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High-performance liquid chromatography with on-line coupled UV, mass spectrometric and biochemical detection for identification of acetylcholinesterase inhibitors from natural products

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Abstract

A high-performance liquid chromatography (HPLC) method with on-line coupled ultraviolet (UV), mass spectrometry (MS) and biochemical detection for acetylcholinesterase (AChE) inhibitory activity has been developed. By combining the separation power of HPLC, the high selectivity of biochemical detection, and the ability to provide molecular mass and structural information of MS, AChE inhibitors can be rapidly identified. The biochemical detection was based on a colorimetric method using Ellman's reagent. The detection limit of galanthamine, an AChE inhibitor, in the HPLC–biochemical detection is 0.3 nmol. The three detector lines used, i.e., UV, MS and Vis for the biochemical detection were recorded simultaneously and the delay times of the peaks obtained were found to be consistent. This on-line post-column detection technique can be used for the identification of AChE inhibitors in plant extracts and other complex mixtures such as combinatorial libraries.

Keywords: Detection, LC; Biochemical detection; Acetylcholinesterase inhibitors; Enzyme inhibitors

1. Introduction

Alzheimer's disease is one of the most common mental health problems in the aged population. Based on the cholinergic hypothesis that memory impairments in patients suffering from this disease result from a defect in the cholinergic system [1], one approach to the treatment for this disease is to enhance the acetylcholine level in the brain [2]. Acetylcholinesterase (AChE) inhibitors are therefore being developed for the symptomatic treatment of this disease [3]. In 1993, tacrine (tetrahydroaminoacridine) was approved by the US Food and Drug Administration (FDA) for mild to moderate Alzheimer's dementias. However, its narrow therapeutic range and hepatotoxicity limit its use [4]. Recently, galanthamine, an alkaloid from plants of the Amaryllidaceae family, has received its first approval for the treatment for this disease in Austria. The search for new AChE inhibitors is still of great

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interest and natural products are an important potential source of such compounds.

In the last decade, strategies for on-line coupling of bioactivity detections to separation methods such as high-performance liquid chromatography (HPLC) have been developed [5]. When a separation technique is coupled with a continuous flow bioassay, it will allow the simultaneous separation and detection of bioactive compounds from complex mixtures. Moreover, if the on-line system is coupled with other detection techniques such as a diode array detection (DAD) and mass spectrometry (MS), the active compounds can be rapidly identified from the additional information obtained. This technique can be useful in natural products-based drug discovery program. In such programs, ubiquitous compounds with certain activities are a major problem. Therefore selectivity of the bioassay is more important than sensitivity. This approach thus allows detecting other active compounds in the presence of the already known active compounds which is a major advantage in dereplication of active extracts. For example, it opens the possibility to rapidly screen galanthamine containing plants for other AChE inhibitors.

A colorimetric assay for AChE [6] is suitable to be developed into a continuous-flow biochemical detection system as the assay is relatively simple, fast and straightforward. Moreover, AChE has satisfactory stability at room temperature. The enzyme and its inhibitors are also commercially available. In this study, the development of the HPLC with on-line coupled UV–MS–biochemical detection for AChE inhibitory activity will be described and the advantages and the problems of this on-line coupling method will be discussed. This hyphenated technique is useful for the search for AChE inhibitors from natural products or combinatorial mixtures as will be demonstrated by the analysis of the fraction from a *Narcissus* ''Carlton'' extract.

2. Experimental

2.1. Chemicals

Acetylthiocholine (ATCI), 5,5′-dithiobis-[2-nitrobenzoic acid] (DTNB), galanthamine, physostigmine, carbachol, AChE, Tween 80 and bovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO, USA). All organic solvents (analytical-reagent grade) were purchased from J.T. Baker (Deventer, The Netherlands). 50 m*M* Tris–HCl, pH 8.0 was used as a buffer for all experiments. 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 dissolved in buffer to obtain a 1130 U/ml stock solution. The enzyme stock solution was kept at −80°C. Further enzyme dilution was obtained by dissolving in 0.1% BSA in buffer. DTNB was dissolved in buffer to which 0.1 *M* NaCl and 0.02 *M* $MgCl_2$ were added. ATCI was dissolved in Millipore water.

2.2. Sample preparation

All reference compounds were dissolved in HPLC eluent before injection. The bulbs of *Narcissus* ''Carlton'' (Amaryllidaceae) were obtained from W.F. Leenen & Zn, Sassenheim, The Netherlands. They were extracted with methanol and prefractionated by centrifugal partition chromatography as previously described [7]. The most polar fraction, which contained alkaloids, was dried and redissolved in HPLC eluent before injection.

2.3. Microplate assay for AChE activity

The microplate assay for measuring AChE activity was modified from the assay described by Ellman et al. [6]. One hundred and twenty-five μl of 3 m*M* DTNB, 25 μl of 15 m*M* 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 Bio-Rad microplate reader Model 3550 UV (Bio-Rad Labs., Richmond, CA, USA) at 405 nm every 13 s 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 s 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 Labs.). 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.

2.4. Flow biochemical detection system for AChE inhibitory activity

The scheme of the system is shown in Fig. 1a. The system consisted of four LKB pumps type 2150 (Bromma, Sweden), an injector with a 2-μl injection loop, a type 119 UV–Vis detector (Gilson, Middleton, WI, USA) set at 405 nm and a recorder (Kipp & Zonen, The Netherlands). Four reagents, i.e., buffer, DTNB solution, AChE solution and ATCI solution were pumped into the reaction coil at flow-rates of 0.04 ml/min each. The reaction coil (volume 300 μl) consisted of a 0.5 mm I.D. knitted perfluoro alkoxy alkane (PFA) tubing (Upchurch Scientific, Oak Harbor, WA, USA).

