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The Isolation of Acetylcholinesterase Inhibitory Constituents from *Lycoris radiata* using On-line HPLC-biochemical Detection System

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Abstract – Bioactivity-guided fractionation using on-line HPLC biochemical detection system on $CHCl_3$-soluble fraction of *Lycoris radiata* led to the isolation of deoxylycorenine (**1**), *O*-demethylhomolycorine (**2**), galanthamine (**3**), lycoramine (**4**), mixture of 6α-and 6β-haemanthidine (**5**), and lycorine (**6**), identified by spectroscopic data and physicochemical property. Among the isolated compounds, **1**, **3** and **6** showed acetylcholinesterase inhibitiory activities with IC_{50} values of 18.0, 12.0 and 16.6 μM, respectively, in *in vitro* colorimetric microplate assay.
Keywords – *Lycoris radiata*, Amaryllidaceae, acetylcholinesterase, galanthamine, deoxylycorenine, lycorine, flow biochemical detection system, on-line HPLC-biochemical detection system

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by progressive and irreversible intellectual dysfunctions such as memory, learning and language, and the most common form of dementia in the elderly population (Blennow *et al.*, 2006). The patients of AD have been suffered from the cognitive dysfunction by the imbalance of the synthesis and release of acetylcholine (ACh) (Terry and Buccafusco, 2003). Thus, to elevate the availability of ACh in central synapses, the inhibitor of acetylcholinesterase (AChE) which the hydrolysis enzyme of ACh has become the main approach for the treatment of AD (Bores *et al.*, 1996). At the moment, galanthamine, a tertiary alkaloid belonging to the amaryllidaceous family, is being used as a symptomatic drug for the treatment of AD, and is known to have less side effects than other inhibitors like tacrine, and physostigmine (Krall *et al.*, 1999).

Bioactivity-guided isolation on the extract of *Lycoris radiata* HERB. (Amaryllidaceae) using an on-line HPLC-biochemical detection system with a few slightly modification of previous system (Ingkaninan *et al.*, 2000) was carried out because it showed AChE inhibitory activity in a conventional microplate assay. Since this on-line HPLC-biochemical detection system consisted of a colorimetric flow assay system and HPLC system, it is an effective and rapid technique for the detection and separation of AChE inhibitory compounds simultaneously.

L. radiata, a perennial plant native to Korea, Japan and China, has been used to treat emetics, dysentery, fever and expectorant (Park and Sung, 2007) in Korean traditional medicines. Literature reviews on this plant provided the occurrence of alkaloids (Kihara *et al.*, 1991; Kobayashi *et al.*, 1980) and pharmacological activity including antiviral (Li *et al.*, 2005), cytotoxic activity (Son *et al.*, 2010) and decrease of blood pressure (Miyasaka *et al.*, 1979). Herein, we described the rapid detection and separation of AChE inhibitory alkaloids from *L. radiata*

Experimental

General – The NMR spectra were recorded on a Jeol JMN-GS 500 spectrometer (Jeol, Tokyo, Japan) operating at 500 MHz. A Jeol JMS-700 mass spectrometer (Jeol, Tokyo, Japan) was used for measuring the ESI or FAB-MS. For flow biochemical detection system, four HPLC pumps (Hitachi L-7100, L-6200, L-6000; Tokyo, Japan, and JASCO PU-975; Easton, MD, USA) were used for the delivery of sample, substrate, enzyme and dye. UV spectra were recorded on a JASCO PU-980 UV/Vis detector set at 405 nm. For on-line HPLC-UV-biochemical detection system, HPLC system was connected to flow biochemical detection system using the micro splitter

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valve (Upchurch Scientific, Oak Harbor, WA, USA). HPLC system consisted of a Gilson 321 pump, UV/Vis-151 detector (Middleton, WI, USA) and an Inertsil ODS-3 (4.6 × 250 mm, i.d.; 5 μm particle size, GL Sciences, Tokyo, Japan) HPLC column. A perfluoro alkoxy alkane (PFA) tubing (Upchurch Scientific) was used for reaction coil in two detection systems. AChE from electric eel (type VI-s, lyophilized powder, 292 U/mL solid, 394 U/mg protein), acetylthiocholine iodide (ACTI), 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), galanthamine bromide, bovine serum albumin (BSA), sodium chloride (NaCl) and magnesium chloride ($MgCl_2$) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Organic solvents used in the isolation of AChE inhibitory compounds from *L. radiata* were purchased from Daejung Chemical Co. (Seoul, Korea).

