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Simultaneous separation, identification and activity evaluation of three butyrylcholinesterase inhibitors from *Plumula nelumbinis* using *on-line* HPLC-UV coupled with ESI-IT-TOF-MS and BChE biochemical detection

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ABSTRACT

We have firstly established a method of high-performance liquid chromatography – ultraviolet analysis coupled with electrospray ionization – ion trap – time-of-flight mass spectrometry and butyrylcholinesterase biochemical detection (HPLC-UV-ESI-IT-TOF-MS-BChEBCD). Applying this *on-line* method to the identification of BChE inhibitors in a *Plumula nelumbinis* sample, three alkaloids, namely liensinine, isoliensinine, and neferine, have been detected as having a strong BChE inhibition activity for the first time; in addition, norisoliensinine and 6-hydroxynorisoliensinine were proposed as two new compounds identified by their UV and MS data. The HPLC fingerprint, the MS fragments of the components, and the BChE activity profile could be simultaneously recorded during real-time analysis of complex samples using this *on-line* approach. Tacrine, a BChE inhibitor, was used as a positive reference compound, and its detection limit in the biochemical detection system was 1 nmol. The BChE activity of 1 g of *P. nelumbinis* sample was equal to that of 127.88 μmol tacrine. The proposed *on-line* method has been validated as having good precision and reproducibility, and could be used to rapidly identify BChE inhibitors and to screen potential drugs for the treatment of Alzheimer's disease in complicated samples.

1. Introduction

Currently, an increasing number of people are suffering from Alzheimer's disease (AD) all over the world. Taking the US as an example, the number of persons with AD in its population is expected to increase almost three-fold, from 4.5 million in 2000 to 13.2 million by 2050 [1]. This disease can lead to a declining quality of life in aged people by undermining cognitive functioning, the capacity for meaningful time use, activities of daily living and social behavior, and the capability of balancing positive and negative emotions [2]. As such, it has become a major challenge for researchers. Although several pathomechanisms have been proposed for AD, the cholinergic projection is considered to be a major pathogenesis [3], and inhibition of cholinesterases (ChEs) to increase the amount of acetylcholine (ACh) has been acknowledged as the most effective treatment against AD [4]. Two cholinesterases coexist in the central nervous system (CNS), namely acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), both of which are capable of efficiently hydrolyzing ACh at different rates [5]. Moreover, BChE can hydrolyze ACh in all hippocampal and temporal neocortical areas known to receive cholinergic input [6], making BChE inhibitors an important therapeutic goal in Alzheimer's disease. However, currently approved drugs (such as tacrine, donepezil, galantamine) mainly possess AChE inhibition activity and have shown side effects, e.g. nausea, vomiting, extrapyramidal symptoms, and sleep disturbances [7]. No drug showing BChE inhibition activity has hitherto been developed for AD [8]. Therefore, identifying new BChE inhibitors is of practical significance for addressing the shortage of drugs for the treatment of AD.

It is known that substrates of ChEs and approved ChE inhibitors are all N-containing compounds, most of which are plant-derived drugs. Moreover, natural plants constitute a large pool of N-containing compounds, and the alkaloids in herbal medicines have attracted much of our attention. *Plumula nelumbinis*, the plumule of *Nelumbo nucifera* Gaertn, has been widely used as an essential Chinese medicine called "Lian Zi Xin" in Chinese. Three alkaloids, liensinine, isoliensinine, and neferine, are accepted as being major components in *P. nelumbinis* [9]. Some studies have suggested that *P. nelumbinis* might be used for treating cancer, tranquilization, lowering

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blood pressure, and so on [10], but many other activities of this Chinese medicine remain unclear.

To rapidly screen active compounds in complex samples, many *on-line* activity detection methods based on chemical reactions have been developed. Generally, these reaction systems, such as luminol chemiluminescence (CL) [11,12] and 2,2'-diphenyl-1-picrylhydrazyl (DPPH·) radical de-coloration [13], have always been coupled with HPLC−MS to achieve *on-line* identification of active ingredients. In recent years, theoretical concepts [14,15] for *on-line* HPLC-biochemical detection (BCD) based on ligands and proteins have been presented, and these have enabled *on-line* detection of estrogenic compounds [16], angiotensin-converting enzyme (ACE) [17], and acetylcholinesterase (AChE) [18] inhibitors.

Tacrine, the first synthetic drug approved by the FDA (Food and Drug Administration) for treatment of AD, was one of the commonly used positive reference compounds during BChE inhibitor studies [19,20]. It was also used in this study not only because of its high inhibition potency of BChE, but also due to its favorable selectivity against BChE over AChE (e.g. tacrine: 3.7-fold selectivity, galanthamine: 0.3-fold selectivity) [20,21]. The half maximal inhibitory concentration (IC_{50}) value of tacrine was calculated and compared with that obtained by a colorimetric method using Ellman's reagent [22].

Based on our previous study [12] concerning *on-line* detection, a rapid *on-line* approach was established and applied to the identification of BChE inhibitors in *P. nelumbinis*. In the present study, five alkaloids were identified, in which three newly identified BChE inhibitors, namely liensinine, isoliensinine, and neferine, have been demonstrated to show strong activity by the *on-line* HPLC-UV-ESI-MS-BChEBCD method. This method has proved to be precise and rapid, and may therefore be useful for the screening of BChE inhibitors in natural medicines and complex samples.

2. Experimental

2.1. Materials

P. nelumbinis sample was purchased from Beijing Tong-Ren-Tang Group Co., Ltd., and identified by Associate Professor Hong Wang, Department of Natural Medicines, School of Pharmaceutical Sciences, Peking University. Liensinine was purchased from Shanghai Tauto Biotech Corporation Limited (Shanghai, China), and its purity was greater than 98% as determined by HPLC. Reversed-phase (RP) C_{18} silica gel (80−100 mesh) was obtained from Tianjin Borui Jianhe Chromatography Technology Co. Ltd. (Tianjin, China).

