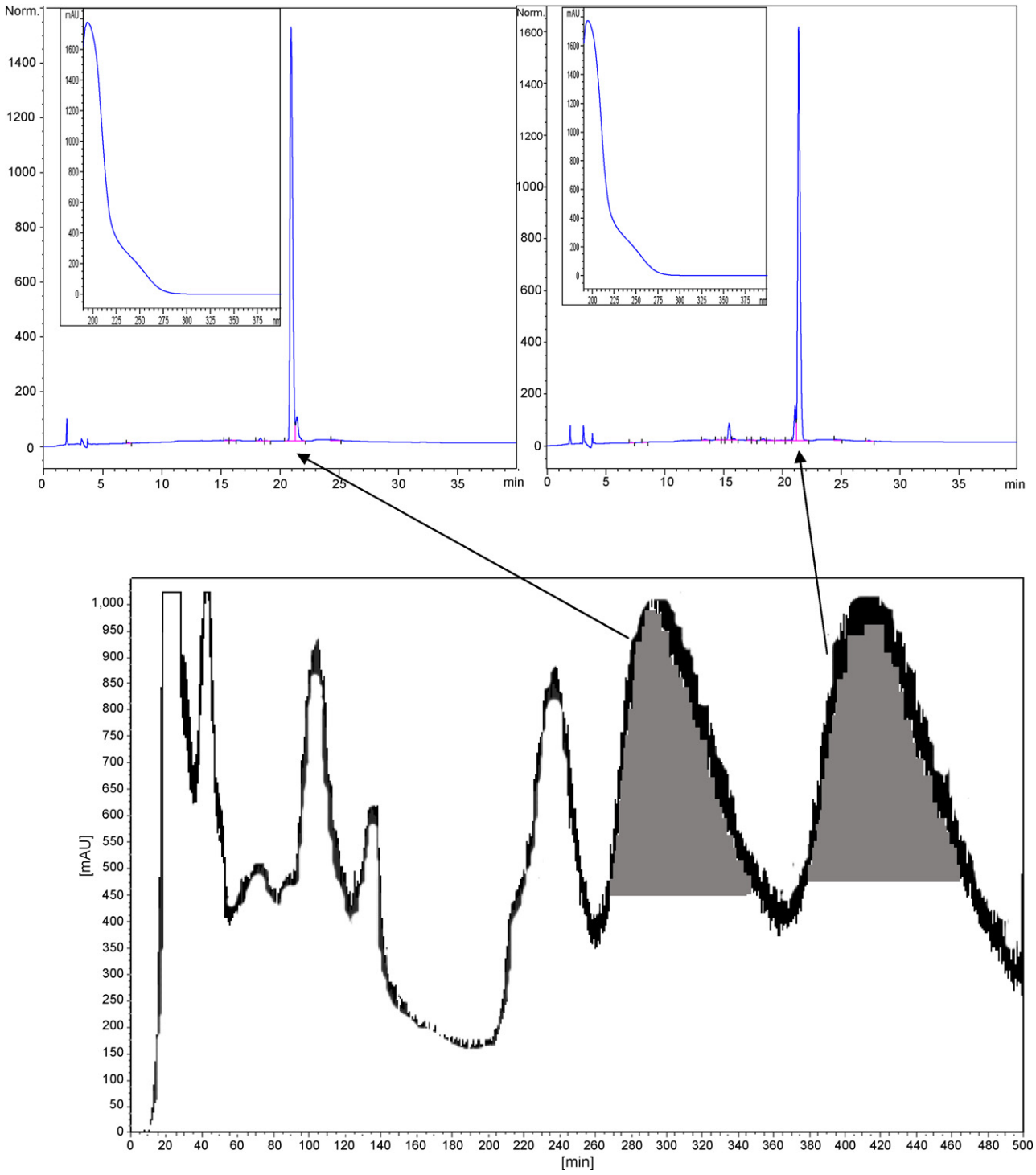

Fig. 3. Chromatogram of HSCCC and the results of HPLC analyses of peak 1 and 2 fractions. Solvent system: ethyl acetate–*n*-butanol–methanol–2% acetic acid (3.5:1.5:2:4.5, v/v/v/v); mobile phase: the lower phase; elution mode: head to tail; flow-rate: 2.0 ml/min; sample size: 1.0 g; injection volume: 10 ml; revolution speed: 850 rpm; retention of the stationary phase: 60%; HPLC condition was same as in Fig. 2.

