

## Achiral and Chiral Determination of Benzophenanthridine Alkaloids from Methanol Extracts of *Hylomecon* Species by High Performance Liquid Chromatography

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A high performance liquid chromatographic (HPLC) method was developed for the qualitative and quantitative determination of benzophenanthridine alkaloids from the methanol extracts of *Hylomecon hylomeconoides* and *H. vernale* (Papaveraceae). Achiral and chiral methods were adapted for the separation of 6-methoxydihydrosanguinarine (**1**), 6-acetyldihydrosanguinarine (**2**) and dihydrosanguinarine (**3**). The achiral reversed phase HPLC method made it possible the simultaneous separation and determination of **1**, **2** and **3** within 20 min on ODS column using acetonitrile-phosphate buffer (50 mM, pH 7.0) (50 : 50, v/v). The separation and determination of **1** and **2** enantiomers was available using chiral columns. The same amount of (+) and (-)-enantiomers of **1** was found from the methanol extract of specimen, indicated that **1** could be the artifact produced by the reaction of sanguinarine with methanol. *H. hylomeconoides* showed higher level of **1** and **3** in compared with *H. vernale*, especially in root samples permitting the possibility of chemical discrimination between two species.

**Key words:** Benzophenanthridine alkaloids, *Hylomecon* species, Enantiomers, Chiral separation, HPLC

### INTRODUCTION

*Hylomecon hylomeconoides* and *H. vernale* are only two *Hylomecon* species growing wild in Korea (Lee, 1996). They belong to Papaveraceae family, which has been reported to produce lots of benzophenanthridine alkaloids. Benzophenanthridine alkaloids exhibit a variety of pharmacological effects including antiinflammatory (Lenfeld *et al.*, 1981), antispasmodic and relaxant activity (Hiller *et al.*, 1998), antimicrobial (Mitscher *et al.*, 1978), anti-tumor (Nakanishi *et al.*, 1999), acetylcholinesterase inhibition (Schmeller *et al.*, 1997), transient hypotension, and so on. In a continuing investigation of biologically active compounds from Korean indigenous plants, three benzophenanthridine alkaloids were isolated from the hexane fraction of the methanol extract of *H. hylomeconoides* roots by repeated silica gel column chromatography. Physicochemical and

spectral data revealed their structures as 6-methoxydihydrosanguinarine (**1**), 6-acetyldihydrosanguinarine (**2**) and dihydrosanguinarine (**3**).

Sanguinarine has been the focus of the recent attention for diverse biological activities such as antimicrobial, antitumor, antimicrotubule activities and antiinflammatory (Chaturvedi *et al.*, 1997). Several reports focused on the production of the alkoxyanguinarine by treatment of sanguinarine free base with an appropriate alcohol (Williams *et al.*, 1993; Konda *et al.*, 1986). Therefore, compound **1** isolated from *H. hylomeconoides* roots might be an artifact to be formed in the methanol extraction procedure, but it showed a potent inhibitory activity against acetylcholinesterase and cytotoxic activity against human cancer cell lines (Cho, 2001). Compound **1** and **2** contain a chiral carbon and it could be expected that the extent of biological activity of each enantiomer would be different.

For the viewpoint of the effective utilization of the natural resources, the achiral and chiral analysis of these compounds in the plant samples could be important. Several reports were published focused on the isolation, structure

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elucidation (Dostal *et al.*, 1996, 1998; Marek *et al.*, 1997) and stability (Suresh *et al.*, 1997) of benzophenanthridine alkaloids from *Papaver somniferum* (Kraml *et al.*, 1993), *Cheilodorum japonicum* (Itokawa *et al.*, 1978), *Dactylicapnos torulosa* (Rucker *et al.*, 1994; Zhang *et al.*, 1995), *Bocconia integrifolia* (Oechslin *et al.*, 1991), *Coptis japonica* var. *dissecta* (Mizuno *et al.*, 1987), and *Argemone ochroleuca* (Fletcher *et al.*, 1993). The benzophenanthridine alkaloids in the crude plant extracts or pharmaceutical preparations were characterized and quantitatively determined by HPLC (Tome *et al.*, 1999; Husain *et al.*, 1999), capillary electrophoresis (Sevcik *et al.*, 2000), MS (1995; Fabre *et al.*, 2000), and TLC (Pothier *et al.*, 1993). All of them were focused on the achiral analysis and there was no information about the enantiomeric contents of the compounds. In this report, the achiral and chiral analysis of **1**, **2** and **3** by HPLC was carried out and the possibilities of the discrimination of *H. hylomeconoides* from *H. vernale* in the point of chemical constituents were discussed.