2.2. Chemicals

BChE (20 U/mg) was obtained from Sigma (St. Louis, MO, USA). BTCI (*S*-butyrylthiocholine iodide), DTNB (5,5'-dithiobis[2–nitrobenzoicacid]), BSA (bovine serum albumin), tacrine, Tris, and SDS (sodium dodecyl sulfate) were all purchased from Suzhou Yacoo Chemical Reagent Corporation (Jiangsu, China). Deionized water (18 MΩ-cm resistance) was prepared by passing distilled water through a Milli-Q system (Millipore, MA, USA). HPLC-grade methanol, analytical grade acetonitrile (ACN), methanol, ammonium acetate (NH_4Ac), triethylamine, HCl, formic acid, and ethyl acetate were purchased from Beijing Chemical Works (Beijing, China).

2.3. Sample preparation

2.0 g of *P. nelumbinis* powder (60 mesh) was accurately weighed and extracted with 90% ethanol (3 × 50 mL) in an ultrasonic water bath for 30 min each time. The extract was concentrated to dryness under reduced pressure, subjected to chromatography on an RP C_{18} silica gel (5 g) column (i.d. 1 cm), and eluted successively with 10% (40 mL), 50% (40 mL), and 90% (100 mL) methanol to afford the corresponding fractions A−C. The eluent of fraction C, containing active compounds, was dried and redissolved in methanol (50 mL). The solution was filtered through a 0.45 μm membrane prior to use, and a 40 μL aliquot was injected into the system for analysis.

2.4. On-line biochemical detection of solutions

0.1 M Tris–HCl at pH 8.0 was used as buffer for all experiments. A 1.6 U/mL BChE stock solution (containing 0.1% BSA) was prepared in buffer and stored in a refrigerator before dilution. A 0.1 U/mL BChE solution was obtained by diluting the stock solution with buffer. Solutions of BTCI (0.2 mM) and DTNB (0.1 mM) were obtained by dissolving BTCI and DTNB in buffer, respectively.

2.5. 96-Well plate method for BChE inhibition activity

The 96-well plate method used was a modification of a previously reported method [22]. 300 μL of 1 mM BTCI and 240 μL of 1.6 U/mL BChE were added to the test tubes followed by 150 μL of sample solution. The tubes were sealed and immediately incubated in a water bath for 20 min at 40 °C. Thereafter, 100 μL of 4% SDS was added to quench substrate conversion followed by 240 μL of 1 mM DTNB. After shaking, a 250 μL aliquot of the final solution was transferred to a 96-well plate, and the absorbance was measured at 405 nm using a photometric microplate reader (DNM-9602, Beijing Pulang Xinjushu Co., Ltd.). Blanks were set up by adding 150 μL of buffer solution instead of sample solutions. Blank control and experiment control were set up by adding 240 μL of buffer solutions instead of enzyme solution in order to enable sample background subtraction. Each experiment was repeated three times to give an average inhibition rate. BChE inhibition activity was calculated as a percentage by the following equation.

$$\text{Inhibition rate}\ (\%) = 1 - 100 \times (\text{experiment} - \text{experiment control})/(\text{blank} - \text{blank control})$$

2.6. Apparatus and conditions of on-line HPLC-UV-ESI-MS-BChEBCD

The instrumentation of the *on-line* system is shown in Fig. 1. The system consisted of three essential parts to enable *on-line* identification of active compounds, namely an HPLC-UV part for sample isolation, ESI-MS for compound identification, and a BChE reaction system for enzyme activity detection. After detection of HPLC effluent by UV1 to give chromatogram, part of the eluent was divided by flow splitter 1 (FS1) and introduced into MS detector to yield MS spectrum of HPLC peaks, the other part was further split by FS2. Except the waste stream from FS2, the other small part was mixed with BCD solutions from pump C, D and E, and then the mixed stream flowed into mixing coil to react with each other. If active compound was reacted with the enzyme solutions, the signal of UV2 should appear a negative peak, corresponding with peaks in MS and UV1 detectors with consistent time differences.

2.6.1. HPLC conditions

The HPLC analyses were performed on a Shimadzu analytical HPLC system (Kyoto, Japan) consisting of two LC-20AD pumps (pump A and pump B), a CTO-20A column oven, a DGU-20A3 degasser, an SPD-20A UV detector (abbreviated as UV1), and an SIL-20AC auto injector. A Diamonsil™ C_{18} column (5 μm, 150 mm × 4.6 mm,