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, gas flow rate, and the split ratio of the splitter. So the influence of these variables on the detection efficiency was studied. Signal-to-noise ratio of compounds **1** and **2** was well achieved when the drift tube temperature was set at 110 °C, nitrogen gas flow rate was 3.0 L min^{-1}, and split ratio was set at 3:1.

3.3. Structural identification

The chemical structures of the peaks in Fig. 3 were identified by their HR-ESI-MS, ^{1}H NMR, ^{13}C NMR, and 2D NMR data.

Compounds **1** and **2** were obtained as colorless powder and had a same molecular formula as $C_{20}H_{25}NO_4$ deduced from ESI–MS ($[M+H]^+$ at *m/z* 344.2). From the ^{1}H, ^{13}C NMR, DEPT, HSQC, HMBC (Fig. 4), and NOESY data, the structure of **1** and **2** were

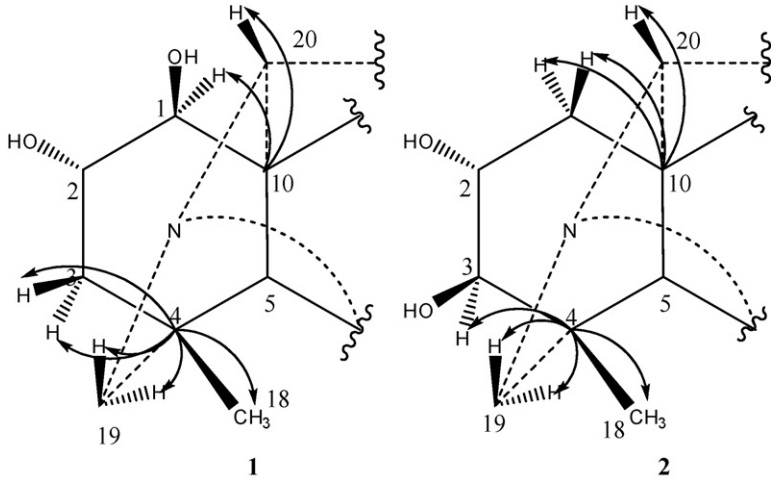

Fig. 4. Key HMBC correlations of compounds **1** and **2**.

established as 13-dehydro-1β,2α,6β-trihydroxyhetisine and 13-dehydro-2α,3β,6β-trihydroxyhetisine.

The ^{13}C NMR (DEPT) spectrum of **1** and **2** exhibited 20 signals attributed to the C_{20} diterpenoid core, it indicated carbons characteristic signals for one angular methyl group (C_{18}), one extreme alkenes (=CH_2, C_{17}), and four quaternary C-atoms (C_4, C_8, C_{10}, C_{16}). The ^{1}H NMR spectrum of **1** and **2** exhibited 25 proton signals, it indicated protons characteristic signals for one Me (H–C_{18}), one C = CH_2 (H–C_{17}), one CH (H–C_{20}), and one CH_2 (H–C_{19}) group. Signals for *N*-methyl, MeO and aromatic ring are absent suggesting that there is no oxazolidine ring in the C_{20} skeleton, it can infer that they are C_{20} hetisine alkaloids [14].

