The Application of HPLC with On-Line Coupled UV/MS−Biochemical Detection for Isolation of an Acetylcholinesterase Inhibitor from *Narcissus* 'Sir Winston Churchill'

K. Ingkaninan,[†] A. Hazekamp,[†] C. M. de Best,[‡] H. Irth,[‡] U. R. Tjaden,[⊥] R. van der Heijden,[†] J. van der Greef,[⊥] and R. Verpoorte*[,†]

Division of Pharmacognosy and Division of Analytical Chemistry, Leiden/Amsterdam Center for Drug Research, Gorlaeus Laboratories, Leiden University, P.O. Box 9502, 2300 RA, Leiden, The Netherlands, and Screentec, Niels Bohrweg 11-13, 2333 CA, Leiden, The Netherlands

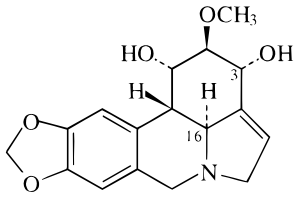
Received November 17, 1999

An HPLC with on-line coupled UV/MS−biochemical detection method for acetylcholinesterase (AChE) inhibitors in natural sources has been developed. The potential of this method is shown by the isolation of a new AChE inhibitor from the alcoholic extract of *Narcissus* 'Sir Winston Churchill'. Combining a prefractionation technique using centrifugal partition chromatography with the on-line HPLC−UV/MS−biochemical detection resulted in the isolation of the active compound that was identified as ungiminorine. This alkaloid shows a mild inhibitory effect on AChE.

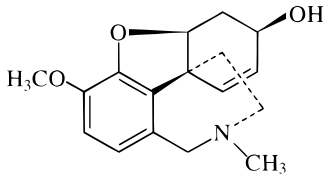
Alzheimer's disease is a disorder associated with progressive degeneration of memory and cognitive function. It is becoming a major health problem in this decade. One of the promising therapeutic strategies is to reverse the cholinergic deficit in the brain by using an acetylcholinesterase (AChE) inhibitor.[1] The AChE inhibitor galanthamine is now being developed for treatment of Alzheimer's disease. Recently, it received the first approval in Austria.[2] This compound is an alkaloid commonly found in the bulbs of snowdrops and daffodils, both belonging to the Amaryllidaceae family. The further search for other AChE inhibitors is of great interest. Nature seems to be a very interesting source of such compounds.

Recently, several strategies to improve the efficiency of drug discovery programs and to minimize time and cost in the search for new leads from natural products have been developed.[3] One approach is prefractionation of the extract before bioactivity screening to improve the quality of the samples and to rapidly identify known active compounds.[4] Another approach is to couple separation techniques to biochemical detection and some other identification techniques such as mass spectrometry (MS) and diode array spectrometry for the simultaneous separation, detection, and identification of active compounds in crude extracts.[5,6] In this study, these two approaches were combined. Centrifugal partition chromatography (CPC) was used in the fractionation process as it offers many benefits for natural product separation, such as high recovery of compounds, short run times, and easy up-scaling. In the general CPC separation procedure for plant extracts previously developed,[7] the fractions obtained from the CPC prefractionation step are tested for bioactivity, and the active fraction is further separated by a second CPC step. The fractions obtained are tested again, and the active fractions are injected into an HPLC with on-line coupled UV/MS−biochemical detection for AChE inhibitory activity.[6] Figure 1 shows the scheme of the on-line system. The biochemical detection was developed from the colorimetric method described by Ellman et al.[8] The product from AChE reaction will react to 5,5′-dithiobis[2-nitrobenzoic acid] (DTNB) and form the yellow substance, which can be detected by a spectrometer. The negative peak will be observed if there is an inhibitory activity to the enzyme from the eluate from HPLC. In this way, known inhibitors such as galanthamine can be easily recognized, and the investigation can be selectively focused on the unknown active compounds.