Plant extracts – The bulbs of *L. radiate* Herb. (3.0 kg) were purchased from Key-chungsan botanical garden, Pohang, Korea in March 2006. A voucher specimen (SNU0817) was deposited at the herbarium of College of Pharmacy, Seoul National University.

Extraction and isolation – The lyophilized powder of dried bulbs (1.6 kg) was extracted with 80% aqueous MeOH (2 *l* × 3) under sonication at room temperature and yielded the crude extract (491 g). This crude extract was then dissolved in H_2O and partitioned with *n*-hexane (3 *l* × 3), $CHCl_3$ (3 *l* × 3) and H_2O in turn, to give *n*-hexane-soluble extract (8.6 g), $CHCl_3$-soluble extract (9.3 g) and H_2O residue (362.5 g), respectively. The H_2O residue was fractionated into H_2O-soluble (292.1 g) and MeOH-soluble (36.5 g) extracts by a Diaion HP-20 column chromatography.

The $CHCl_3$-soluble extract and MeOH-soluble fraction, showing the potent AChE inhibitory activities on microplate assay (Fig. 1), were further fractionated into ten sub-fractions (C1-C10) and two sub-fractions (M1-M2), respectively, by using a silica gel column chromatography with a gradient mixtures of $CHCl_3$-MeOH (25 : 1 → 10 : 1 → 5 : 1 → 3 : 1).

Conventional Microplate assay – The microplate assay method for measuring AChE inhibitory activities of extracts or individual compounds of *L. radiata* was based on modified Ellman's method (Kiely *et al.*, 1991). Briefly, in each well of the 96-well plates, 25 μl of extracts (10 mg/ml dissolved in MeOH) or compounds dissolved in MeOH were mixed with 25 μl of 15 mM ACTI dissolved in H_2O, 125 μl of 3 mM DTNB dissolved in a buffer with 0.1 M NaCl and 0.02 M $MgCl_2$, and 50 μl of 50 mM Tris-HCl buffer (pH 8.0). The absorbance of each well was read by an ELISA reader at 405 nm after 60s. Then,

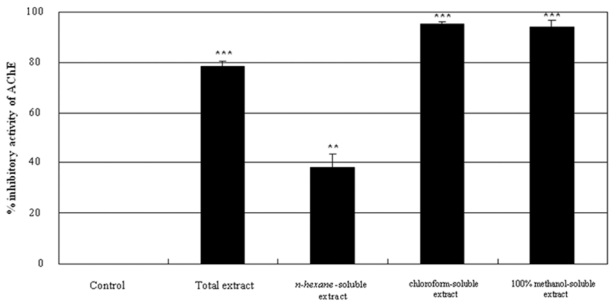

Fig. 1. Acetylcholinesterase inhibitory activity (%) of each extracts from *L. radiata* on a microplate. Each value represents the mean ± SD of three experiments. Each extract-treated value significantly differs from the control (MeOH) at a level of $^{**}P < 0.01$, $^{***}P < 0.001$.

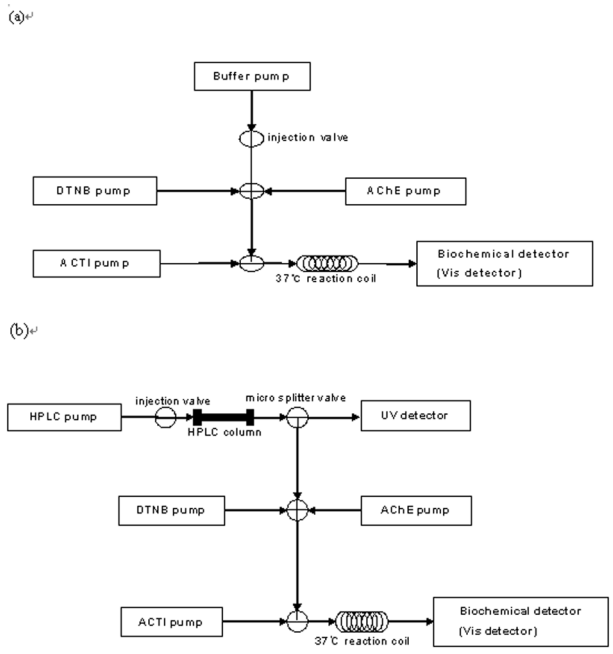

Fig. 2. (a) Flow biochemical detection system and (b) On-line HPLC-biochemical detection system.

