Journal of Chromatography A, 1218 (2011) 3695–3698

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Preparative separation of alkaloids from *Gelsemium elegans* Benth. using pH-zone-refining counter-current chromatography

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ARTICLE INFO

Article history:
Received 21 January 2011
Received in revised form 5 April 2011
Accepted 8 April 2011
Available online 16 April 2011

Keywords:
Gelsemium elegans
pH-zone-refining counter-current chromatography
Alkaloids
Gelsemine
Koumine
Gelsevirine

ABSTRACT

Alkaloids in *Gelsemium elegans* possess a variety of therapeutic properties, including tumor suppression, analgesic and anti-inflammatory effects. In China, *G. elegans* has been used for centuries to treat a variety of medical conditions, including chronic pain and skin ulcer. Methods currently used to separate the active components of *G. elegans* are time-consuming and have low recovery. In the present study, we used pH-zone-refining counter-current chromatography to separate major alkaloids from a crude extract of *G. elegans*. The two-phase solvent system was methyl *tert*-butyl ether (MtBE)/acetonitrile/water (3:1.5:4, v/v). Triethylamine (20 mM) was added to the upper organic stationary phase as a retainer. Hydrochloric acid (10 mM) was added to the lower aqueous phase as an eluter. From 1.5 g of crude extract, we obtained 312 mg gelsemine, 420 mg koumine and 195 mg gelsevirine, with purities at 94.8%, 95.9% and 96.7%, respectively, which were determined by HPLC at 256 nm. The chemical identity of the isolated compounds was verified by electrospray ionization-mass spectrometry (ESI-MS), ^{1}H NMR and ^{13}C NMR. These results demonstrated that pH-zone-refining counter-current chromatography is an effective method to separate and purify major alkaloids from *G. elegans*.

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1. Introduction

Gelsemium elegans Benth. (Loganiaceae), referred to as "Gou-Wen" or "Duan-Chang-Cao" in China, has been used for centuries to treat a variety of medical conditions, including chronic pain and skin ulcer [1]. The reported pharmacological effects mainly include tumor suppression [2,3], analgesic and anti-inflammatory actions [4]. However, the crude extract or mixed alkaloids from this plant are highly toxic [1,5,6]. More than fifty kinds of alkaloids have been isolated from this plant to date [7–10]. Gelsemine, koumine and gelsevirine are three major active components of gelsemium (Fig. 1) [11,12]. Separation and purification of these alkaloids from *G. elegans* is typically achieved with conventional silica gel column chromatography followed by recrystallization [13]. Such processes are time-consuming. More importantly, the recovery is low.

pH-zone-refining counter-current chromatography (CCC) was first introduced by Ito and co-workers in the mid-1990s [14,15]. This technique is typically used to separate ionizable compounds, particularly alkaloids [16–23] and organic acids [24–26]. Briefly, a retainer base (or acid) is used in the stationary phase to retain the compounds of interest. An eluter acid (or base) consistent with the pK_a values and hydrophobicity of the compounds is used for elution. The process produces a succession of rectangular peaks with minimum overlap. The advantages of this method over standard high-speed CCC include high loading capacity, high concentration of the compounds in fractions and minor impurities [14]. Also, separation can be monitored by the pH of the effluent when there are no chromophores [27].

In this study, alkaloids were successfully separated from *G. elegans* using pH-zone-refining CCC for the first time.

2. Materials and methods

2.1. Reagents and materials

Methyl *tert*-butyl ether (MtBE), hydrochloric acid (HCl), triethylamine (TEA), acetonitrile (CH_3CN), chloroform, and methanol (for recrystallization) all of analytical grade were obtained from Sinopharm Chemical Reagents (Shanghai, China). Methanol (for HPLC analysis; Sinopharm) was of chromatographic grade. Double distilled water was used for all solutions and dilutions.

The roots and stems of *G. elegans* were obtained from a commercial source and authenticated by the Department of Pharmacognosy, College of Pharmacy, Fujian Medical University [28].