In this study, the isolation and identification of an AChE inhibitor from *Narcissus* 'Sir Winston Churchill' (Amaryllidaceae) is described using the above methods. *Narcissus* spp. are well-known for AChE inhibitory activity because of the presence of the alkaloid, galanthamine. However, there might be other compounds that exhibit the same activity. In a preliminary screening for AChE inhibitory activity, the ethanolic extract from *Narcissus* 'Sir Winston Churchill' showed mild activity in the microplate assay. It was chosen to be a model for the study to show that the separation and detection procedure developed could lead to the rapid identification of known active compounds or to the isolation of new, perhaps minor, active compounds.



ungiminorine



galanthamine

Results and Discussion

The scheme of the separation, detection, and identification of the AChE inhibitor from *Narcissus* 'Sir Winston

* To whom correspondence should be addressed. Tel.: +31-71-5274528. Fax: +31-71-5274511. E-mail: verpoort@LACDR.leidenuniv.nl.
[†] Division of Pharmacognosy.
[‡] Screentec.
[⊥] Division of Analytical Chemistry.

10.1021/np9905719 CCC: $19.00
Published on Web 05/03/2000

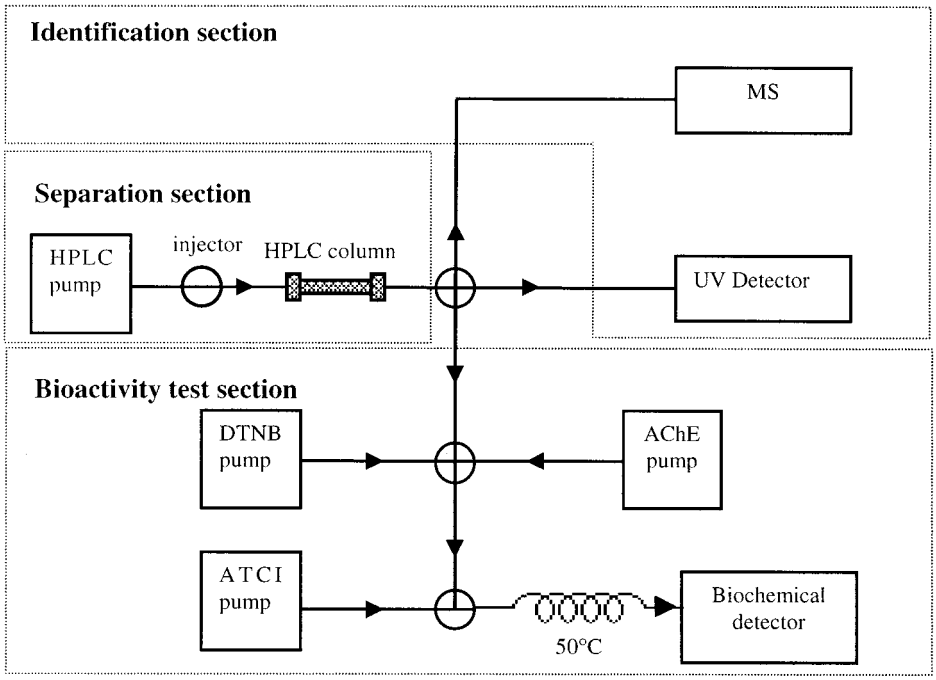


Figure 1. A scheme of the HPLC coupled on-line with UV/MS−biochemical detection.

Churchill' is shown in Figure 2. After the two-step fractionation,[7] the microplate assay screening showed that the activity was concentrated in fraction 6/12. This active fraction was analyzed by the on-line HPLC−UV/MS−biochemical detection of AChE inhibitors. The active peak from the biochemical detector at 15.2 min corresponded to the UV peak at 12.9 min and the MS peak at 13.1 min. The activity detected did not belong to galanthamine, as galanthamine showed a different retention time and molecular weight from the active peak detected (galanthamine has retention time of 8.16 min and a molecular weight of 287). However, MS showed that there were two compounds present at 13.1 min having molecular weights of 265 ([M + H] of 266) and 317 ([M + H] of 318). The results from the less active neighboring fractions in this on-line system could give useful information for the identification of the active peak. However, in the neighboring CPC fractions, these two molecular peaks were also always eluted simultaneously. Therefore, a change in the HPLC separation conditions (such as the column or the eluent) was necessary to separate these two compounds from each other.

The second separation by CPC was repeated to obtain a larger amount of the active fraction. The active fraction was further separated by preparative HPLC, and the collected fractions were tested by the microplate assay. This resulted in the isolation of a pure active compound with a MW 317, while the fraction having the compound with MW 265 did not show inhibitory activity. This active compound was determined to be ungiminorine by comparison of the MS and ^{1}H NMR spectra with the literature[9,10] and the reference compound. The proton assignments and the stereochemistry of the compound were confirmed by COSY and NOESY experiments.