25 μl of 0.2 U/ml AChE dissolved in a buffer with 0.1% BSA was added to each well and the absorbance was measured again at the same wavelength after 120s. The increase in absorbance due to spontaneous hydrolysis of the substrate was calculated by subtracting the absorbance of the reaction before adding the enzyme from the absorbance after adding the enzyme. Inhibition of enzyme activity by sample was calculated as a percentage compared to an assay using MeOH (control) without any inhibitor. All tests were performed in triplicate for each concentration, and were repeated three times.

Flow biochemical detection system – Flow biochemical activity detection system is modified from the assay

reported previously (Ingkaninan *et al*., 2000). The overall scheme of this system is shown in Fig. 2a. Four reagents, i.e., buffer (0.05 M Tris-HCl, pH 8.0), 0.2 mM ACTI, 0.2 mM DTNB and 0.2 U/ml AChE were introduced at a flow rate of 0.3 mL/min into the system and met in the reaction coil (inner volume: 300 μl) consisted of PFA tubing. The reaction coil was put into a water bath set at 37 °C. A solution of galanthamine used as reference chemical (10 μl of 0.005 μg/ml to 1 mg/ml; dissolved in MeOH) or each aliquot (10 mg/ml dissolved in MeOH) of twelve sub-fractions (C1-C10, M1-M2) was injected via a manual injector connected at the buffer pump. After the reaction coil, the final product of Ellman's reaction, 5-thio-2-nitrobenzoate, is detected at 405 nm.

Galanthamine was used as the reference standard to validate the limit of detection on flow biochemical detection system. As shown in Fig. 3, the AChE inhibitory activity of galanthamine was detected until 1 μg/ml. The AChE inhibitory activities of higher concentrations than 1 μg/ml were distinguished from that of a control (100% MeOH). Thus, the limit of detection on this system was determined until not less than 1 μg/ml.

On-line HPLC-Biochemical Detection System – This system consists of HPLC-UV system and flow biochemical detection system (Fig. 2b). A solution of galanthamine used as reference standard (10 μl of to 1 mg/ml; dissolved in MeOH), or an aliquot of C4 sub-fraction (10 μl of 10 mg/ml; dissolved in MeOH) of $CHCl_3$-soluble extracts was injected into the HPLC column (flow rate: 1 ml/min; 0.03 M ammonium carbonate and MeOH = 60 : 40, v/v). After HPLC column, the eluate introduced at a flow rate of 1 mL/min was split into UV detector (HPLC system; wavelength: 280 nm) at a flow rate of 0.7 ml/min, and into flow biochemical detection system at a flow rate of 0.3 ml/min using the micro splitter valve. The accordance of the retention time between the chromatogram of HPLC system and the chromatogram of flow biochemical detection system was determined as adjusting the length of PEEK tubing into each system, and controlling the micro splitter valve. A solution (20 μl of 1 mg/ml) of galanthamine was injected via a manual injector into this system to synchronize between the chromatogram of the HPLC system and the chromatogram of flow biochemical detection system (Fig. 4). The mobile phase was split into two ways; one way is connected to detect the AChE inhibitory compounds (flow rate: 0.7 ml/min, wavelength: 280 nm), the other way to reaction coil (flow rate: 0.3 ml/min, wavelength: 405 nm). A flow rate into reaction coil was faster than it described previously to shorten the detection time of the AChE inhibitory activity. Split ratio into each

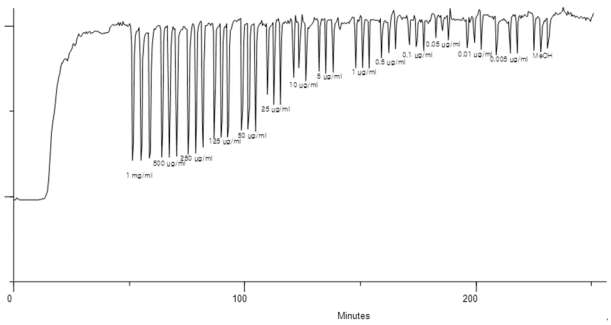

Fig. 3. The chromatogram of galanthamine was obtained from flow biochemical detection system. A solution of galanthamine (10 μl of 0.005 μg/ml to 1 mg/ml; dissolved in MeOH) was injected via a manual injector connected at the buffer pump.