In the HMBC spectrum of **1**, the correlations of C_{10} (δC: 53.3)/H–C_1 (δH: 3.28, 1H, d, J = 3.0 Hz) and C_{10} (δC: 53.3)/H–C_{20} (δH: 3.42, 1H, br. s), and the correlations of C_4 (δC: 36.3)/H–C_3 (δH: 1.43, 1.68, 2H, m), C_4 (δC: 36.3)/H–C_{19} (δH: 2.78, 2.94, 2H, m) and C_4 (δC: 36.3)/H–C_{18} (δH: 1.23, 3H, s) were found. It verified that the OH group was connected to C_1. In the HMBC spectrum of **2**, the correlations of C_{10} (δC: 42.8)/H–C_1 (δH: 1.17, 1.58, 2H, m) and C_{10} (δC 42.8)/H–C_{20} (δH: 3.51, 1H, br. s), and the correlations of C_4 (δC: 43.0)/H–C_3 (δH: 3.48, 1H, m), C_4 (δC: 43.0)/H–C_{19} (δH: 3.10, 3.38, 2H, d, J = 12.2 Hz) and C_4 (δC: 43.0)/H–C_{18} (δH: 1.50, 3H, s), can strongly suggest that the C_3 connected with OH group. The difference between their HMBC spectrums were shown in Fig. 4, the conformation and other suitable groups were confirmed by 2D NMR analyses.

Data of each compound were given as follows:

Compound **1**: HR–ESI–MS (*m*/*z*): 344.1857 ([M + H]$^+$). ^{1}H NMR (500 MHz, DMSO-d6, 303 K): δ in ppm, J in Hz: H–C_1 (δ3.28, 1H, d, J = 3.0), H–C_2 (δ3.70, 1H, br. s), H–C_3 (δ1.43, 1.68; 2H, m), H–C_5 (δ1.65, 1H, s), H–C_7 (δ1.72, 1.81; 2H, d, J = 13.1), H–C_9 (δ1.90, 1H, m), H–C_{11} (δ1.65, 1.87; 2H, m), H–C_{12} (δ2.76, 1H, br. s), H–C_{14} (δ2.10, 1H, br. s), H–C_{15} (δ2.22, 2.46; 2H, d, J = 17.3), H–C_{17} (δ4.76, 4.89; 2H, br. s), H–C_{18} (δ1.23, 3H, s), H–C_{19} (δ2.78, 2.94; 2H, m), H–C_{20} (δ3.42, 1H, br. s). ^{13}C NMR (125 MHz, DMSO-d6, 303K): δ in ppm: C_1 (δ67.8), C_2 (δ70.0), C_3 (δ37.5), C_4 (δ36.3), C_5 (δ57.2), C_6 (δ98.7), C_7 (δ42.4), C_8 (δ42.5), C_9 (δ46.2), C_{10} (δ53.3), C_{11} (δ22.4), C_{12} (δ52.9), C_{13} (δ210.3), C_{14} (δ60.2), C_{15} (δ32.8), C_{16} (δ143.9), C_{17} (δ109.6), C_{18} (δ30.9), C_{19} (δ60.2), C_{20} (δ65.7). Compound **1** was named as Guan Fu base T (GFT, 13-dehydro-1β,2α,6β-trihydroxyhetisine).