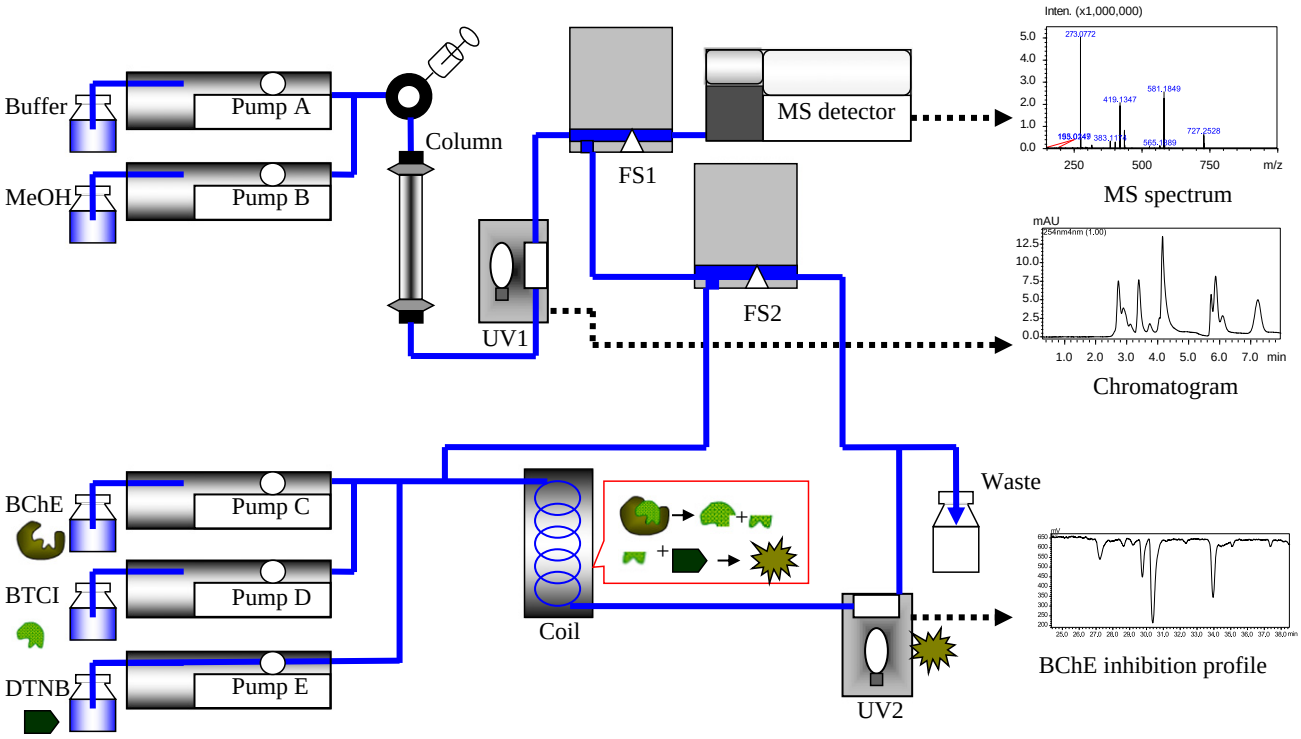

Fig. 1. Instrumentation for the *on-line* HPLC-UV-ESI-MS-BChEBCD method (UV: ultraviolet detector, FS: flow splitter (split ratio of FS1, MS: FS2=0.2:0.8 mL/min; split ratio of FS2, BCD: Waste=0.1:0.7 mL/min), MS: mass spectrometer, BChE: butyrylcholinesterase, BTCI: *S*-butyrylthiocholine iodide, DTNB: 5,5'-dithiobis[2-nitrobenzoicacid]).

Dikma, NY, USA) with a column temperature of 40 °C was used for sample separation. The HPLC mobile phase was water/methanol (40:60, v/v) pumped by pump A and pump B, respectively; the water phase contained 0.01 M ammonium acetate at pH 8.0 (adjusted by triethylamine), and the flow rate was 1.0 mL/min. The *on-line* chromatogram of the *P. nelumbinis* sample was recorded by UV1 at 282 nm in the HPLC system; for tacrine, the chromatogram was recorded at 240 nm.

2.6.2. ESI-IT-TOF-MS analysis

The above HPLC system was connected to a hybrid IT-TOF mass spectrometer (Shimadzu LCMS-IT-TOF, Kyoto, Japan) via an ESI interface. The effluent from the HPLC system was split by the first adjustable flow splitter (FS1) (QuickSplit post-column splitter, Dikma Technologies Inc., Beijing, China) and introduced into the ESI source at a flow rate of 0.2 mL/min. The capillary voltage, CDL voltage, and detector voltage were fixed at 4.5 kV, 10 V, and 1.7 kV, respectively. The curved desolvation line (CDL) temperature and block heater temperature were both maintained at 200 °C. The flow rate of nitrogen as nebulizer gas was 1.5 L/min. For full-scan MS analyses, spectra were recorded in the range m/z 100–700. Data-dependent acquisition was set such that the most abundant ions in full-scan MS would trigger tandem mass spectrometry (MS^n, $n=3$). All ions were first accumulated in the octopole and then rapidly pulsed into the ion trap for MS^n analyses in the automatic mode. The collision energy for MS^n was adjusted to 50% in the LC/MS analysis, and the isolation width of precursor ions was 3.0 Th. All ions produced were finally introduced into the TOF instrument for accurate mass determination. The entire mass range from m/z 50 to 650 was calibrated using the sodium trifluoroacetate cluster as an external reference to obtain accurate mass values. Data were processed later by LC/MS solution software (version 3, Shimadzu, Kyoto, Japan), which included a formula predictor.

2.6.3. Conditions of the BChE biochemical detection method

The BChE biochemical detection system consisted of three LC-20AD analytical pumps (Shimadzu, Japan), a reaction coil (length: 10 m, i.d. 0.25 mm) in a CTO-20A column oven, and an SPD-20A UV detector (abbreviated as UV2). A solution stream of the enzyme reaction obtained by mixing solutions of BChE (0.04 mL/min), BTCI (0.05 mL/min), and DTNB (0.04 mL/min) was generated by means of pumps C, D, and E; the three solutions merged at T-joints. A constant flow rate (0.1 mL/min) of the HPLC eluate stream from the first splitter (FS1) was introduced by the second flow splitter (FS2) to merge with the mixed enzyme reaction solutions. This combined stream was then immediately introduced into the reaction coil maintained at 40 °C throughout the detection. The mixture finally arrived at UV2 for recording the absorbance at 405 nm to give the *on-line* inhibition profile.

Different parts of the instrumentation were linked by polyether ether ketone (PEEK) tubes (i.d. 0.25 mm) of different lengths, and adjustable flow splitters and T-joints were applied either to interconnect the PEEK tubes or to ensure an accurate and stable split ratio among the different parts. The solutions of BChE, BTCI, and DTNB were protected from light in a blackbox, and they were kept at 0 °C in an ice-water bath to maintain their stability during the detection process.

3. Results and discussion

3.1. Inhibition effects of different solvents by the 96-well plate method

Due to the incompatibility between HPLC mobile phases and the BChE reaction system, the inhibition effects of several solvents and pH modifiers that are commonly used in HPLC systems were tested by the 96-well plate method. Before coupling to BChE biochemical detection, different concentrations of acetonitrile (ACN), MeOH, formic acid, triethylamine, and ammonium acetate

were used to test their effects on BChE activity (see Fig. 2). Their inhibition rates are presented with their standard deviations.