2.2. Apparatus

pH-zone-refining CCC was carried out using a column consisting of 110-m multilayer coil (1.8 mm I.D., Model TBE-300A; Shang-

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doi:10.1016/j.chroma.2011.04.025

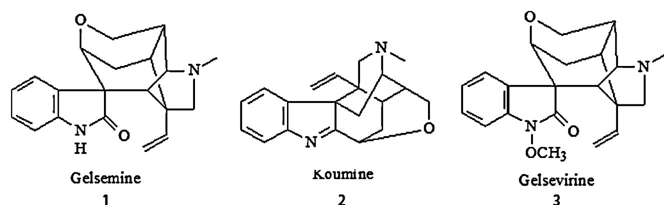

Fig. 1. Chemical structures of gelsemine, koumine and gelsevirine.

hai Tauto Biotech, Shanghai, China). The total capacity was 280 ml. The β value ($\beta = r/R$, where r is the rotation radius or the distance from the coil to the holder shaft, and R is the revolution radius or the distances between the holder axis and central axis of the centrifuge) ranged from 0.57 (internal) to 0.76 (external). Solvent was pumped into the column with a constant-flow pump (Model TBP-50A; Shanghai Tauto Biotech). The effluent was continuously monitored with a UV detector at 254 nm (Model TBD-23; Shanghai Tauto Biotech). The sample was injected into the column manually via a 20-ml loop. The pH of the fractions was measured by a pH-meter (Model RHS-4CT; Shanghai Dapu Instruments, Shanghai, China). The alkaloids were evaluated using HPLC (Agilent 1100; Agilent Technologies, USA).

2.3. Preparation of crude extract

Preparation of the crude extract was carried out as previously described [29]. Briefly, *G. elegans* dry powder (0.5 kg) was refluxed in 2500 ml of 95% ethanol for 3 h and the extraction was repeated, three times. The extracts were combined and the solvent eliminated using a rotavapor (Model RE-5299; Yuhua Instrument, Gongyi, China). The residue was dissolved with 300 ml of 2% HCl, and extracted again with 100 ml of ethyl ether, three times. The pH of the resulting extract was adjusted to 11 with 100 ml of 5 M NaOH prior to extraction of total alkaloids with 100 ml of chloroform for three times. Vacuum-evaporization of the chloroform extract yielded 2.4 g of crude total alkaloids.

2.4. Preparation of the two-phase solvent system and of the sample solution

A number of two-phase solvent systems with varying solvent ratio were tested to achieve optimal composition. Solvent systems were based upon a reference [27], and included MtBE/CH_3CN/water (5:0:5, v/v), (4:1:5, v/v), (3:1.5:4, v/v) and (2:2:3, v/v); MtBE/n-butyl alcohol/CH_3CN/water (4:2:3:8, v/v) and (2:2:1:5, v/v). The partition coefficient (K) values of the solvent systems in both acidic (K_{acid}) and basic (K_{base}) conditions were also determined as described in the reference.

MtBE/CH_3CN/water (3:1.5:4, v/v) was equilibrated in a separatory funnel prior to separation of the two phases for use. Triethylamine was added to the upper organic phase at a concentration of 20 mM. The lower aqueous phase was acidified with hydrochloric acid at 10 mM final concentration.

The sample solution was prepared by dissolving 1.5 g of crude alkaloid extract in a mixture of 5 ml upper phase and 5 ml lower phase. The pH of the sample solution was adjusted to 6.0 with HCl [16].

2.5. Separation procedure

The sample was injected into a column filled with the organic phase through the sample port. The aqueous phase was then pumped into the column at 2 ml/min in a head-to-tail mode while the column was rotating at 850 rpm. The effluent was continuously monitored with a UV detector set at 254 nm and collected with a fraction collector at 3-min intervals. The pH of the fractions was measured manually. After the separation, the column content was collected into a graduated cylinder with pressurized nitrogen gas. The retention of the stationary phase relative to the total column capacity was calculated. The fractions were brought to dryness and were analyzed with HPLC.

2.6. Analyses and identification of pH-zone-refining CCC fractions

Alkaloids were analyzed with HPLC using a Hypersil ODS2 column (250 mm × 4.6 mm I.D.) at 256 nm at a temperature of 25 °C. The mobile phase, consisting of methanol and 0.05% butyl amine in water (1:1, v/v), was set at a flow-rate of 1 ml/min. The effluent was monitored using a diode array detector (Model G1315B; Agilent Technologies, USA).