2.5. HPLC with on-line coupled UV–biochemical detection for AChE inhibitory activity

The scheme of this on-line system is shown in Fig. 1b. The LC system consisted of an LKB pump type 2150, an autosampler type WISP 710B (Waters, Milford, MA, USA), a diode array detector type 990 (Waters) and a Waters 5200 printer plotter. The column used was a 125×4.6 mm I.D. LiChrospher RP SelectB (Merck, Darmstadt, Germany). The HPLC mobile phase was methanol–water–diethylamine (40:60:0.05, v/v/v), adjusted to pH 7.0 by 85% phosphoric acid. It was pumped at a flow-rate of 1.2 ml/min. The eluate was split into the diode array detector at a flow-rate of 1.16 ml/min, and into the biochemical detection system at a flow-rate of 0.04 ml/min. The splitter consisted of a T-shaped union where two split ratios were determined using two polyether ether ketone (PEEK) capillaries (64 μm I.D.) with different lengths as restrictors. Three reagents, i.e., 0.1 m*M* DTNB, 0.113 U/ml AChE and 0.1 m*M* ATCI were pumped into the reaction coil together with eluent from HPLC at flow-rates of 0.04 ml/min each. The three pumps for bioassay-reagent were of the LKB type 2150. The reaction coil (volume 300 μl) consisted of 0.5 mm I.D. knitted PFA tubing. The enzyme reaction product was detected by a 119 UV–Vis detector (Gilson) set at 405 nm.

2.6. HPLC with on-line coupled UV–MS–biochemical detection for AChE inhibitory activity

The LC system consisted of an LKB pump type 2150, an injector with a 50-μl loop, and a variable-wavelength detector Model 2158 LKB set at 215 nm. The column used was a 125×4.6 mm I.D. LiChrospher RP SelectB. The HPLC mobile phase was methanol–water (30:70, v/v) with 0.01 *M* ammonium acetate in total (pH 7.0). 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 system 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 from Knauer (Berlin-Zehlendolf, Germany). They were connected as shown in Fig. 1c and used at flow-rates of 0.04 ml/min. The reaction coil as described above was put into a water bath set at 50°C. The product from the reaction was detected by a 119 UV–Vis detector (Gilson) set at 405 nm. The UV measurements from the biochemical detector and from the HPLC system were recorded by a DAx Voltage measurement software version 5.0 (Prince Technologies, Emmen, The Netherlands). Electrospray ionization (ESI) MS was carried out on a MAT900 apparatus (Finnigan, San Jose, CA, USA).

3. Results and discussion

3.1. Flow biochemical detection system for AChE inhibitory activities

A flow biochemical detection system was optimized before being coupled to the HPLC column. The system was modified from the microplate assay as described in Experimental. The principle of the reaction is as follows.

$$\text{acetylthiocholine} + H_2O \xrightarrow{\text{AChE}} \text{acetate} + \text{thiocholine}$$

$$\text{thiocholine} + \text{DTNB} \leftrightarrow \text{5-thio-2-nitrobenzoate} +$$

2-nitrobenzoate-5-mercaptothiocholine

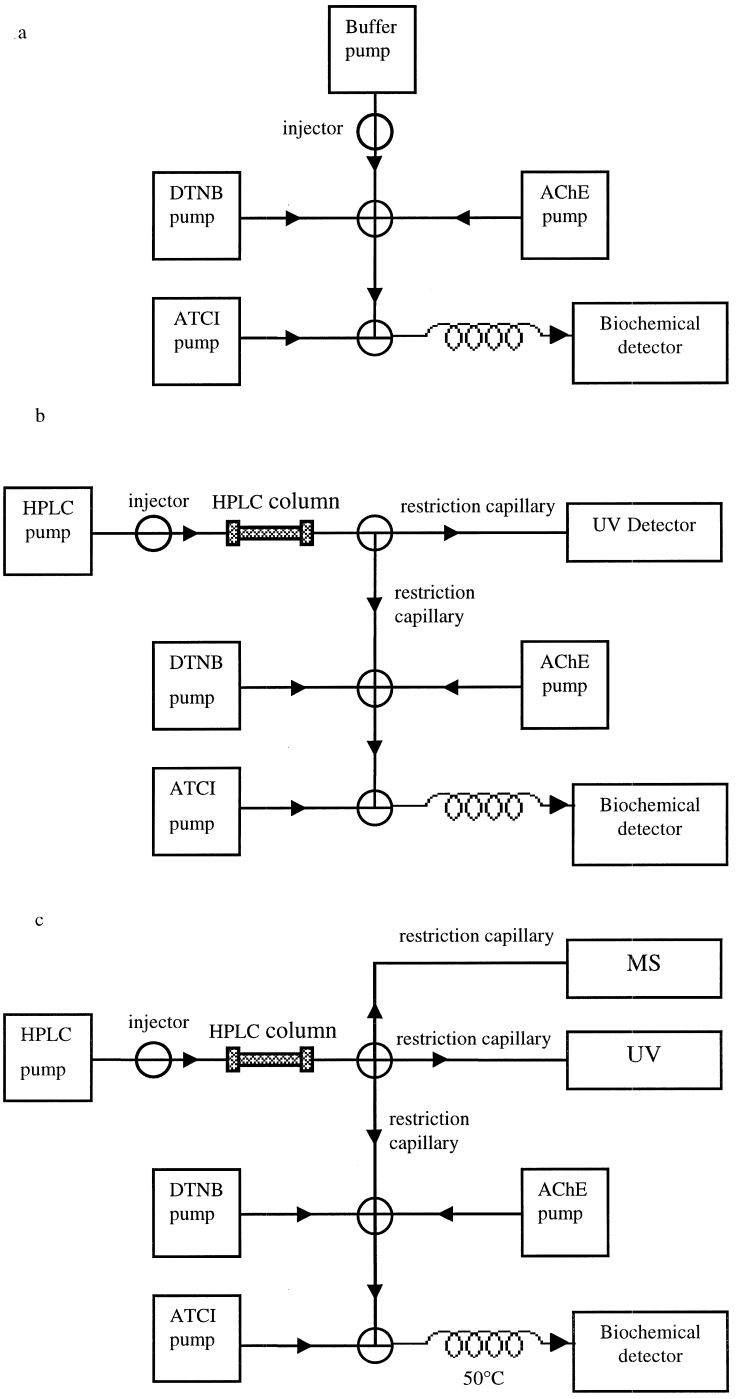

Fig. 1. Schemes of (a) the flow biochemical detection system, (b) the on-line HPLC–UV–biochemical detection system, (c) the on-line HPLC–UV–MS–biochemical detection system.