As a potential HPLC organic phase, 50% acetonitrile inhibited almost 60% of the BChE activity, while methanol showed only a 30% inhibition rate, indicating that ACN showed stronger inhibitory activity than methanol. Methanol was thus more compatible with the BChE reaction system. For pH modifiers, concentrations of formic acid higher than 0.6% might be unsuitable for use in the HPLC water phase for analysis considering that 0.8% formic acid inhibited the BChE activity by more than 85%. Triethylamine and ammonium acetate displayed little inhibitory effects on enzyme activity at low concentrations. The inhibition rate of ammonium acetate showed a decreasing trend as its concentration was increased, possibly because of an enhanced pH buffering capacity. Although formic acid was somewhat helpful for the analysis of alkaloids by HPLC, an alkaline environment created by triethylamine and ammonium acetate could significantly improve the shape of the alkaloid peaks. From what we discussed above, 0.01 M ammonium acetate (adjusted to pH 8.0 by triethylamine) and methanol (40:60, v/v) were used as mobile phase after optimization for this study.

3.2. Optimization of the on-line detection method

3.2.1. Design of the on-line BChE activity detection

A colorimetric assay for BChE activity has always been used to detect active compounds for evaluation of their BChE inhibition activity. The yield of the final product reflects the residual activity of the enzyme. The reaction for BChE activity detection is as follows.

$$\text{BChE} + \text{BTCI(substrate)} + H_2O \rightarrow \text{butyrate} + \text{thiocholine} \quad (1)$$

$$\text{thiocholine} + \text{DTNB} \leftrightarrow \text{2-nitrobenzoate-5-mercaptothiocholine} + \text{5-thio-2-nitrobenzoate} \quad (2)$$

when BChE, BTCI, and DTNB were pumped at a constant flow rate and reacted continuously in the coil, the absorbance of 5-thio-2-nitrobenzoate as a yellow product could be detected at 405 nm by UV2 to give the baseline. When the HPLC eluent containing active compounds was introduced into the BChE reaction system by FS2, the rate of formation of the yellow product was retarded. This inhibition was manifested in a negative peak detected by UV2, and thus enabled *on-line* detection of BChE inhibitors. Moreover, if part of the HPLC eluent was also introduced into the MS detector by FS1, the BChE inhibitors could be rapidly identified *on-line* by this method combining HPLC, MS, and BChE biochemical detection.

3.2.2. Optimization of BChE biochemical detection

When only the BCD system was used, different factors affecting the intensity at UV2 were optimized before coupling with HPLC. According to Fig. 3, when each of the enzyme solutions was set at the same flow rate, a flow rate of less than 0.05 mL/min for each solution was conducive to higher peak intensity, which is, producing more yellow product. However, when the flow rate was set at 0.03 mL/min or slower, a fluctuating baseline was detected, even though the HPLC pumps were employed to give constant streams; this may have been due to either poor blending or inaccuracy of the flow rate from the pumps. To obtain a favorable baseline and high intensity, different ratios among these reagents were compared, and eventually BChE:BTCI:DTNB = 0.04:0.05:0.04 mL/min was chosen for detection.

As regards the reaction temperature, a higher temperature of the reaction coil could improve the reaction rate. To avoid an inactivation effect of the enzyme, 40 °C was selected as the reaction temperature in the BCD system. As shown in Fig. 3, higher concentrations of BChE and BTCI could significantly increase the yield of the final product, but DTNB did not give rise to an evident increase. In order to obtain a low background absorption and slow hydrolysis of the BCD system, 0.1 U/mL, 0.2 mM, and 0.1 mM were used as the concentrations of BChE, BTCI, and DTNB, respectively.

3.2.3. Optimization of the on-line HPLC-BChEBCD conditions

Considering the conditions used for the biochemical detection, pH 8.0 and 40 °C were selected as the pH value of the HPLC inorganic phase and the column temperature, respectively, so as to generate an HPLC eluent compatible with the BCD system. The dilution effect and shortening of the reaction time caused by the introduction of either buffer solution or HPLC mobile phase both contributed to producing a lower intensity of the baseline. A 0.1 mL/min flow rate of 60% methanol from the post-column to the BCD system reduced the baseline level by more than 55%.

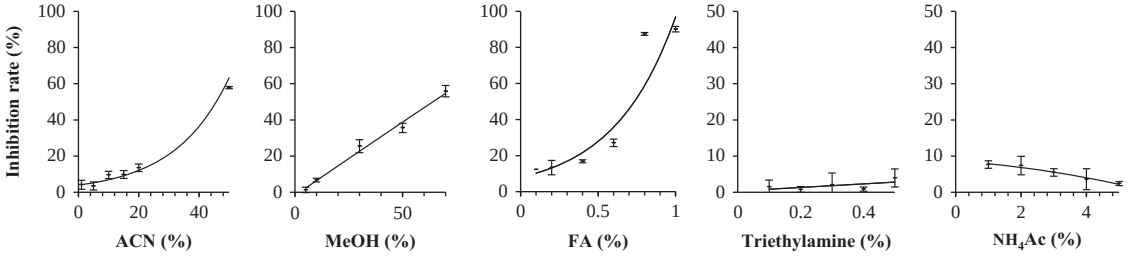

Fig. 2. The BChE inhibition effects of ACN, MeOH, formic acid, triethylamine, and ammonium acetate by 96-well plate method ($n=3$).

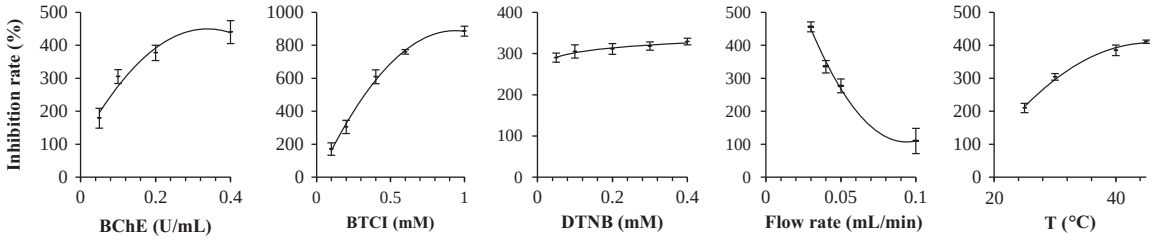

Fig. 3. Effects of reagent concentrations, flow rate of each reagent, and temperature on the intensity of biochemical detection ($n=3$).