The AChE inhibitory activities of ungiminorine and galanthamine were measured by the microplate assay. The IC_{50} value of ungiminorine was 86 ± 7 μM, while galanthamine was more than 80-fold more potent (IC_{50} value of 0.98 ± 0.07 μM) (Figure 3). The tertiary amine function of ungiminorine might bind with an anionic site of the enzyme. The inhibitory effect of ungiminorine on AChE was not reported before. As the chemical structure of ungiminorine is quite different from galanthamine, it might be useful for further structure−activity relationship study of alkaloidal AChE inhibitors.

This study demonstrates the usefulness of CPC fractionation in combination with on-line HPLC−UV/MS−biochemical detection in the search for new leads from natural products. The general fractionation procedure by CPC allows the rapid separation of bioactive compounds. The on-line system leads to the rapid detection and identification of the active compounds. Also, these two techniques can dereplicate the known active compounds during the study. It is to be noted that only a small amount of the eluate from the HPLC is split to the biochemical detector, therefore, on-line fraction collection can be performed. To have more separation capacity, this on-line system can be applied to a preparative separation technique such as preparative HPLC. To gain more information for the identification of active peaks, it would be useful to couple other detection lines, such as a photodiode array detector or an NMR, to this on-line system.

Experimental Section

General Experimental Procedures. Acetylthiocholine iodide (ATCI), AChE, bovine serum albumin (BSA), carbachol, DTNB, galanthamine, and physostigmine were obtained from Sigma (St. Louis, MO). All organic solvents (analytical reagent grade) were purchased from J. T. Baker (Deventer, The Netherlands); 50 mM Tris-HCl pH 8.0 was used as a buffer through the experiment unless otherwise stated. AChE used in the assay was from electric eel (type VI-S lyophilized powder, 480 U/mg solid, 530 U/mg protein). The lyophilized enzyme was prepared in buffer to obtain 1130 U/mL stock solution. The enzyme stock solution was kept at −80 °C. The further enzyme-dilution was dissolved in 0.1% BSA in buffer. DTNB was dissolved in the buffer containing 0.1 M NaCl and 0.02 M $MgCl_2$. ATCI was dissolved in Millipore water. The reference compound, ungiminorine, was from Professor Belkis Gözler, Ege University, Turkey.

A modular Sanki (Kyoto, Japan) centrifugal partition chromatograph (type LLN) was used. It consisted of a power supply (model SPL), a triple-head constant-flow pump (model LBP-V), and a centrifuge (model NMF). The centrifuge could contain