## MATERIALS AND METHODS

### Chemicals and instruments

Methanol (J. T. Baker, NJ, USA), isopropanol (Merck, Germany) and hexane (Fisher Chem., UK) used in this work were of HPLC grade and other reagents were of analytical grade. Milli-Q (Millipore, MA, USA) treated water was used throughout the experiment. The chromatographic system for achiral analysis was consisted of SCL-10A system controller, LC-10AD pump, SPD-10MVP diode array detector and CTO-10AS column temperature controller (Shimadzu, Japan). The chiral analysis was carried out on a chromatographic system consisted of HPLC Pump 64 (Knauer, Germany), Variable Wavelength UV Monitor 655A (Hitachi, Japan) and Chromato-Integrator D-2500 (Hitachi, Japan). The OR-990 polarimetric detector (Jasco, Japan) was connected to HPLC for the on-line monitoring of optical rotation of separated peaks. For qualitative analysis a QP8000 LC/MS (Shimadzu, Japan) with an atmospheric pressure chemical ionization (APCI) interface, LC-10AD pump and SPD-10A UV/VIS detector was used.

### Plant materials

*H. hylomeconoides* were collected from Chiri mountain (H01) in September, 1999 and Okcheon (H02) in September, 2001. *H. vernale* were collected from Okcheon (H03) in September, 2001. They were identified by Prof. KiHwan Bae in the College of Pharmacy, Chungnam National University, Daejeon, Korea. The plant material was separated in leaves, roots and flowers and stored at a cool and dark place. The voucher specimens were deposited at the herbarium in the College of Pharmacy, Chungnam National University.

### Isolation of sanguinarine derivatives

Compound **1**, **2** and **3** were isolated from roots of *H. hylomeconoides* as reported previously (Cho, 2001) and identified by <sup>1</sup>H-NMR, <sup>13</sup>C-NMR and MS. The purities were more than 99% by HPLC analysis.

### Sample preparation

The dried material was coarsely powdered and 1 g of sample was extracted on ice bath with 5 mL of methanol for 4 times. Methanol fractions were collected and extracted on ice bath with 10 mL of hexane for 4 times. The *n*-hexane fraction was then evaporated under vacuum and the residue was dissolved in 10 mL of methanol. Separately, samples were extracted on ice bath with *n*-hexane only. The extract was then evaporated and the final volume was adjusted to 10 mL with *n*-hexane. The solution was ready for HPLC analysis by being filtered through membrane filter. When the concentration of the compounds in sample was higher than the upper calibration range, the filtered solution was diluted with suitable solvent.

### Chromatography and identification

The achiral analysis of the sanguinarine derivatives was performed on a Nova-Pak C<sub>18</sub> (3.9×150 mm) column (Waters, CA, USA) with the eluent of acetonitrile-phosphate buffer (50 mM, pH 7.0) (50 : 50, v/v) in the flow rate of 1.0 mL/min. The injected volume was 10 μL and detected at UV 280 and 320 nm simultaneously. The identification of the components in the sample was carried out by analyzing the mass spectrum of chloroform extract of fraction corresponding to **1**, **2** and **3** in the HPLC effluent.

The chiral analysis of **1** and **2** was performed using Chiralcel OD (4.6×250 mm) from Daicel Chem. (Japan) with the eluent of isopropanol-*n*-hexane-diethylamine (20 : 80 : 0.1, v/v/v) and Chiralcel OJ-H (4.6×150 mm) from Daicel Chem. with the eluent of methanol in flow rate of 1.0 mL/min. The system was operated at room temperature. The direction of optical rotation was on-line monitored with a polarimetric detector.

### Calibration and quantitation

To prepare the calibration curves for the quantitative determination, different volumes of a mixed standard solution which contains 1 mg/mL of racemic **1** and **3** and 0.1 mg/mL of racemic **2** in methanol, were spiked to 1 g leaves of H01, which contain the least detectable amounts of **1**, **2** and **3**. The spiked samples were then extracted with methanol and *n*-hexane consecutively and analyzed by HPLC. Linear regression was used for all calibrations and the limit of detection was determined for each compound. For recovery test, known amounts of standards were spiked to the leaves of H01. The samples were extracted and then analyzed by HPLC.