The pH-zone-refining CCC peaks were identified with electrospray ionization-mass spectrometer (ESI-MS) (Model DECAX-30000 LCQ Deca XP, Thermo Finnigan, USA) and with ^{1}H NMR and ^{13}C NMR spectrometer (Model Unity-500, Varian, USA) with chloroform ($CDCl_3$) as solvent and tetramethylsilane (TMS) as internal standard.

3. Results and discussion

3.1. Selection of the pH-zone-refining CCC solvent systems

pH-zone-refining CCC requires suitable partition coefficient ($K_{acid} \ll 1$ and $K_{base} \gg 1$) as well as good solubility of the sample in the solvent system [27,30]. MtBE/CH_3CN/water (5:0:5, v/v) and (4:1:5, v/v), MtBE/n-butyl alcohol/CH_3CN/water (4:2:3:8, v/v) and (2:2:1:5, v/v) were eliminated based on K_{acid} or K_{base}. MtBE/CH_3CN/water (3:1.5:4, v/v) and (2:2:3, v/v) produced suitable K values for the main constituents. Due to the cost of CH_3CN,

Table 1
Partition coefficients (K) of major components of *G. elegans* in different solvent systems.

Solvent system (v/v)		Compound 1	Compound 2	Compound 3
MtBE/CH_3CN/water (5:0:5)	K_{acid}	$\ll 1$	$\ll 1$	$\ll 1$
	K_{base}	4.22	3.87	10.73
MtBE/CH_3CN/water (4:1:5)	K_{acid}	$\ll 1$	$\ll 1$	$\ll 1$
	K_{base}	7.00	5.31	5.70
MtBE/CH_3CN/water (3:1.5:4)	K_{acid}	0.09	0.02	0.10
	K_{base}	11.23	7.77	11.63
MtBE/CH_3CN/water (2:2:3)	K_{acid}	0.10	0.04	0.10
	K_{base}	7.99	6.74	14.47
MtBE/n-butyl	K_{acid}	0.36	0.16	0.21
alcohol/CH_3CN/water (4:2:3:8)	K_{base}	5.01	4.65	7.13
MtBE/n-butyl	K_{acid}	0.26	0.21	0.20
alcohol/CH_3CN/water (2:2:1:5)	K_{base}	7.84	9.66	12.72

Note: Because the K_{acid} of these alkaloids in MtBE/CH_3CN/water (5:0:5, v/v) and (4:1:5, v/v) were very small, they were recorded as "$\ll 1$".

MtBE/CH_3CN/water (3:1.5:4, v/v) was selected for further use. The K values were presented in Table 1. As described earlier [25], the solubility of the sample was substantially improved by adding CH_3CN. 1.5 g of crude alkaloids could be dissolved in the mixture of 5 ml upper phase and 5 ml lower phase.

3.2. Separation of crude alkaloids by the pH-zone-refining CCC

Representative results obtained with pH-zone-refining CCC are presented in Fig. 2A. A total of 1.0 g crude alkaloid extract of *G. elegans* was separated under the reverse-displacement mode [31]. The retention of the stationary phase was 58.8%. Alkaloids were eluted as irregular rectangular peaks. The pH of the fractions was consistent with the absorbance. Minor components presented as multiple peaks at the front and rear boundaries.

Fig. 2B shows the chromatogram obtained from the pH-zone-refining CCC separation of 1.5 g crude alkaloid extract. The retention of the stationary phase was 58.3%. Major alkaloids were eluted as irregular peaks. The three major alkaloids eluted as irregular rectangular peaks at the retention times of 78–100, 105–132, and 138–159 min, respectively. The retention time of these compounds was 10.72, 16.42 and 18.10 min as determined by HPLC at 256 nm, with content of 23.3%, 29.2% and 13.9%, respectively (Fig. 3A). Compound 1 (312 mg, fraction 1 in Fig. 2), Compound 2 (420 mg, fraction 2 in Fig. 2) and Compound 3 (195 mg, fraction 3 in Fig. 2) were obtained by combining fractions. The purities were 94.8%, 95.9% and 96.7% as determined by HPLC (Figs. 3B, C and D), respectively.