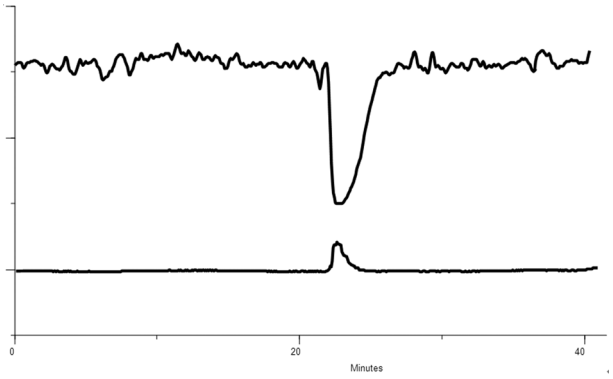

Fig. 4. The chromatogram of galanthamine was obtained from on-line HPLC-biochemical detection system. A negative peak of inhibition was observed at 22.3 min; enzyme inhibition chromatogram (top line), HPLC chromatogram (bottom line).

way was determined by using the micro splitter and adjusting the length of its connected PEEK capillaries. The peak of galanthamine from the HPLC system (bottom trace) was identical with that of the chromatogram in flow biochemical detection system (top trace) at 22.3 min (Fig. 4).

Isolation of Standard Compounds – Sub-fraction C4 (5.5 g), showing the most significant AChE inhibitory activity (Fig. 5), was subjected to a silica gel column chromatography by using a mixture of $CHCl_3$ and MeOH (30 : 1 → 15 : 1 → 10 : 1 → 5 : 1 → 1 : 1) and gave five sub-fractions (C4-1-C4-5). Compound **1** (23 mg) and **2** (190 mg) were isolated from sub-fraction C4-2 (2.1 g) by repeated semi-preparative HPLC (Inertsil ODS-3, 10 × 250 mm, 0.03 M ammonium carbonate-MeOH; 50 : 50, v/v, 4 ml/min). Compounds **3** (70 mg) and **4** (27 mg) were purified from sub-fraction C4-3 (0.9 g) using a HPLC separation (0.03 M ammonium carbonate-MeOH; 55 : 45, v/v, 4 ml/min). HPLC fractionation (0.03 M ammonium carbonate-MeOH; 60 : 40, v/v, 4 ml/min) on sub-fraction

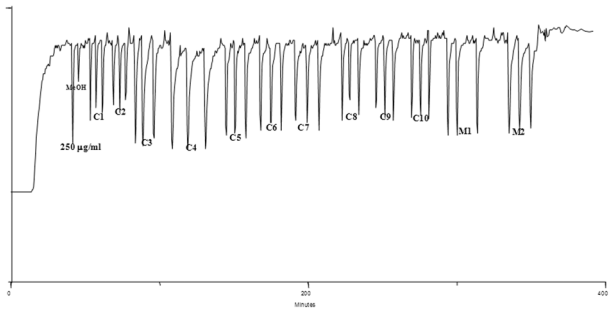

Fig. 5. The chromatogram of C1-C10 and M1-M2 sub-fractions obtained from flow biochemical detection system. An aliquot of each sub-fraction (10 µl of 10 mg/ml; dissolved in MeOH) was injected via a manual injector connected to the buffer pump.

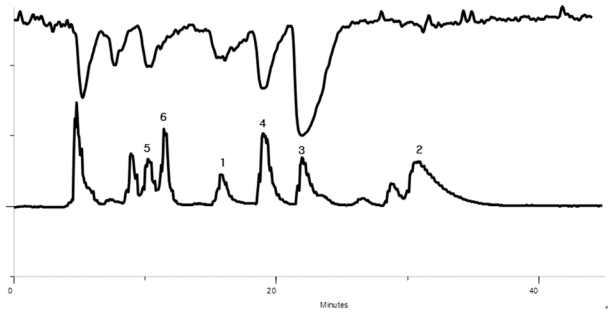

Fig. 6. The chromatogram of C4 sub-fraction obtained from on-line HPLC-biochemical detection system. Negative peaks of inhibition were observed at 10.1, 18.0, 19.6, and 22.3 min, respectively, and isolated compounds were presented tentatively on HPLC chromatogram according to their retention times; enzyme inhibition chromatogram (top line), HPLC chromatogram (bottom line).