When the reagents are mixed continuously, absorbance at 405 nm of the yellow product, 5-thio-2-nitrobenzoate will be detected. If an inhibitor for AChE is injected into the system, less product will be formed. This inhibitory effect will be reflected by a negative peak. The length of the reaction coil set the reaction time at approximately 2 min. By using a randomly-knitted reaction coil, the mixing effect of the compounds during flow will be increased.

Fig. 1a demonstrates the flow biochemical detection system. After the buffer, AChE and DTNB were separately pumped into the biochemical detection system, they were mixed together in a low-dead volume mixing union and later mixed again with the substrate, ATCI. The mixture then traveled through the reaction coil for approximately 2 min to the VIS detector where the absorbance of the product was detected at 405 nm. Different concentrations of enzyme, DTNB and ATCI were tested in order to optimize the system regarding background absorption by DTNB and the spontaneous hydrolysis of ATCI as well as to minimize the cost of the reagents used. DTNB and ATCI at the concentration of 0.1 m*M* and AChE at the concentration of 0.113 U/ml proved to be the optimum concentrations for the assay.

Fig. 2 shows plots obtained from the flow biochemical detection system. When only DTNB, ATCI and buffer were introduced into the system, there was a low steady absorption at 405 nm. As AChE was pumped into the system, the products of the enzymatic reaction were formed causing higher absorbance. A gradually increasing slope of the baseline was observed indicating non-specific binding of the enzyme in the system. Physostigmine and galanthamine in different concentrations were injected into the system. These compounds inhibited the enzyme causing a negative peak in the plot. The results showed the reproducibility of the detection system when several samples with similar concentration were injected. When physostigmine was injected at high concentration (4 m*M*, 0.4 m*M* and 0.12 m*M*), the negative peaks reached the baseline indicating total inhibition. Similar results were obtained when 4 m*M* of galanthamine were injected into the flow system. This agreed with the results from a microplate assay for AChE inhibitory activity showing that galanthamine was slightly less potent than physostigmine (Fig. 3). The IC_{50} values of galanthamine and physostigmine calculated from means±SD of three individual determinations each performed in duplicate were 0.98±0.07 μ*M* and 0.69±0.06 μ*M*, respectively. The microplate assay was more sensitive than the flow biochemical detection system as lower level of the inhibitors can be determined in the microplate assay. Moreover, the tailing peaks indicated binding of the enzyme in the system. Therefore, several attempts have been made to solve the problem of non-specific binding and to improve the sensitivity of the flow bioassay.

Some strategies to reduce the non-specific binding have been undertaken. The first attempt was to continuously use buffer with 0.1% BSA, in order to saturate the possible non-specific binding sites in the coil. The second was to use Tween 80 in buffer as a surfactant to solubilize protein remaining in the coil. However both did not significantly improve the performance of the baseline. Furthermore, different kinds of tubings for the reaction coils were tested. The results showed that the slopes of the baselines from the system using the PEEK, PFA and PFTE were approximately 1.5-fold less compared to those of plastic, fluorinated ethylene propylene (FEP) and Tefzel tubings. Therefore, we used only PEEK, PFA or PFTE in the flow biochemical detection system.

3.2. HPLC with on-line coupled UV–biochemical detection

The pilot experiment in a microplate assay showed that the presence of some organic modifiers affected AChE affinity (Fig. 4). Ten percent acetonitrile inhibited almost 90% of enzyme activity while 0.25% of acetic acid caused the same effect. Methanol was relatively well compatible with the AChE assay used considering that 10% of methanol reduced the activity of the enzyme less than 50%. The use of organic modifiers in the on-line HPLC–biochemical detection system is thus limited, although the HPLC eluent is diluted four times in the reaction coil.

The biochemical detection system was coupled to an HPLC column as described in Experimental. As shown in Fig. 5, after the mobile phase had been changed from 25% methanol to 40% methanol, not only the level of the baseline but also the slope