## RESULTS AND DISCUSSION

### Achiral separation and identification of compounds

The achiral separation of **1**, **2** and **3** on the reversed system with the eluent of acetonitrile-phosphate buffer was optimal for either standard mixture or plant extract (Fig. 2). The pH of the phosphate buffer was adjusted to 7.0 to maintain the stability of **1** during the separation process. The peaks on the chromatogram were identified as **1**, **2** and **3** by analyzing the mass spectrum of chloroform extract of fraction corresponding to each compound in the HPLC effluent. Fig. 2(c) showed the chromatogram of sample extracted only with hexane. The measured amount of **1** in *n*-hexane extract was about 1.5% of that in methanol-*n*-hexane extract. This value is very low in comparison with the extraction rate of **3** (61%) indicating that **1** was possibly an artifact formed from the reaction of methanol with sanguinarine or equivalent compound during the extraction process. Generally the C-6 on sanguinarine is

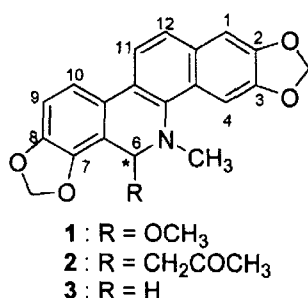


Fig. 1. Structure of sanguinarine derivatives isolated from *Hylomecon hylomeconoides*. The chiral center is marked with asterisk.

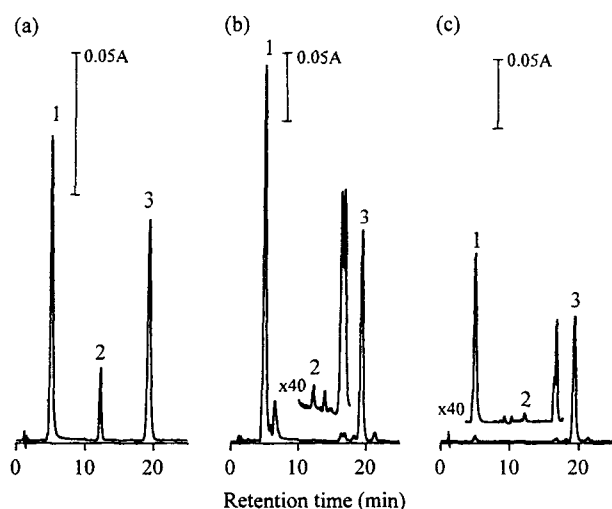


Fig. 2. Representative chromatogram of racemic 6-methoxydihydrosanguinarine (**1**), 6-acetyldihydrosanguinarine (**2**) and dihydrosanguinarine (**3**) standards in methanol (a), H02 roots extracted with methanol-hexane (b) and the same specimen extracted with hexane only (c) analyzed by the achiral method.

reactive and undergoes rapidly the nucleophilic reaction with alcohol to produce alkoxy compound (Williams *et al.*, 1993). This means that the measured amounts of **1** in methanolic extracts of *H. hylomeconoides* and *H. vernale* could be directly corresponded to the amounts of sanguinarine.

### Achiral determination and calibration

The calibration curves of three compounds calculated with peak area showed good linearity over the concentration range 0.01 to 1 mg/mL for **1** and **3** and 0.001 to 0.1 mg/mL for **2**. The  $R^2$  values of the calibration were 0.998, 0.998 and 0.996 for **1**, **3** and **2**, respectively. The efficiency of recovery in the HPLC analysis was more than 94% as shown in Table I. The internal standard was not used in this analysis hence the coefficient of variation for interday and intraday analysis was relatively high. The observed linearity and the results of recovery testing indicate that this HPLC method is suitable and applicable for quantitative evaluation of the *Hylomecon* species. The detection limits were 1  $\mu$ g/g for **1** and **3** and 0.5  $\mu$ g/g for **2**, at a signal to noise ratio of 2.

The variation of the benzophenanthridine alkaloids in *Hylomecon* species was presented in Table II. A sample with high level of **3** showed high concentration of **1**, or vice versa. The correlation between the concentration of **1** and **3** was relatively high ( $R^2 = 0.939$ ), supporting that **3** is the biosynthetic precursor of sanguinarine (Schumacher *et al.*, 1988). Generally, the concentration of benzophenanthridine alkaloids in roots of specimen was higher than that in leaves. Also *H. hylomeconoides* showed higher level of **1** and **3** in comparison with *H. vernale*, especially in roots. This fact could be used on the chemical discrimination of the *H. hylomeconoides* from *H. vernale*, because the classification of the *Hylomecon* species from the appearance was not so easy.