The chromatograms demonstrated features characteristic of pH-zone-refining CCC. When the K value of the retainer fall between K_{acid} and K_{base} of the analytes, the analytes are behind the border and form solute zones, and are eluted in a train of rectangular peaks. The peak width broadens with increasing sample size [12]. As a result, the sample loading capacity is much higher than high-speed CCC. Apparently, peaks 1, 2 and 3 in Fig. 2A and B satisfied the above requirement and therefore formed characteristic rectangular peaks at appropriate pH. Increasing the load from 1.0 to 1.5 g resulted in broadening of the target peaks. HPLC analysis of the fractions corresponding to each zone boundary revealed that the mixing zones were no more than several milliliters. The limit between peaks 2 and 3 in the chromatogram was not clear when the load increase to 1.5 g, but there was no loss of resolution. With increasing sample size, the concentration of minor components was improved. The total time required for separation using pH-zone-refining CCC was dramatically shorter (4 h) than that required for conventional silica gel column chromatography (typically at more than one month for a crude extract) [13].

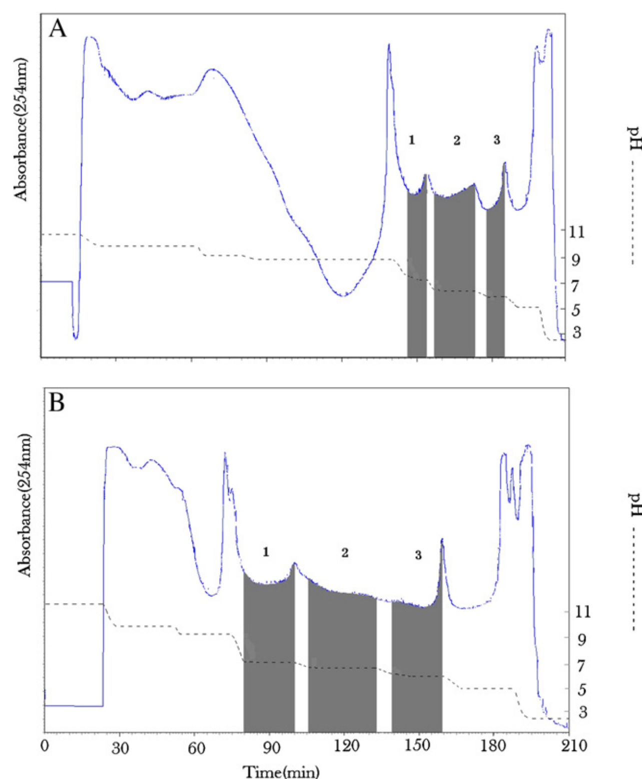

Fig. 2. pH-zone-refining CCC separation of *G. elegans* extract. Solvent system: MtBE/CH_3CN/water (3:1.5:4, v/v), 20 mM TEA in the upper organic stationary phase and 10 mM HCl in the lower aqueous phase; sample size: 1.0 g (A) and 1.5 g (B); flow-rate: 2 ml/min; detection: 254 nm; revolution speed: 850 rpm; retention of stationary phase: 58.8% (A) and 58.3% (B).