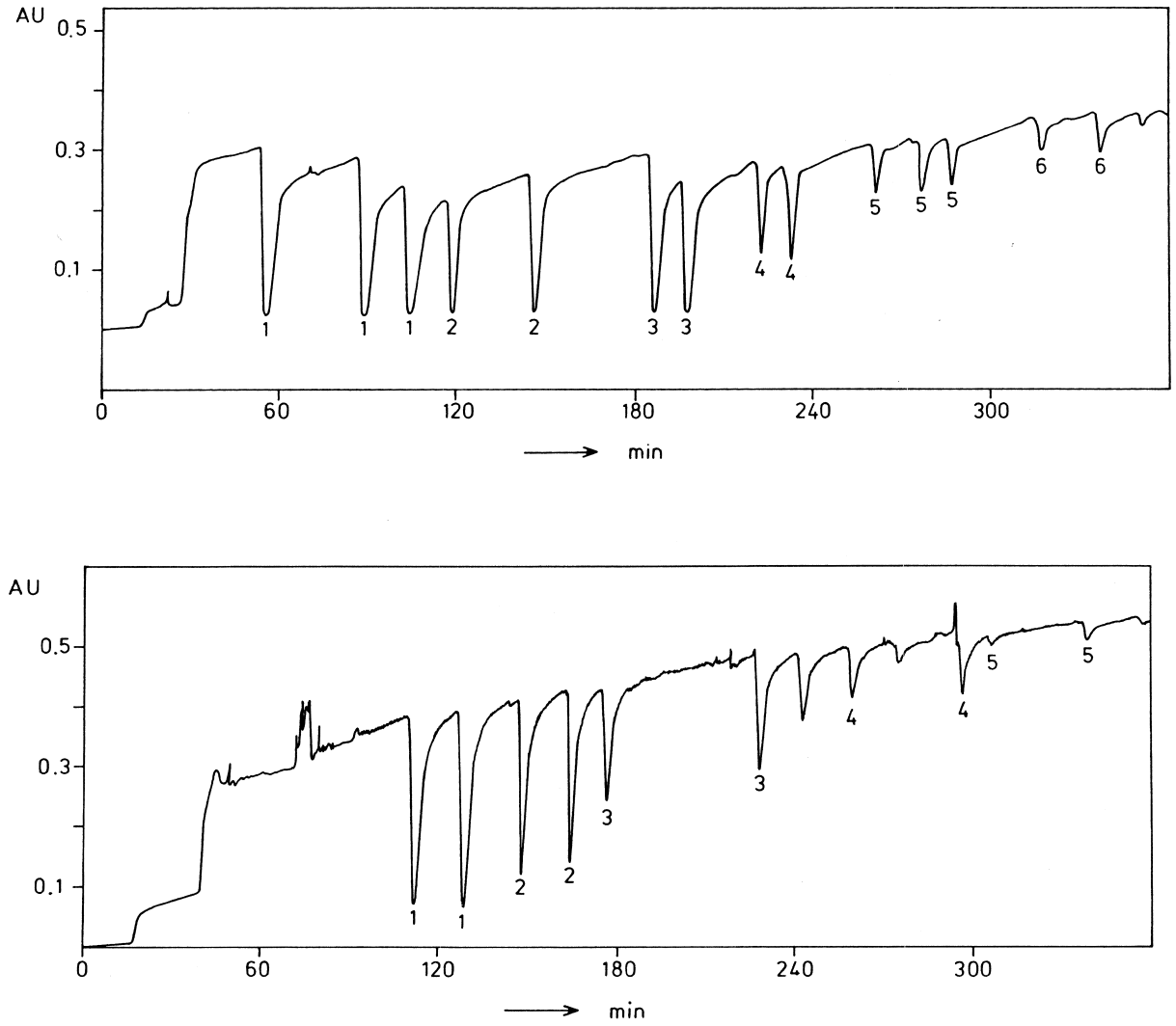

Fig. 2. Plots obtained from the flow biochemical detection system before coupling to an HPLC column. (Top) The baseline at t_0 was set when only buffer was in the system. At t_{13}, 0.2 m*M* DTNB, 0.2 m*M* ATCI and buffer were introduced into the system. At t_{27}, 0.09 U/ml of AChE was pumped into the system. A 2-μl volume of physostigmine in different concentrations, i.e., (1) 4 m*M*, (2) 0.4 m*M*, (3) 0.12 m*M*, (4) 40 μ*M*, (5) 12 μ*M*, (6) 4 μ*M* was then injected into the system. (Bottom) The baseline at t_0 was set when only buffer was in the system. At t_{17}, 0.2 m*M* DTNB, 0.2 m*M* ATCI and buffer were introduced into the system. At t_{39}, 0.09 U/ml of AChE was pumped into the system. A 2-μl volume of galanthamine in different concentrations, i.e., (1) 4 m*M*, (2) 0.4 m*M*, (3) 0.12 m*M*, (4) 40 μ*M*, (5) 12 μ*M* was then injected into the system.

decreased significantly. This demonstrated that methanol decreased AChE activity and at the same time decreased the non-specific binding in the system. Using methanol–water–diethylamine (40:60:0.05, v/v/v) at pH 7.0 as HPLC mobile phase gave a satisfactory baseline in the biochemical detector. This HPLC system was modified from the HPLC analysis for galanthamine described by Tencheva et al. [8]. Diethylamine was used in this system to reduce peak tailing of nitrogen containing compounds [9].

The detection limit (signal-to-noise ratio of 3) for galanthamine in the biochemical detector coupled to HPLC was 0.9 nmol. We tried to improve the

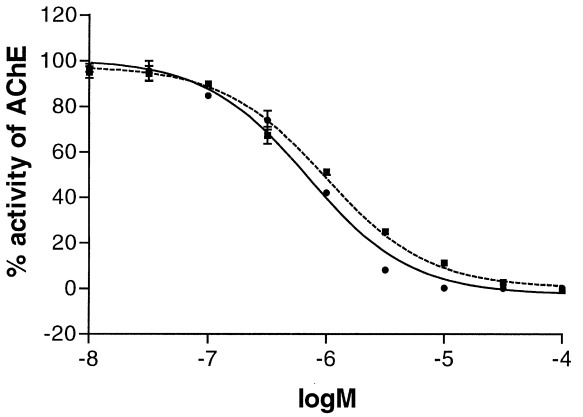

Fig. 3. The dose–response curves of galanthamine (■) and physostigmine (●) on AChE measured by microplate assay. The activities of the enzyme were determined as described in the Experimental section. Values are means of one typical experiment performed in triplicate.

sensitivity by reducing the concentration of the substrate [10]. However, when the concentration of substrate was reduced 10-times, there was no significant change in the detection limit. Either increasing the reaction time in the coil or decreasing the flow-rate may cause broader peaks at the biochemical detector. The presence of diethylamine affected the activity of AChE. When the HPLC mobile phase was changed to methanol–water with 0.01 *M* ammonium acetate (pH 7.0), the peak height of galanthamine in the biochemical detector was improved. Moreover, higher sensitivity was achieved when the reaction coil was put in a waterbath of 50°C to increase the rate of the enzymatic reaction. The detection limit for galanthamine then decreased to 0.3 nmol.