Table I. The recovery of **1**, **2** and **3** by the achiral method

Compounds	Added <sup>1)</sup> (mg)	CV (%) n = 4		Recovery (%)
		Interday	Intraday	
<b>1</b>	0.50	6.7	6.4	95
	1.00	5.7	5.5	97
	2.50	5.2	5.1	98
<b>2</b>	0.05	8.5	8.2	94
	0.10	7.8	7.4	95
	0.25	6.5	6.4	97
<b>3</b>	0.50	5.4	5.0	96
	1.00	5.1	4.9	99
	2.50	4.9	4.4	101

<sup>1)</sup>The amounts of each benzophenanthridine alkaloids were added to 1 g powdered roots of H01.

**Table II.** Concentration<sup>1)</sup> of **1**, **2** and **3** in the methanol extracts of *Hylomecon* species determined by achiral method

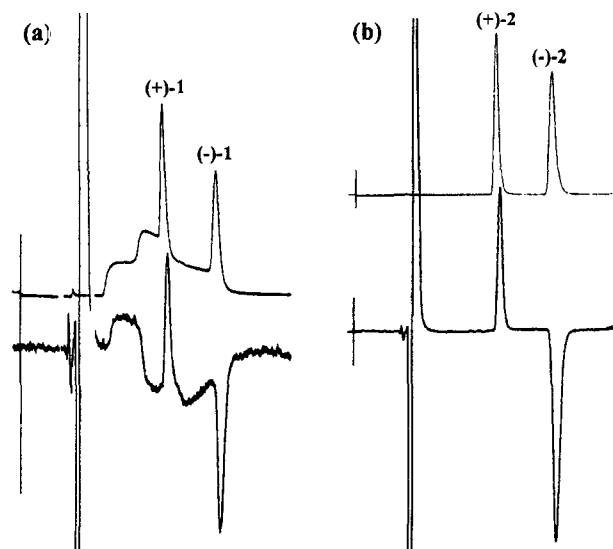
Specimen		<b>1</b>	<b>2</b>	<b>3</b>	
<i>H. hylomeconoides</i>	H01	roots	2.07	0.002	1.87
		leaves	0.03	n.d. <sup>2)</sup>	0.04
	H02	roots	2.44	0.002	1.75
		leaves	0.18	0.003	0.46
	flowers	0.37	0.002	0.52	
<i>H. vernale</i>	H03	roots	0.27	n.d.	0.04
		leaves	0.05	n.d.	0.04

<sup>1)</sup>Concentration was presented as mg/g dried specimen.

<sup>2)</sup>Not determined; when the concentration was lower than 2 µg/g or the peak was not fully separated.

### Chiral separation of **1** and **2** enantiomers

The racemic **1** and **2** in methanolic solution was separated into its enantiomer on chiral stationary phase Chiralcel OD and the direction of optical rotation was monitored with a polarimetric detector. Fig. 3 showed that the enantiomers of **1** and **2** were sufficiently separated on Chiralcel OD and the first eluted compounds were (+)-enantiomers for both compound. The separation of the standard mixture of **1**, **2** and **3** gave five peaks on Chiralcel OD shown as Fig. 4a. The elution order was **3**, (+)-**2**, (+)-**1**, (-)-**2** and (-)-**1** and the quantitative determination of the enantiomers was available for the methanol extracts of *Hylomecon* species (Fig. 4b). When the specimens were extracted with hexane, only about 1.5% of **1** were found in comparison with that extracted with methanol-hexane. In both cases the amounts of (+) and (-)-enantiomers were about the same. This suggests that **1** could be an artifact formed

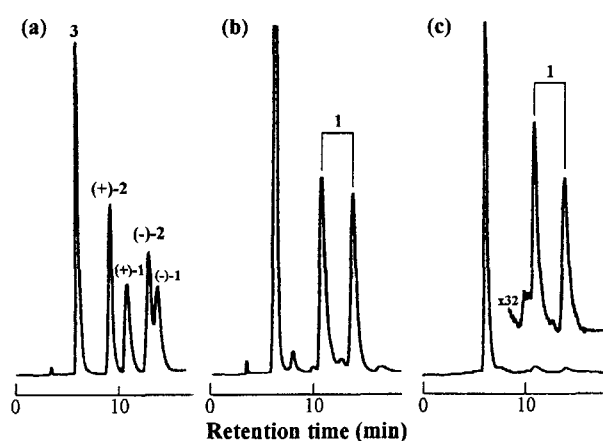


**Fig. 3.** Separation of enantiomers of **1** (a) and **2** (b) on Chiralcel OD with isopropanol-*n*-hexane-diethylamine (20:80:0.1, v/v/v) monitored by UV 320 nm (upper trace) and optical rotation (lower trace).