The proportion of methanol in the HPLC mobile phase had a double-edged effect on the *on-line* analysis; on the one hand, increasing methanol content could shorten the analysis time of the sample, but enzyme activity was favored at a low proportion of methanol. To make a balance between separation efficiency in the HPLC system and residual BChE activity in biochemical detection, 60% methanol was chosen as the mobile phase for the *on-line* HPLC-BCD system. When one and/or more BCD solvents were replaced by corresponding buffers to perform experiment control for evaluating the solvent effects, the baseline signal of the *on-line* system will dramatically drop to almost zero, indicating that absence of each BCD solvent was destructive to the *on-line* detection system.

3.2.4. Optimization of the on-line HPLC-MS-BChE biochemical detection

According to a previous literature report [23], a mobile phase of acetonitrile/water (containing 0.2% acetic acid and 0.1% triethylamine) was applied to analyze the alkaloids in *Plumula nelambinis*. Based on these conditions, 0.01 m NH_4Ac (pH 8.0 adjusted by triethylamine)/methanol (40:60, v/v) was selected for HPLC separation because both NH_4Ac and triethylamine are quite compatible with MS analysis. Triethylamine was also employed to improve the HPLC peak shape as well as to strengthen the abundant ion peaks of alkaloids as potential BChE inhibitors in the sample. To obtain better precision and identification, both positive- and negative-ion modes were utilized to give protonated and deprotonated ions. Due to the characteristics of the alkaloids in the sample, protonation was found to be a more effective mode than deprotonation.

3.3. Identification of tacrine as a reference active compound

Tacrine was used as a positive reference compound in the HPLC-MS-BChEBCD method. Its chromatogram, activity profile, and mass spectra are shown in Fig. 4, in which the peaks of $[M+H]^+$ at m/z 199.1228 (error = −1.00 ppm) and $[M-H]^-$ at m/z 197.1082 (error = −1.01 ppm) may be attributed to tacrine, which has a molecular weight of 198.1157. Although negative-ion mode was not sufficiently sensitive to give the deprotonated ion of tacrine in its MS^1, MS^2, and MS^3 total ion current (TIC), $[M-H]^-$ was detected, as shown in Fig. 4 (F).

It has been reported [18] that larger delay volumes and non-specific binding of the enzyme are responsible for the broader

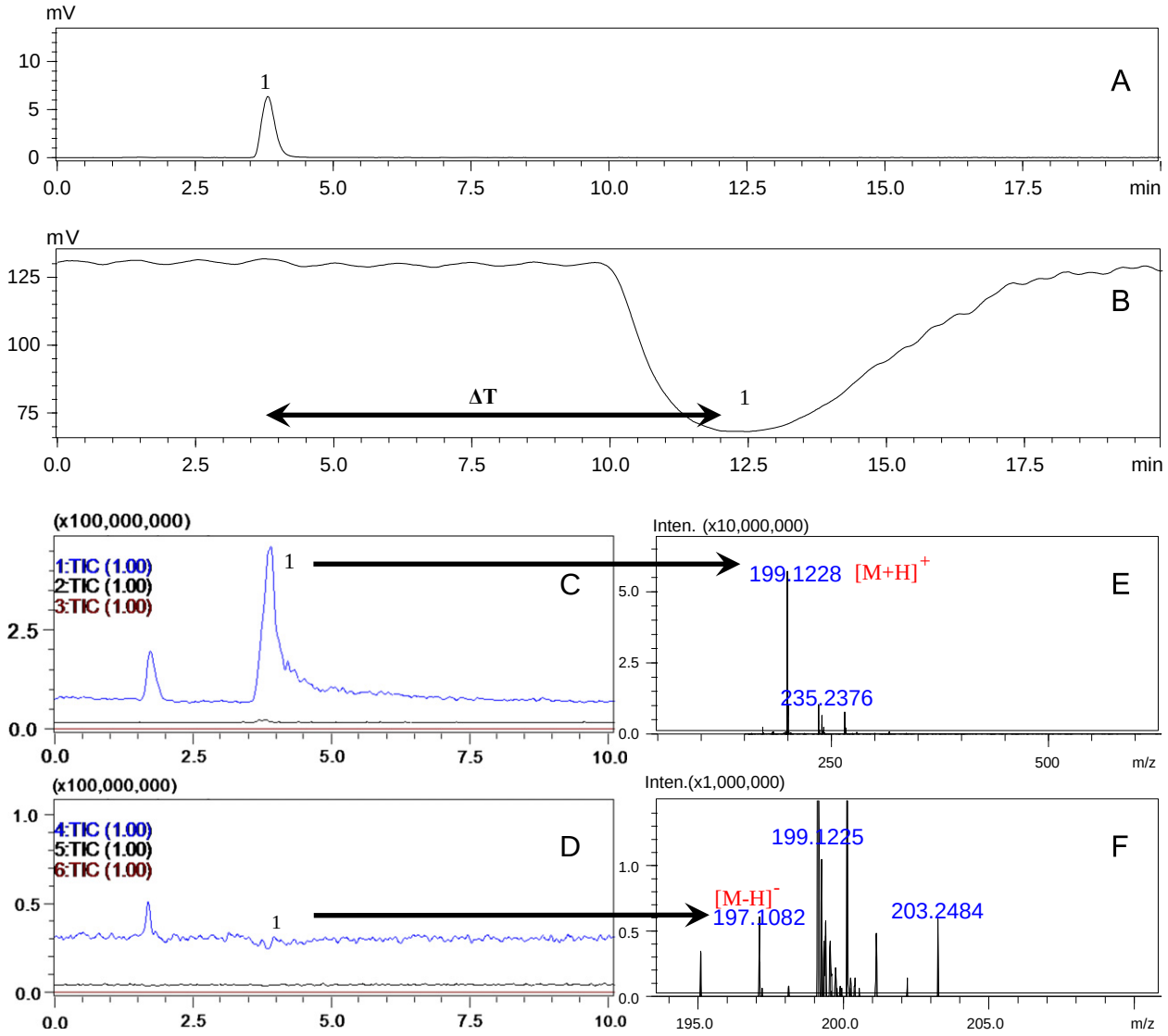

Fig. 4. The chromatogram (A), activity profile (B), TIC in positive- (C) and negative-ion (D) mode, and mass spectra of protonated (E) and deprotonated (F) tacrine (1) in the HPLC-MS-BCD method.