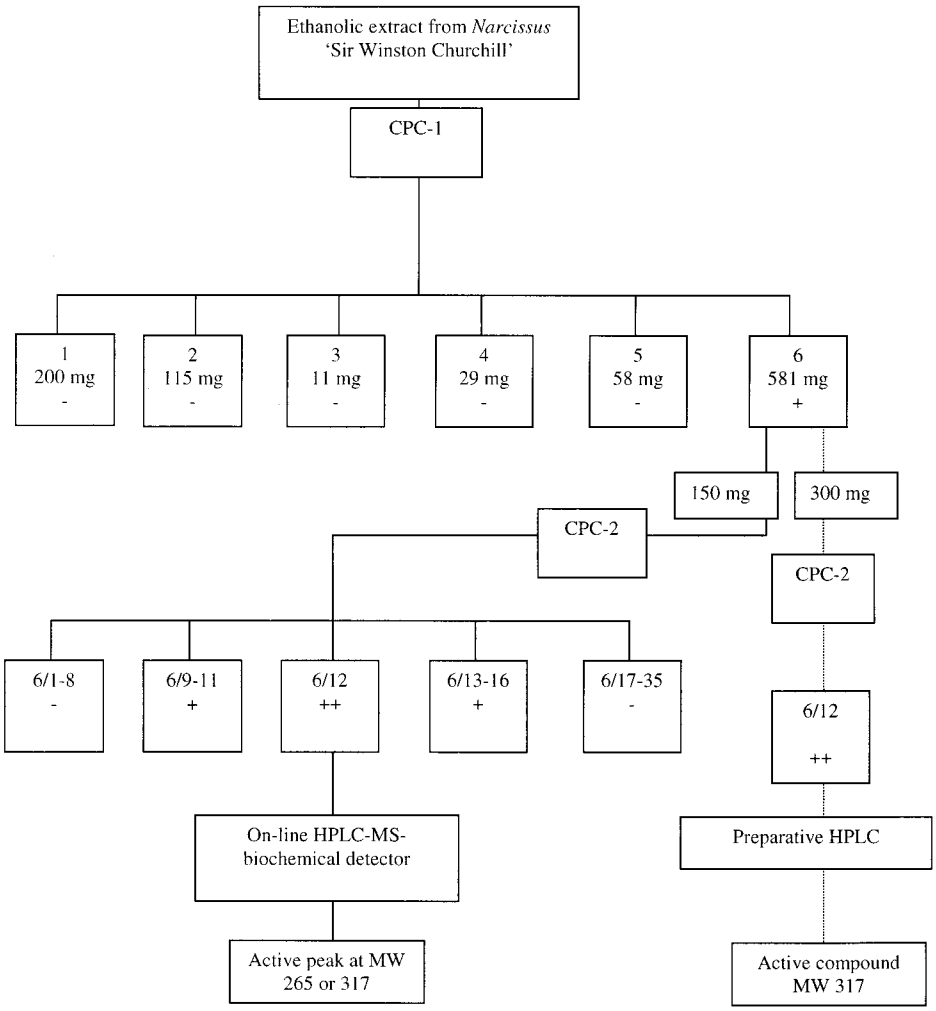


Figure 2. A scheme of the separation and detection of the active compound from *Narcissus* 'Sir Winston Churchill' extract using general fractionation procedure in combination with the microplate assay screening for AChE inhibitory activity. The activity of the fractions at a concentration of 0.1 mg/mL are presented as follows: − = less than 60% inhibition, + = 60−80% inhibition, ++ = more than 80% inhibition. The on-line coupling of HPLC−UV/MS−biochemical detection was used for the rapid identification of the active peak. The off-line preparative HPLC was used at the final separation step.

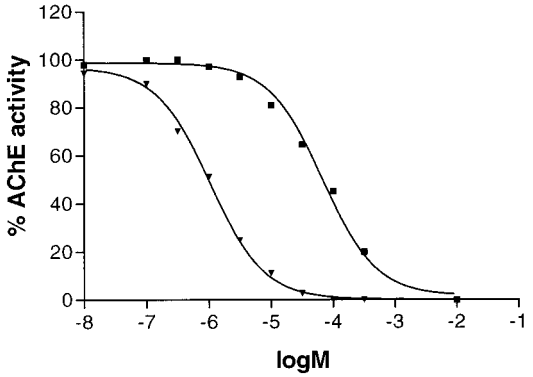


Figure 3. Inhibition effects of ungiminorine (■) and galanthamine (▲) on AChE measured by the microplate assay. Percentage inhibition was assayed by the standard procedure described in the text. The points are averages of one typical experiment done in triplicate.

up to 12 cartridges with a total volume of 250 mL. A Panasonic Pen-recorder (model VP 67222A) was connected to a UVIS 200 detector (Linear Instruments, Reno, NV). For the repeat of the second separation of fraction 6, a Sanki high performance centrifugal partition chromatograph (HPCPC type LLB-M) was used. The centrifuge contains 12 cartridges with a total volume of 101.2 mL.

The on-line HPLC−UV/MS−biochemical detection for AChE activities was set as described previously.[6] The HPLC mobile phase was 0.01 M ammonium acetate in MeOH/H_2O 30:70 (v/v). It was pumped at a flow rate of 1.2 mL/min and then split, resulting in flow rates of 1.12 mL/min to the UV detector, 0.04 mL/min to the MS, and 0.04 mL/min to the biochemical detection system. The splitter consisted of a cross-shaped union where three split ratios were adjusted using three PEEK capillaries (64 μm i.d.) with different lengths as restrictors. The three bioassay-reagent pumps were connected as shown in Figure 1 and used at flow-rates of 0.04 mL/min. The reaction coil, consisting of 0.5 mm i.d. knitted PFA tubing, was put into a water bath set at 50 °C. The product from the reaction was detected by a 119 UV/vis detector (Gilson, Middleton, WI) set at 405 nm. The UV measurements from the biochemical detection and from the HPLC were recorded by a DAx Voltage measurement software version 5.0 (Prince Technologies, Emmen, The Netherlands). ESIMS was measured by a MAT900 apparatus (Finnigan, San Jose, CA). The delay times of the on-line system were calibrated by five injections of three AChE inhibitors, that is, 0.3 mM galanthamine, 0.3 mM physostigmine, and 3 mM carbachol.