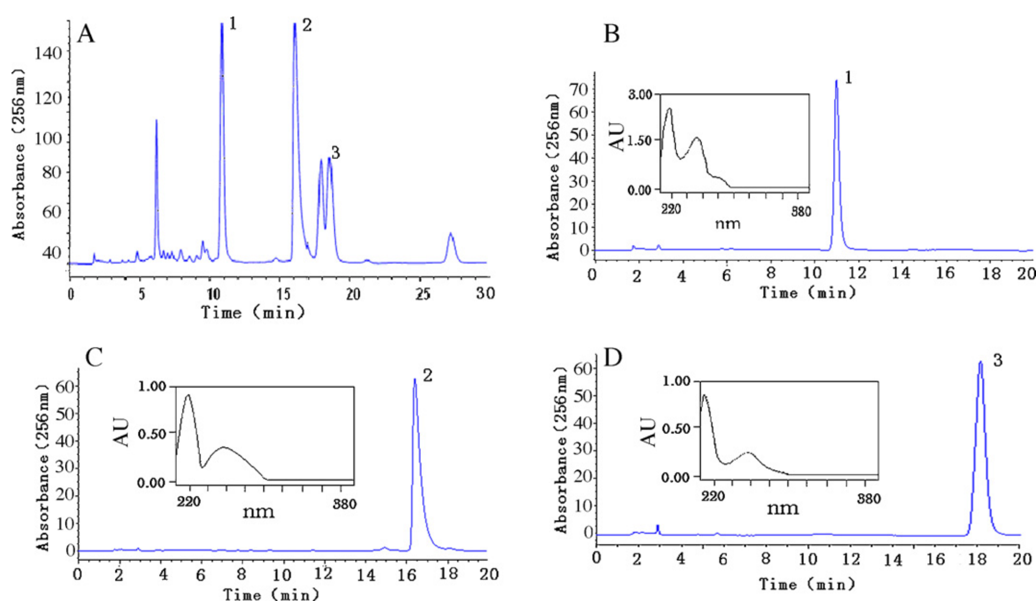

Fig. 3. HPLC and UV spectrometry analyses of crude alkaloids extracted from *G. elegans* and the purified fractions obtained in Fig. 2B. Experimental conditions: Hypersil ODS2 column (250 mm × 4.6 mm I.D.); column temperature: 25 °C; mobile phase: methanol–0.05% butyl amine in water (1:1, v/v); flow rate: 1.0 ml/min; detection: 256 nm; injection volume: 5 μl. (A) crude sample; (B) peak 1 in Fig. 2B; (C) peak 2 in Fig. 2B; (D) peak 3 in Fig. 2B.

3.3. Identification of the isolated compounds

Compound 1 was a white crystalline material with the following properties: positive ESI-MS *m/z* 323.4 $[M+H]^+$, ^1H NMR ($CDCl_3$, TMS) δppm: 3.85 (1H, s, 3-H), 2.37 (3H, m, N_b–CH_3), 4.13 (1H, dd, 5-H, *J* = 11.2, 2.0 Hz), 2.19 (1H, s, 6-H), 7.45 (1H, t, 9-H, *J* = 7.2 Hz), 7.04 (1H, t, 10-H, *J* = 7.4 Hz), 7.23 (1H, t, 11-H, *J* = 7.4 Hz), 6.82 (1H, d, 12H, *J* = 7.7 Hz), 2.85 (1H, dd, 14α-H, *J* = 14.5, 2.8 Hz), 2.04 (1H, m, 14β-H), 2.37 (1H, m, 15-H), 2.98 (1H, s, 16-H), 3.95 (2H, d, 17-H, *J* = 10.9 Hz), 5.14 (1H, d, 18a-H, *J* = 11.0 Hz), 4.98 (1H, d, 18b-H, *J* = 17.5 Hz), 6.27 (1H, dd, 19-H, *J* = 17.8, 11.0 Hz), 3.51 (1H, s, 21α-H), 2.79 (1H, d, 21β-H, *J* = 9.8 Hz). ^{13}C NMR ($CDCl_3$, TMS) δppm: 178.95 (2-C), 69.48 (3-C), 72.16 (5-C), 50.67 (6-C), 54.08 (7-C), 131.95 (8-C), 128.35 (9-C), 121.93 (10-C), 128.00 (11-C), 108.99 (12-C), 140.22 (13-C), 22.88 (14-C), 35.75 (15-C), 38.32 (16-C), 61.59 (17-C), 112.29 (18-C), 138.63 (19-C), 54.06 (20-C), 66.25 (21-C), 40.81 (N_b–CH_3). The ^1H NMR and ^{13}C NMR profile of Compound 1 matched with the reported NMR data for gelsemine [32].