negative peaks in biochemical detection. Several strategies, such as adding BSA to the solution or increasing the reaction temperature, have been examined with a view to improving the peak shape in the activity profile; however, no significant improvements have been achieved. The time delay of tacrine between HPLC (3.827 min) and BCD (12.384 min) was 8.557 min (ΔT), and the time difference from MS (3.920 min) to BCD was 8.464 min. As these time delays were constant, peaks in HPLC, BCD, and MS could be readily correlated to one another.

3.4. On-line identification of BChE inhibitors in the Plumula nelambinis sample

The structures of the alkaloids in the *Plumula nelambinis* sample were identified and confirmed by the following procedures. First, the alkaloids gave their protonated ions $[M+H]^+$ and deprotonated ions $[M-H]^-$ in positive and negative modes. These ions provided their molecular weights. Second, authentic molecular formulas of these compounds were predicted by the formula predictor software based on the mass data recorded by the IT-TOF-MS detector (error < 5 ppm). Third, the MS^n fragmentation behaviors could be used to confirm and elucidate the fragmentation of the alkaloids. In this study, the HPLC retention behavior and abundant MS fragments proved very helpful in confirming the structures according to previous studies.

Three major alkaloids, namely liensinine ($C_{37}H_{42}N_2O_6$), isoliensinine ($C_{37}H_{42}N_2O_6$), and neferine ($C_{38}H_{44}N_2O_6$), were rapidly identified as possessing strong BChE inhibition activity by the established *on-line* method. The chromatogram, BChE inhibition profile, and TIC in positive- and negative-ion modes of the *Plumula nelambinis* sample are shown in Fig. 5. The retention times of liensinine, isoliensinine, and neferine were 14.843, 25.522, and 38.491 min respectively; the delay times between HPLC and BCD (liensinine: 23.375, isoliensinine: 34.159, neferine:

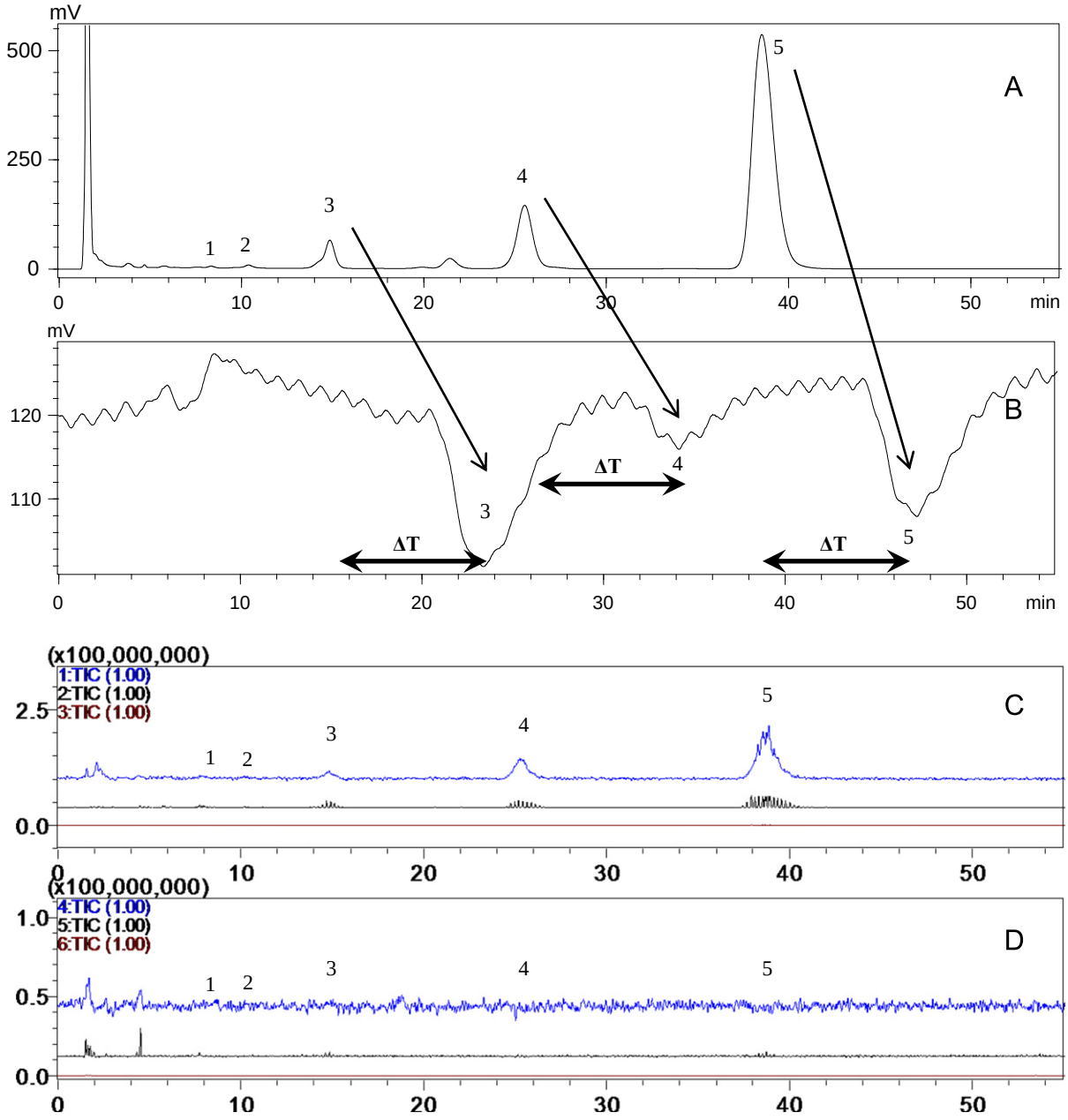

Fig. 5. Liensinine (1), isoliensinine (2), and neferine (3) identified as BChE inhibitors in the chromatogram (A), BChE inhibition profile (B), and TIC in positive- (C) and negative-ion (D) modes of the *Plumula nelambinis* sample by the *on-line* HPLC-MS-BCD method.