3.3. HPLC with on-line coupled UV–MS–biochemical detection

On-line HPLC–biochemical detection was coupled to ESI-MS in order to obtain information for the molecular mass of the active compounds (Fig. 1c). The HPLC eluent was changed to methanol–water (30:70, v/v) with 0.01 *M* ammonium acetate (pH 7.0) as diethylamine was incompatible with MS. The delay times between the three detectors were determined. Three AChE inhibitors, i.e., galanthamine, physostigmine and carbachol were used as reference compounds. Each was injected into the HPLC column five times. Table 1 shows the retention times of peaks from each detector. The small standard deviations indicate the reproducibility of the separation and the detection steps. The time difference between the peaks from the UV to the biochemical detection was 2.3 min and from MS to the biochemical detection was 2.1 min. It was noted that the peaks obtained from biochemical detection were broader than those from MS and from UV because of the larger delay volume and the nonspecific binding of

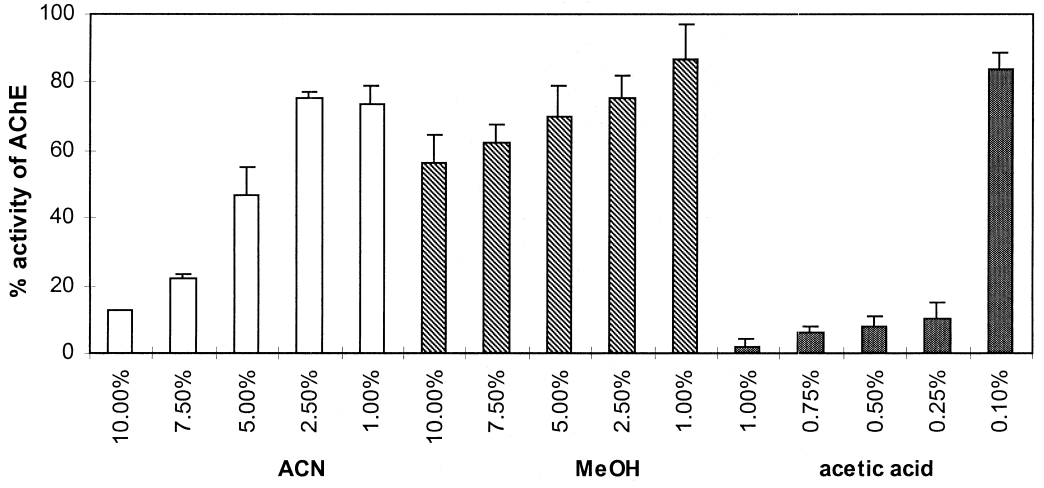

Fig. 4. The effects of acetonitrile, methanol and acetic acid in the microplate assay for AChE. The activity of the enzyme was determined as described in the Experimental section. Values are averages from three separate experiments presented with their standard deviations.