Compound 2 was a white crystalline material with the following properties: positive ESI-MS *m/z* 307.4 $[M+H]^+$, ^1H NMR ($CDCl_3$, TMS) δppm: 4.94 (1H, m, 3-H), 2.65 (3H, s, N_b–CH_3), 2.93 (1H, s, 5-H), 2.45 (2H, m, 6-H), 7.56 (1H, d, 9-H, *J* = 7.6 Hz), 7.35 (1H, t, 10-H, *J* = 7.2 Hz), 7.42 (1H, t, 11-H, *J* = 7.2 Hz), 7.66 (1H, d, 12H, *J* = 7.2 Hz), 1.89 (1H, d, 14α-H, *J* = 14.8 Hz), 2.76 (1H, dt, 14β-H, *J* = 14.8, 3.7 Hz), 2.45 (1H, m, 15-H), 2.90 (1H, d, 16-H, *J* = 12.5 Hz), 3.65 (1H, d, 17α-H, *J* = 11.9 Hz), 4.34 (1H, dd, 17β-H, *J* = 11.9, 4.2 Hz), 4.90 (2H, m, 18-H), 4.72 (1H, dd, 19-H, *J* = 17.8, 11.3 Hz), 3.02 (1H, d, 21α-H, *J* = 11.9 Hz), 3.28 (1H, d, 21β-H, *J* = 11.8 Hz). ^{13}C NMR ($CDCl_3$, TMS) δppm: 186.59 (2-C), 70.52 (3-C), 56.35 (5-C), 29.11 (6-C), 57.68 (7-C), 143.29 (8-C), 123.22 (9-C), 126.32 (10-C), 128.10 (11-C), 120.05 (12-C), 153.64 (13-C), 24.58 (14-C), 32.64 (15-C), 37.27 (16-C), 60.59 (17-C), 115.45 (18-C), 136.69 (19-C), 45.26 (20-C), 56.84 (21-C), 41.48 (N_b–CH_3). This profile was consistent with the reported NMR data for koumine [33].

Compound 3 was a white crystalline material with the following properties: positive ESI-MS *m/z* 353.2 $[M+H]^+$, ^1H NMR ($CDCl_3$, TMS) δppm: 4.01 (3H, s, N_a–OCH_3), 3.85 (1H, s, 3-H), 2.73 (3H, s, N_b–CH_3), 4.08 (1H, s, 5-H), 2.18 (1H, s, 6-H), 7.41 (1H, d, 9-H, *J* = 7.6 Hz), 7.16 (1H, t, 10-H, *J* = 7.6 Hz), 7.41 (1H, d, 11-H, *J* = 7.6 Hz), 7.03 (1H, d, 12H, *J* = 7.6 Hz), 2.88 (1H, d, 14α-H, *J* = 12.8 Hz), 2.15 (1H, m, 14β-H), 2.65 (1H, s, 15-H), 3.51 (1H, s, 16-H), 4.08 (2H, s, 17-H), 5.37 (1H, d, 18a-H, *J* = 11.2 Hz), 5.14 (1H, d, 18b-H, *J* = 17.6 Hz), 6.28 (1H, dd, 19-H, *J* = 17.6, 11.2 Hz), 3.76 (1H, m, 21α-H), 2.88 (1H, d, 21β-H, *J* = 12.8 Hz). ^{13}C NMR ($CDCl_3$, TMS) δppm: 170.99 (2-C), 68.87 (3-C), 73.36 (5-C), 48.92 (6-C), 51.47 (7-C), 125.82 (8-C), 127.98 (9-C), 123.63 (10-C), 129.40 (11-C), 107.81 (12-C), 139.34 (13-C), 22.39 (14-C), 34.28 (15-C), 39.35 (16-C), 60.32 (17-C), 116.86 (18-C), 133.54 (19-C), 36.72 (20-C), 63.80 (21-C), 63.31 (N_a–O–CH_3), 42.89 (N_b–CH_3). This profile matched with the reported NMR data for gelsevirine [32].

In summary, the current study demonstrated that pH-zone-refining CCC is a useful method to isolate major active alkaloids from *G. elegans*. From 1.5 g crude extract, we obtained 312 mg gelsemine, 420 mg koumine and 195 mg gelsevirine with ≥94.8% purity in a single run.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No. 30973520), the Exploitation Program of Industrial Technology of Fujian Development and Reform Commission of China, the Key Program of Scientific Research of Fujian Medical University (No. ZD009) and the Nursery Foundation of Fujian Medical University (No. 2010MP045).

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