47.158 min) were 8.532, 8.637, and 8.667 min, respectively; the time differences between BCD and MS (liensinine: 14.932, isoliensinine: 25.645, neferine: 38.596 min) were 8.532, 8.514, and 8.562 min, respectively. The time differences of HPLC-BCD and MS-BCD were in agreement with that of tacrine detected by the same method.

The structures and MS^n spectra of liensinine, isoliensinine, and neferine are shown in Fig. 6. In the MS^1 spectra in ESI (+), peak 3 showed $[M+H]^+$ at m/z 611.3110 (error = −0.98 ppm), and the molecular formula of this compound was predicted as $C_{37}H_{42}N_2O_6$, based on the accurate mass data recorded by the TOF mass spectrometer. The MS^2 spectrum yielded characteristic peaks at m/z 283, 206, and 190 ($206-CH_4^+$), and this compound was unambiguously identified as liensinine by comparing the experimental data with those of a reference standard as well as literature data [20]. For peak 4 and peak 5, the precursor ions at m/z 611.3120 (error = 0.65 ppm) and 625.3278 (error = 0.95 ppm) yielded characteristic fragments at m/z 503.2484 (error = −12.32 ppm), 297.1711 (error = −6.06 ppm), 206.1159 (error = −10.67 ppm), and 192.1020 (error = −2.60 ppm), which are illustrated in the structural cleavage of the three alkaloids in Fig. 6. Peaks at m/z 177 ($192-CH_3$) for peak 4 and at m/z 190 ($206-CH_3$) for peak 5 in ESI (+) were also in agreement with previous research [23], thus peak 4 and peak 5 were tentatively identified as isoliensinine and liensinine. Moreover, peaks at m/z 609.2966 $[M-H]^-$ (error = −0.65 ppm) for liensinine, m/z 609.2967 $[M-H]^-$ (error = −0.49 ppm) for isoliensinine, and m/z 623.3120 $[M-H]^-$ (error = −1.12 ppm) for neferine in negative-ion mode further confirmed the proposed structures.

In addition, another two trace ingredients, compounds 1 (t_R = 7.692 min) and 2 (t_R = 10.156 min), were tentatively identified by their similar UV and MS^n data (see Fig. 7) as two new alkaloids. The λ_{max} in UV at 285 nm of them indicated their similar structures to that of compound 3, 4 and 5. In the positive mode, both of them showed their $[M+H]^+$ ions at m/z 597.2951(error = −2.34 ppm) and 597.2952 (error = −2.17 ppm), whose molecular formulae were predicted to be $C_{36}H_{40}N_2O_6$, indicating one less methoxyl group than that of isoliensinine. In MS^2 spectrum, m/z at 192.1021 (error = −2.34 ppm) for compound 1 and 192.1013 (error = −6.24 ppm) for compound 2 were predicted as $C_{11}H_{14}NO_2$, the same elemental composition as that of m/z at 192 in isoliensinine. The losses of $C_{25}H_{26}NO_4$ were consistent with the proposed structure cleavage of compounds 1 and 2. In addition, the presence of m/z at 177.0798 ($192-CH_3$, error = 4.51 ppm) indicated the presence of tetrahydroisoquinoline moiety but with different positions of methoxyl substitution. Moreover, m/z at 595, 297 and 282 in the negative mode provided further evidence for the