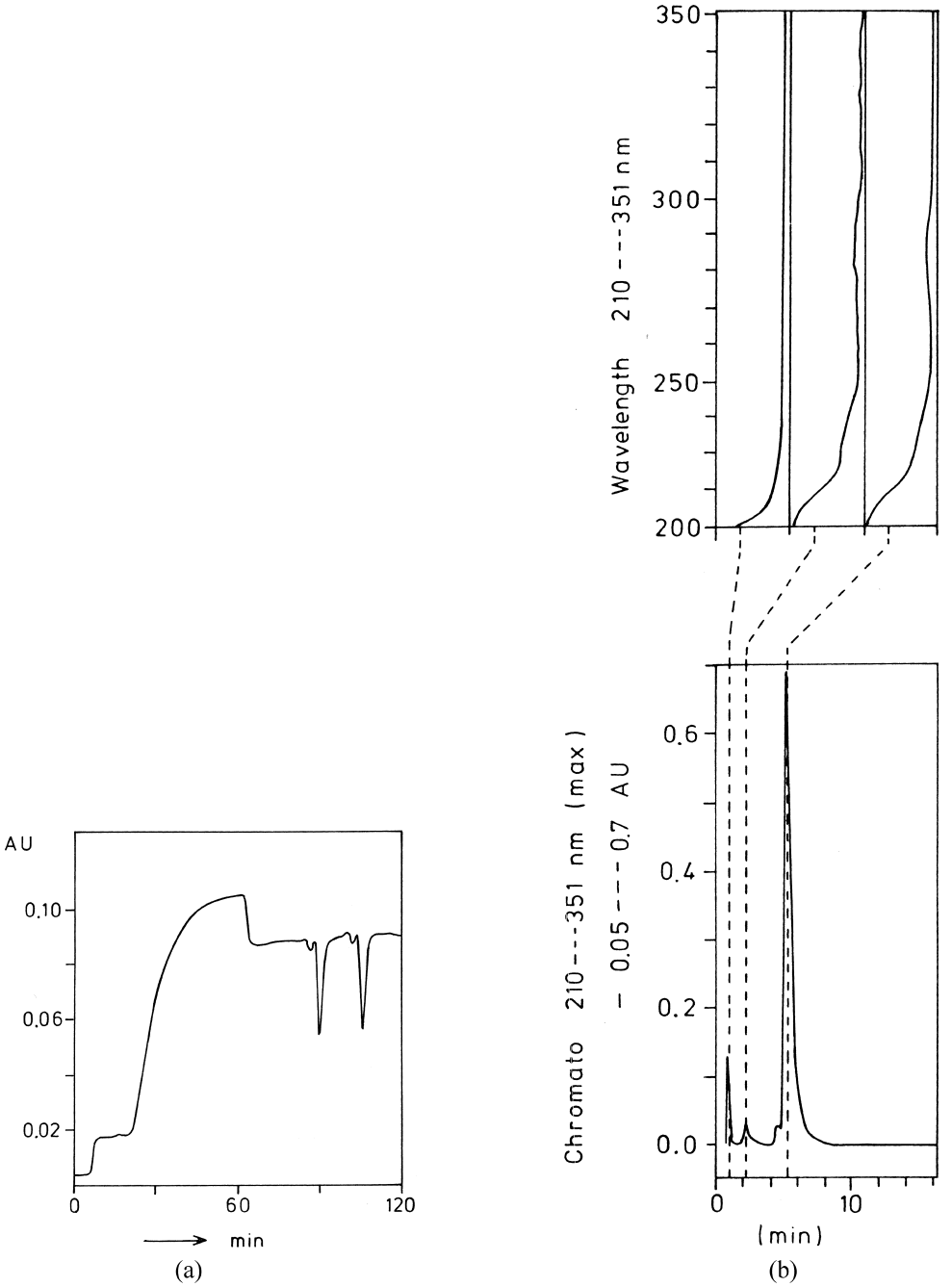

Fig. 5. The chromatograms obtained from the on-line HPLC–biochemical detection system. (a) The chromatogram obtained from the Vis detector of the biochemical detection system. At t_0, only buffer and HPLC mobile phase (methanol–water–diethylamine, 25:75:0.01, v/v/v) were in the system. At t_5, 0.02 m*M* of ATCI and 0.02 m*M* of DTNB were introduced into the system. At t_{21}, 0.113 U/ml of AChE was pumped into the system. At t_{62}, the mobile phase was changed to methanol–water–diethylamine (40:60:0.01). A 30-μl volume of 0.1 m*M* galanthamine was injected twice at t_{81} and t_{96}. (b) The chromatogram of 0.3 m*M* galanthamine from the diode array detector connected to the HPLC system.

Table 1
The retention times of three reference compounds in the on-line HPLC–UV–MS–biochemical detection system[a]

	UV (min)	MS (min)	BC (min)	BC–UV (min)	BC–MS (min)
Galanthamine, 0.3 m*M*	8.16±0.02	8.32±0.08	10.47±0.07	2.31±0.06	2.15±0.08
Physostigmine, 0.3 m*M*	12.26±0.05	12.46±0.06	14.55±0.17	2.30±0.18	2.08±0.21
Carbachol, 0.3 m*M*	–	1.16±0.02	3.32±0.04	–	2.16±0.02

[a] The injection volume was 30 μl. Values are averages from five separate experiments presented with their standard deviations. The retention times from the biochemical detection (BC) and from UV detection were calculated with software DAX. Carbachol was undetectable at UV 215 nm.

enzyme in the system. Therefore, there was a bigger variation of the retention from biochemical detection compared to those from the MS and UV detectors. Fig. 6a–c shows the peaks of the three reference compounds in each detector. Although carbachol could not be seen by UV detection, it is clearly shown to have activity in biochemical detection.

Fig. 7 shows the chromatogram of the mixture of two inhibitors, galanthamine and physostigmine in the on-line system. The difference between the

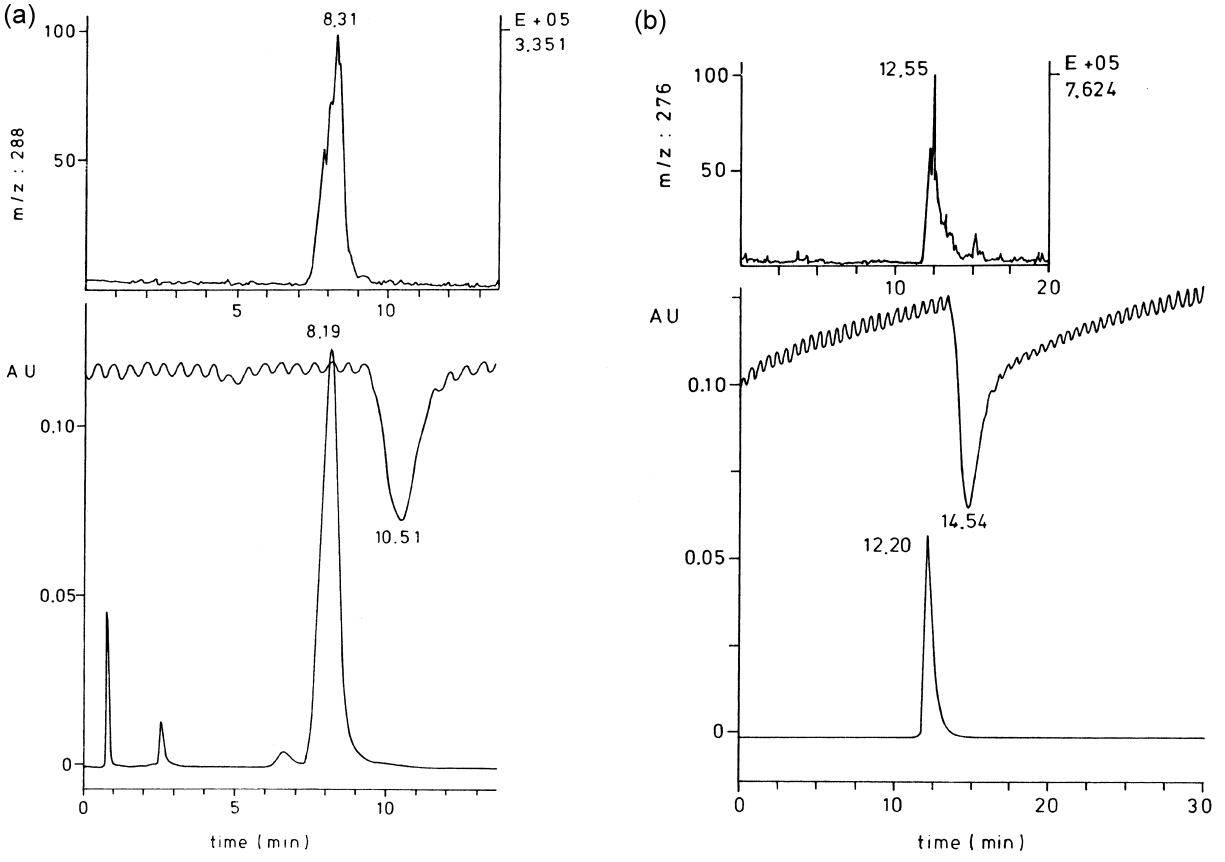

Fig. 6. The ESI-MS spectra and the chromatograms of three reference compounds (30 μl) from the on-line HPLC–UV–MS–biochemical detection (BC) system using methanol–water (30:70) with 0.01 *M* ammonium acetate (in total) as a HPLC eluent: ESI-MS (top), chromatogram from Vis for biochemical detection (middle), and chromatogram from UV (bottom). (a) 0.3 m*M* Galanthamine, (b) 0.3 m*M* physostigmine, (c) 3 m*M* carbachol.