Compound **2**: HR–ESI–MS (*m*/*z*): 344.1859 ([M + H]$^+$). ^{1}H NMR (500 MHz, DMSO-d6, 303 K): δ in ppm, J in Hz: H–C_1 (δ1.17, 1.58; 2H, m), H–C_2 (δ3.00, 1H, m), H–C_3 (δ3.48, 1H, m), H–C_5 (δ2.02, 1H, s), H–C_7 (δ2.16, 2.24; 2H, d, J = 14.0), H–C_9 (δ2.12, 1H, m), H–C_{11} (δ1.74, 1.82; 2H, m), H–C_{12} (δ2.90, 1H, d, J = 3.7), H–C_{14} (δ2.78, 1H, br. s), H–C_{15} (δ2.33, 2.59, 2H, m), H–C_{17} (δ4.86, 4.99; 2H, br. s), H–C_{18} (δ1.50, 3H, s), H–C_{19} (δ3.10, 3.38; 2H, d, J = 12.2), H–C_{20} (δ3.51, 1H, br. s). ^{13}C NMR (125 MHz, DMSO-d6, 303K): δ in ppm: C_1 (δ33.1), C_2 (δ77.9), C_3 (δ67.7), C_4 (δ43.0), C_5 (δ57.0), C_6 (δ100.5), C_7 (δ39.8), C_8 (δ48.7), C_9 (δ46.2), C_{10} (δ42.8), C_{11} (δ22.0), C_{12} (δ51.6), C_{13} (δ206.9), C_{14} (δ57.0), C_{15} (δ31.4), C_{16} (δ141.5), C_{17} (δ111.2), C_{18} (δ25.5), C_{19} (δ54.2), C_{20} (δ65.5). Compound **2** was named as Guan Fu base U (GFU, 13-dehydro-2α,3β,6β-trihydroxyhetisine).

4. Conclusion

An efficient HSCCC method was developed for the purification and separation of two new isomeric diterpenoid alkaloids from the *A. coreanum*. In present study, the addition of acetic acid instead of HCl can affect partitioning coefficient values of the target components. When ethyl acetate–*n*-butanol–methanol–2% acetic acid (3.5:1.5:2:4.5, v/v/v/v) was used as the two-phase solvent system, the suitable K values of the two isomeric compound was got for the HSCCC separation. Highly pure diterpenoid alkaloids could be obtained from the crude extract in a one-step separation; 25.4 mg of compound **1** (peak 1 collected during 270–350 min) and 18.3 mg of compound **2** (peak 2 collected during 370–465 min) were yielded from 1 g crude extract. The purity of two compounds was 96.9% and 95.7% as determined by HPLC. Their structural identifications were carried out by MS, ^{1}H NMR, ^{13}C NMR, and 2D NMR analysis.

References

[1] Editorial Committee of Chinese Bencao of the State Administration of Traditional Chinese Medicine, Selected Works of Chinese Bencao, vol. 1, Shanghai Science and Technology Press, Shanghai, 1998, p. 499.
[2] W.Z. Chen, Y.L. Dong, Y.F. Zhang, Acta Pharmacol. Sin. 4 (1983) 247.
[3] Y. Jiang, H.Q. Wu, J.H. Liu, Chin. Trad. Herb. Drugs 18 (1986) 23.
[4] Y. Ito, R.L. Bowman, Science 167 (1970) 281.
[5] Y. Wei, T.Y. Zhang, Y. Ito, J. Chromatogr. A 1033 (2004) 373.
[6] R.M. Liu, A.F. Li, A.L. Sun, J.C. Cui, L.Y. Kong, J. Chromatogr. A 1064 (2005) 53.
[7] R.M. Liu, L. Feng, A.L. Sun, L.Y. Kong, J. Chromatogr. A 1055 (2004) 71.
[8] H.T. Lu, Y. Jiang, F. Chen, J. Chromatogr. A 994 (2003) 37.
[9] Q.E. Wang, F.S.C. Lee, X.R. Wang, J. Chromatogr. A 1048 (2004) 51.
[10] Q.F. Tang, C.H. Yang, W.C. Ye, J.H. Liu, S.X. Zhao, J. Chromatogr. A 1144 (2007) 203.
[11] Q.F. Tang, C.H. Yang, W.C. Ye, J.H. Liu, S.X. Zhao, Phytochem. Anal. 19 (2008) 155.
[12] D.E. Schaufelberger, T.G. McCloud, J.A. Beutler, J. Chromatogr. 538 (1991) 87.
[13] F. Yang, Y. Ito, J. Chromatogr. A 943 (2002) 219.
[14] I.A. Bessonova, S.H. Saidkhodzhaeva, Chem. Nat. Compd. 36 (2000) 419.