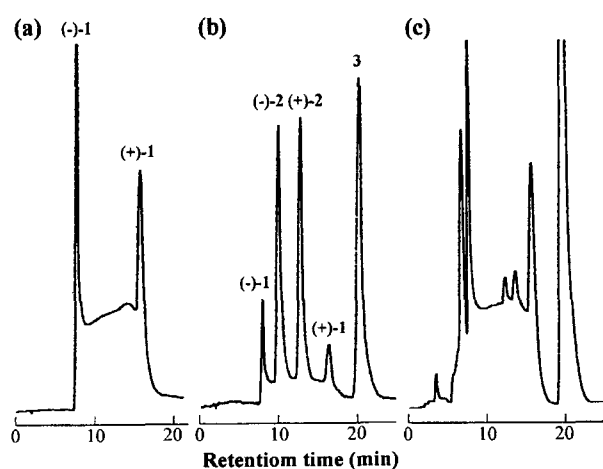
during extraction process.

The better resolution of the enantiomers could be achieved using the Chiralcel OJ-H column instead of Chiralcel OD column (Fig. 5b). In this case, contrary to the case of Chiralcel OD column, (-)-enantiomers were eluted prior to (+)-enantiomers for both **1** and **2**. When **1** was separated on Chiralcel OJ-H column, a plateau was observed between (-) and (+)-**1** peaks (Fig. 5a) caused by the inversion of (+)-**1** to (-)-**1** and vice versa during the chromatographic process. In spite of high resolution of enantiomers on Chiralcel OJ-H column, the elevation of baseline during the separation hindered the accurate determination of the enantiomers of **1** and **2** (Fig. 5c). On the other hand the determination of **3** could be carried out more accurately on Chiralcel OJ-H column.

The results of chiral analysis of **1** on Chiralcel OD and **3**



**Fig. 4.** Representative chromatogram of racemic **1**, **2** and **3** standards in methanol (a), H02 roots extracted with methanol-*n*-hexane (b) and the same specimen extracted with *n*-hexane only (c) separated on Chiralcel OD with isopropanol-*n*-hexane-diethylamine (20:80:0.1, v/v/v).



**Fig. 5.** Separation of enantiomers of **1** (a), standard mixture of **1**, **2** and **3** (b) and H02 roots extracted with methanol-*n*-hexane (c) on Chiralcel OJ-H with methanol.

**Table III.** Concentration<sup>1)</sup> of **1** and **3** in the methanol extracts of *Hylomecon* species determined by chiral method<sup>2)</sup>

Specimen	1			3		
	(+)	(-)	(+):(-)			
<i>H. hylomeconoides</i>	H01	roots	1.09	1.16	0.94	1.66
		leaves	0.02	0.02	1.00	0.03
	H02	roots	1.33	1.43	0.93	1.64
		leaves	0.11	0.10	1.10	0.55
		flowers	0.24	0.22	1.09	0.65
	<i>H. vernale</i>	H03	roots	0.16	0.14	1.14
leaves			0.04	0.03	1.14	0.04

<sup>1)</sup>Concentration was presented as mg/g dried specimen.

<sup>2)</sup>**1** was analyzed on Chiralcel OD and **3** on Chiralcel OJ-H.

on Chiralcel OJ-H were represented at Table III. The total amount of **1** analyzed by chiral method was a little higher than that by achiral method. This could be occurred by the instability of **1** in aqueous medium used by achiral method. The ratio of (+)-**1** to (-)-**1** measured in the methanol extracts of *Hylomecon* species ranged over 0.93 to 1.14, showed no significant differences indicating that **1** could be a racemic mixture. The enantiomers of **2** could not be determined by the chiral method because of the high detection limit of this method (5 µg/g). Compound **3** could be analyzed by chiral method as well as by achiral method. However there are no significant differences in the concentration **3** analyzed by two methods.

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