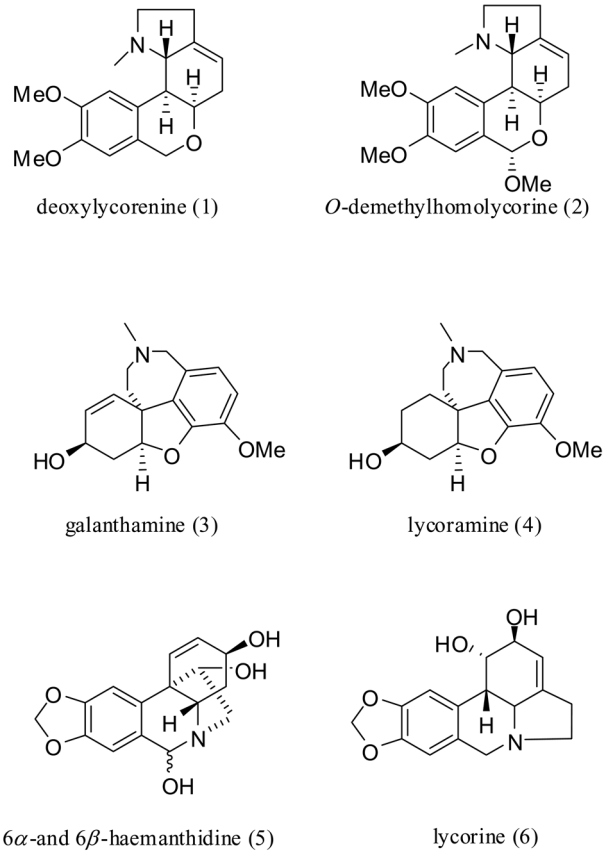

Fig. 7. The structures of compounds **1** - **6**.

C4-4 (0.7 g) yielded compound **5** (44 mg). Compound **6** (213 mg) was obtained from sub-fraction C4-5 (1.8 g) by recrystallization in MeOH.

Results and Discussion

In *in vitro* microplate assay, $CHCl_3$-soluble and 100% MeOH-soluble fractions of the roots of *L. radiata* were found to inhibit AChE activity 95% and 94%, respectively (Fig. 1). Chromatographic sub-fractions of these active fractions, C1-C10 and M1-M2, were tested in flow biochemical detection system. Among the 12 sub-fractions tested, sub-fraction C4 exhibited the most potent inhibitory effect against AChE. This C4 sub-fraction was further subjected to on-line HPLC-biochemical detection system and displayed the chromatographic peaks (lower chromatogram) corresponding to inhibitory activity (upper chromatogram) as shown in Fig. 6. Each peak in the HPLC chromatogram was isolated by repeated chromatographic separation work and identified by spectroscopic measurements as deoxylycorenine (**1**) (Codina *et al.*, 1993), *O*-demethylhomolycorine (**2**) (Codina *et al.*, 1993), galanthamine (**3**) (Jegorov *et al.*, 2006), lycoramine (**4**) (Jegorov *et al.*, 2006), mixture of 6α and 6β-haemanthidine (**5**) (Pabuççuoglu *et al.*, 1989), and lycorine (**6**) (Likhitwitayawuid *et al.*, 1993) (Fig. 7). All these isolates were evaluated for their AChE inhibitory activity in a conventional 96 well microplate assay (Table 1). Along with galanthamine (**3**) (IC_{50} 12.0 ± 4.8 µM) which was known as a famous AChE inhibitor, deoxylycorenine (**1**) and lycorine (**6**) (IC_{50} 18.0 ± 5.0, 16.6 ± 4.5 µM, respectively) were isolated as significant AChE inhibitory constituents in this plant. Even though the roots of *L. radiata* are known to contain galanthamine, this on-line HPLC biochemical detection system enabled to detect active galanthamine and its derivatives successfully. Therefore, the current on-line HPLC-biochemical detection system may be a useful method to simultaneously detect and isolate AChE inhibitory compounds from natural sources.

Table 1. The inhibitory activity of compounds **1 - 6** against acetylcholinesterase

Compound	IC_{50} (μM)	Inhibitory activity (%)	
		10 μM	100 μM
1	18.0 ± 5.0	39.96 ± 2.72	86.73 ± 10.65
2	92.3 ± 6.3	6.30 ± 1.01	52.68 ± 4.62
3	12.0 ± 4.8	47.02 ± 1.73	92.68 ± 6.73
4	76.3 ± 8.2	7.54 ± 8.56	60.19 ± 3.96
5	107.6 ± 15.3	2.16 ± 5.80	48.60 ± 3.59
6	16.6 ± 4.5	41.81 ± 1.32	87.02 ± 4.67

Each value represents the mean ± SD of three experiments. Each extract-treated value significantly differs from the control (MeOH) at a level of $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

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