An LKB pump type 2150 (Bromma, Sweden), a Waters 710B WISP autosampler, and a Waters 990 photodiode array detector were used for preparative HPLC experiments. MS was measured by a Finnigan MAT TSQ-700 triple quadrupole equipped with a custom-made Electro Spray Interface (ESI). The UV spectrum (in MeOH) was measured on a Cary 1Bio UV−vis spectrophotometer. The NMR spectra were measured on a Bruker DPX-600 spectrometer. A Bio-Rad microplate

reader model 3550 UV (Bio-Rad Laboratories, Richmond, CA) for an assay for AChE activity.

Plant Material. The bulbs of *Narcissus* 'Sir Winston Churchill' (Amaryllidaceae) were obtained from W. F. Leenen & Zn, Sassenheim, The Netherlands.

Extraction. Bulbs of *Narcissus* 'Sir Winston Churchill' were peeled and cut into small pieces and dried at 65 °C. Then they were macerated with 5 mL ethanol per gram for 4 days. The suspension was then filtered under vacuum, and the filtrate was evaporated to dryness under reduced pressure.

Isolation of Active Compounds. The scheme of the isolation of the active compound from *Narcissus* 'Sir Winston Churchill' is shown in Figure 2. CPC was used in the first and second separations. In both experiments, six cartridges (total internal volume 125 mL) were used. The pressure was limited to 60 bar. The flow rate was set to 2 mL/min. The subfraction size was 8 mL. The sample was dissolved in 4 mL of each of the two phases before injection. Twenty subfractions (160 mL including the void volume) were collected in ascending mode, using the organic phase as a mobile phase. Then, in the same mode of elution, the mobile phase was changed from the organic phase to the aqueous phase to collect another 20 subfractions.

The ethanolic extract of *Narcissus* 'Sir Winston Churchill' (1.5 g) was prefractionated by CPC using the solvent system heptane/ethyl acetate/MeOH/H_2O 6:1:6:1 (v/v/v/v). All fractions from CPC were analyzed by TLC using Si gel plates, F_{254} No. 5554 (Merck, Darmstadt, Germany). Two solvent systems, $CHCl_3$/MeOH 9:1 (v/v) and ethyl acetate/formic acid/acetic acid/water 100:11:11:27 (v/v/v/v), were used. Subfractions that showed similarity on TLC were combined (six fractions) and evaporated under reduced pressure. They were tested by a microplate assay for AChE inhibitory activity. Then, the active fraction (fraction 6, 150 mg) was fractionated again by CPC using the solvent system, ethyl acetate/MeOH/H_2O 43:22:35 (v/v/v). Each subfraction was tested for AChE inhibitory activity by the microplate assay, and the active fractions were injected into the HPLC with on-line coupled UV/MS−biochemical detection.

To obtain enough material for isolation and structure elucidation, 300 mg of fraction 6 from the CPC prefractionation was fractionated again by the HPCPC using the solvent system ethyl acetate/MeOH/H_2O 43:22:35 (v/v/v), as described before. The fraction that showed a TLC pattern and with AChE inhibitory effect similar to the active fraction obtained before was combined with the previous one and called fraction 6/12 (7 mg).

The final purification of the active compound from fraction 6/12 was done by means of preparative HPLC using a 10 μm Bondapack C_{18} 300 × 7.8 mm (i.d.) preparative column (Waters, M. A.). The mobile phase used was 0.01 M ammonium acetate in MeOH/H_2O 30:70 (v/v). The flow rate was set at 4.5 mL/min, and UV detection was in the range of 190−349 nm. Fractions were collected manually for every peak detected under UV 215 nm. The fractions were tested for an inhibitory effect to AChE. The active fraction 6/12/5 (1 mg) was analyzed by means of MS and NMR.