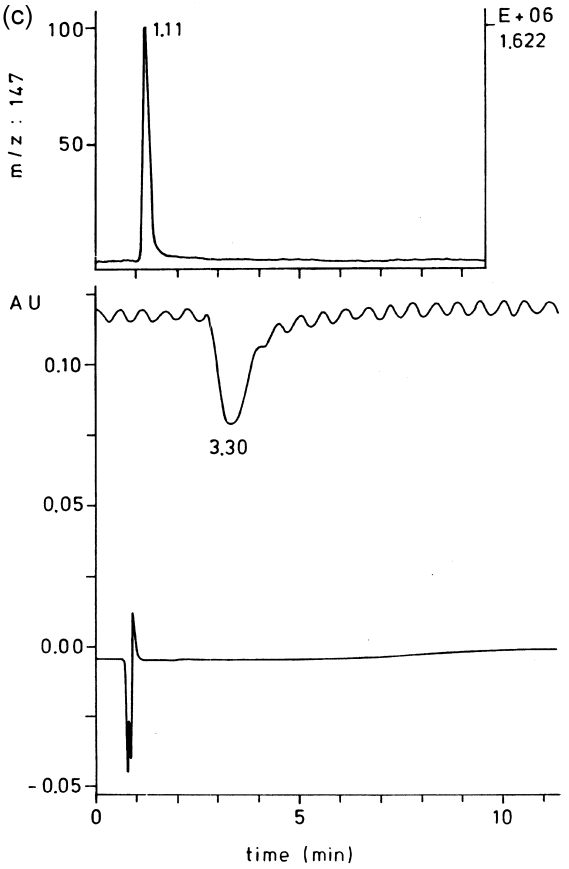

Fig. 6 (*continued*).

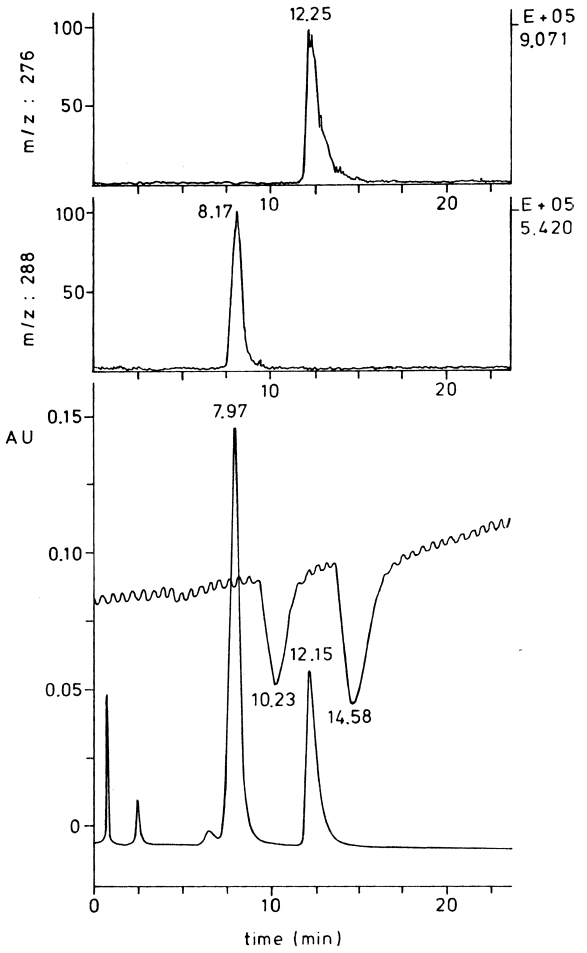

Fig. 7. The ESI-MS spectra and the chromatograms of the mixture of 0.3 m*M* galanthamine and 0.3 m*M* physostigmine (30 μl) in the on-line HPLC–UV–MS–biochemical detection system using methanol–water (30:70) with 0.01 *M* ammonium acetate (in total) as a HPLC eluent: ESI-MS (top), chromatogram from Vis for biochemical detection (middle), and chromatogram from UV (bottom).

biochemical detector and the UV detector of HPLC as well as the difference between the biochemical detector and MS agreed with the values measured before. The increase of the baseline level was still observed but did not interfere with the results.

Finally, the system was applied for the analysis of a plant extract. *Narcissus* species are known to contain galanthamine. Therefore the alkaloid-rich fraction from *Narcissus* ''Carlton'' was tested for AChE inhibitory effect. Five mg/ml of the fraction dissolved in methanol was injected into the system. Fig. 8a shows that although the UV chromatogram from HPLC is very complex, the biochemical detection system shows a high selectivity for the AChE inhibitors. Two broad negative peaks from the biochemical detector proved that there were at least two inhibitors in the extract. The retention time of the main active peak and the molecular mass derived from MS data (Fig. 8b) corresponded to those of galanthamine, a well-known AChE inhibitor. However, MS showed that there was at least one more molecule (at [M+H] of 290) present in the same peak. It could be interesting to further identify this compound and test it for inhibitory effect. The other peak in the chromatogram detected by the biochemical detector might be caused by a new AChE