Microplate Assay for AChE Activity. The assay for measuring AChE activity was modified from the assay described by Ellman et al.:[8] 125 mL of 3 mM DTNB, 25 μL of 15 mM ATCI, and 50 μL of buffer were added to the wells followed by 25 μL of sample dissolved in buffer. The microplate was then read by a microplate reader at 405 nm every 13 sec for five times. Then, 25 μL of 0.226 U/mL AChE solution was added to the wells, and the microplate was read again at the same wavelength every 13 sec for eight times. The increase of absorbance measured was linear for more than 2 min. The velocities of the reactions before and after adding enzyme were calculated by a Microplate Manager software version 4.0 (Bio-Rad Laboratories). The results were corrected for spontaneous hydrolysis of the substrates. Enzyme activity was calculated as a percentage compared to an assay using buffer without any inhibitor. The AChE inhibitory date were analyzed with the software package Prism (Graph Pad Inc., San Diego). IC_{50} values are means ± S.D. of three individual determinations each performed in triplicate.

Ungiminorine (1): colorless solid; UV (MeOH) λ_{max} 210, 238, 285; ^{1}H NMR (MeOD, 600 MHz) δ 6.93 (1H, s, H-11), 6.78 (1H, s, H-8), 5.92 (each 1H, br s OCH_2O), 5.75 (1H, br d, J = 1.7 Hz, H-4), 4.69 (1H, br s, H-1), 4.60 (1H, br s, H-3), 4.15 (2H, br d, J = 12.9 Hz, H-7β, H-5β), 4.09 (1H, m, H-16), 3.69 (3H, m, H-7α, H-2, H-5α), 3.44 (3H, s, OCH_3), 2.84 (1H, br d, J = 11.3 Hz, H-15); ESIMS m/z 318 $[M + H]^+$, MS/MS m/z (%) 318 (64), 300 (10), 282 (5).

Acknowledgment. We are grateful to the Royal Thai Government for the Ph.D. scholarship awarded to K. Ingkaninan. We also thank W.F. Leenen & Zn, Sassenheim, The Netherlands, for providing the bulbs of *Narcissus* 'Sir Winston Churchill'. Professor Belkis Gözler from Faculty of Pharmacy, Ege University, Turkey, is gratefully acknowledged for providing a reference sample of ungiminorine.

References and Notes

(1) Enz, A.; Amstutz, R.; Boddeke, H.; Gmelin, G.; Malonowski, J. *Prog. Brain Res.* **1993**, *98*, 431−445.
(2) Harvey, A. L. *Pharmacol. Ther.* **1995**, *68*, 113−128.
(3) Cordell, G. A. *Phytochemistry* **1995**, *40*, 1585−1612.
(4) Ingkaninan, K.; Hermans-Lokkerbol, A. C. J.; Verpoorte R. *J. Liq. Chromatogr. Relat. Technol.* **1999**, *22*, 885−896.
(5) Irth, H.; Oosterkamp, A. J.; Tjaden, U. R.; van der Greef, J. *Trends Anal. Chem.* **1995**, *14*, 355−361.
(6) Ingkaninan, K.; Best, C. M.; van der Heijden, R.; Hofte, A. J. P.; Karabatak, B.; Irth, H.; Tjaden, U. R.; van der Greef, J.; Verpoorte, R. *J. Chrom. A* **2000**, in press.
(7) Ingkaninan, K.; Hazekamp, A.; Hoek, A. C.; Balconi, S.; Verpoorte, R. *J. Liq. Chromatogr. Relat. Technol.* **2000**, in press.
(8) Ellman, G. L.; Courtney, K. D.; Andres, V.; Featherstone, R. M. *Biochem. Pharmacol.* **1961**, *7*, 88−95.
(9) Kobayashi, S.; Kiharu, M.; Yuasa, K.; Imakura, Y.; Shingu, T.; Kato, A.; Hashimoto, T. *Chem. Pharm. Bull.* **1985**, *33*, 8−5263.
(10) Suau, R.; Gomez, A. I.; Rico, R.; Vazquez Tato, M. P.; Castedo, L.; Riguera, R. *Phytochemistry* **1988**, *27*, 3285−3287.

NP9905719