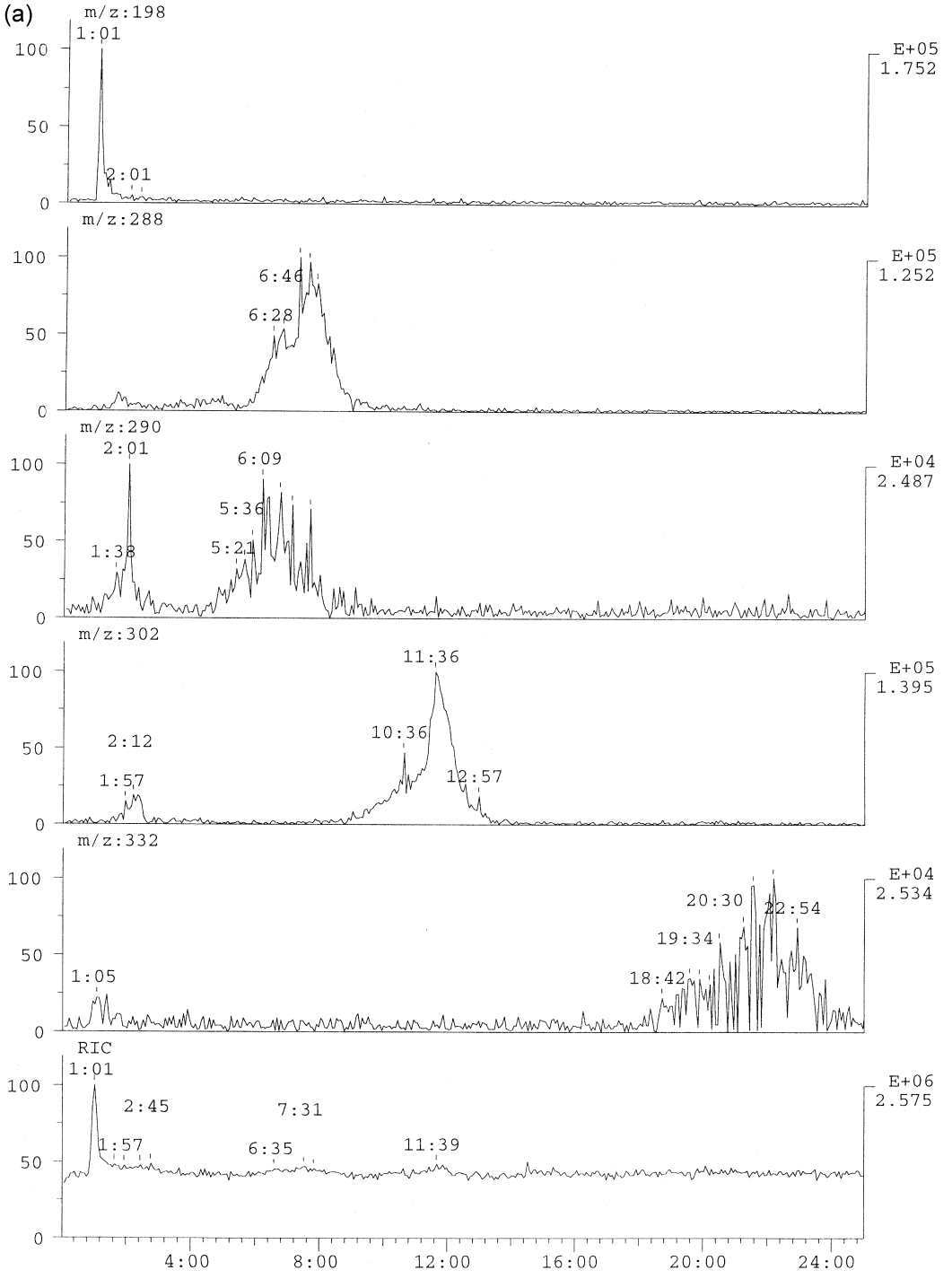

Fig. 8. The ESI-MS spectra and the chromatograms when 5 mg/ml of the fraction from the extract of *Narcissus* ''Carlton'' (30 μl) was injected into the HPLC with on-line coupled UV–MS–biochemical detection system, using methanol–water (30:70) with 0.01 *M* ammonium acetate (in total) as a HPLC eluent. (a) ESI-MS, (b) chromatograms from Vis for biochemical detection (upper line), and from UV (lower line).

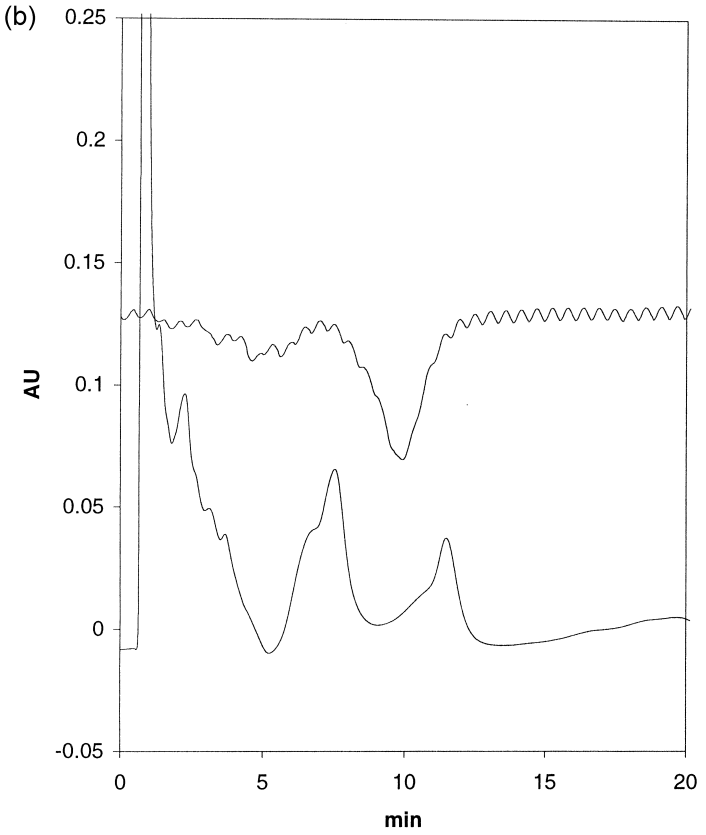

Fig. 8 (*continued*).

inhibitor. Further studies are necessary for the isolation and identification of this active compound.

4. Conclusions

HPLC with on-line coupled UV–MS–biochemical detection proved to be successful in simultaneous separation, detection and identification of AChE inhibitors. The detection limit for galanthamine in an on-line HPLC–biochemical detection is 0.3 nmol. Reduction of baseline noise and band-broadening and the development of fluorescence-based biochemical methods may increase the sensitivity of the biochemical detection. This on-line system can be a major tool for rapid dereplication and search for new AChE inhibitors from natural products.

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