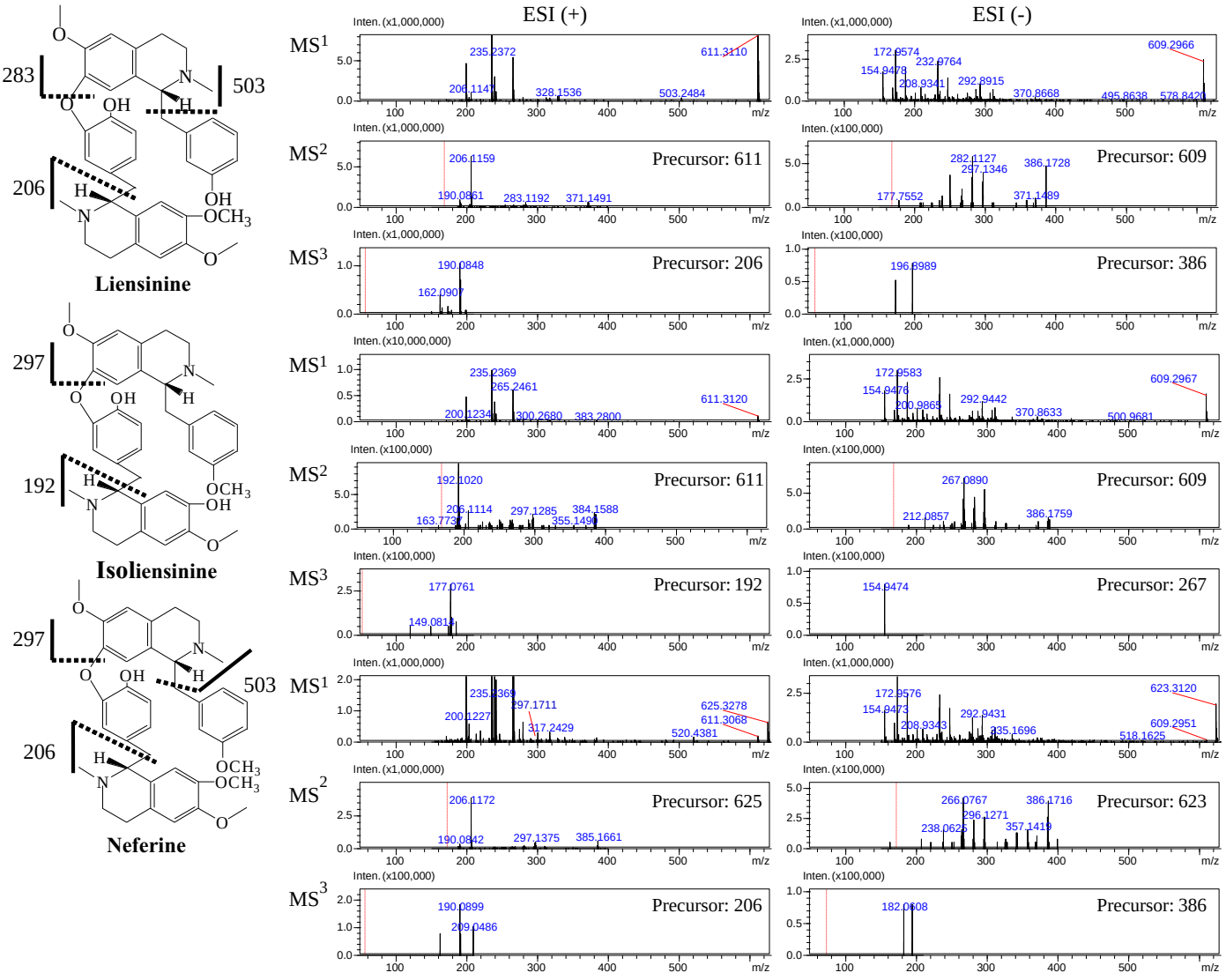

Fig. 6. The ESI (+) and ESI (−) data for liensinine, isoliensinine, and neferine.

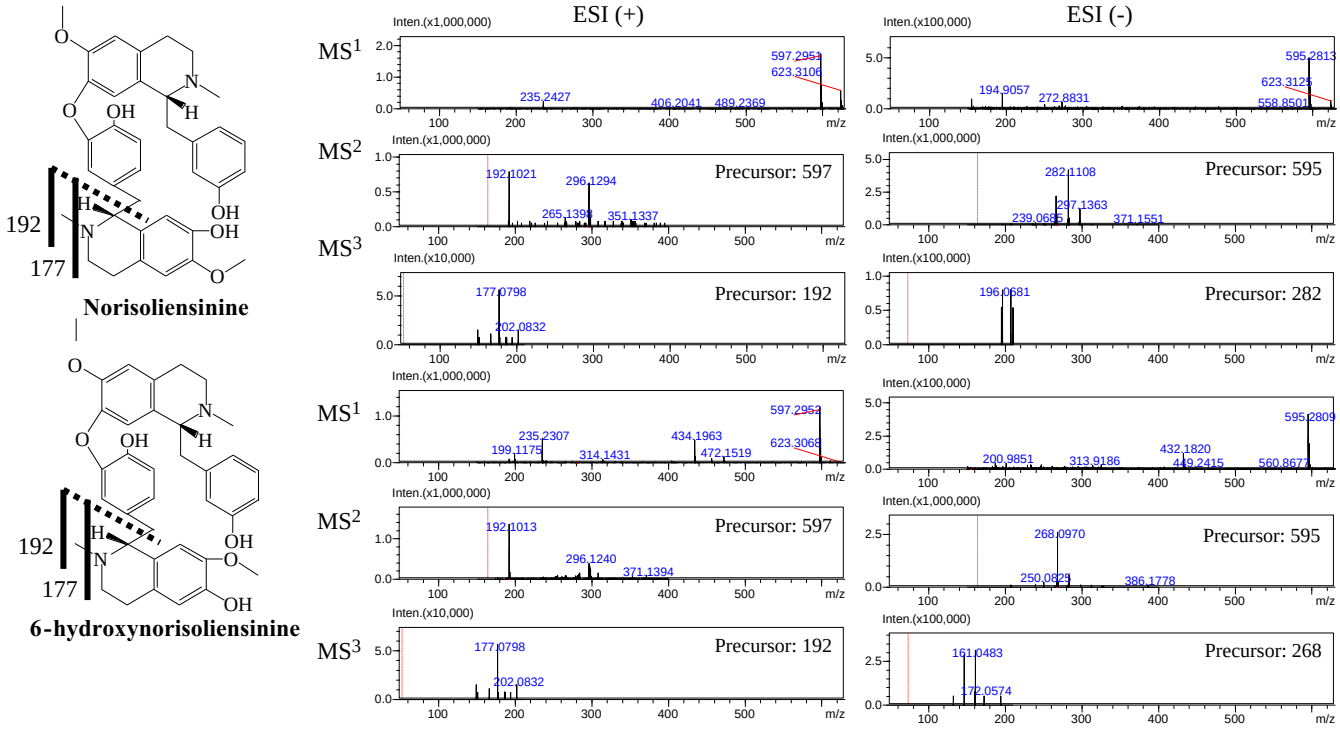

Fig. 7. The ESI (+) and ESI (−) data for norisoliensinine and 6-hydroxynorisoliensinine.

proposed structures. Therefore, the structures of compounds 1 and 2 were proposed in Fig. 7 and they were given the trial names norisoliensinine and 6-hydroxynorisoliensinine, respectively. Further identification and confirmation of these two new compounds using various spectroscopic techniques have already been underway.

3.5. *Method validation and enzyme activity calculation of Plumula nelambinis*

This analytical method has been validated with regard to several aspects, as shown in Fig. 8 (precision, reproducibility, and detection limit), before activity calculation of *Plumula nelambinis*. The validation was evaluated on the basis of relative standard deviations (RSDs) for retention times in chromatograms, activity profiles and TIC, peak areas in HPLC, and activity analysis, as well as time differences of HPLC to BCD and MS to BCD (see Table 1). Tacrine (0.1 mM) was injected into the system five times (see Fig. 8A) to validate the precision of the *on-line* method, and the *Plumula nelambinis* sample (see Fig. 8B) was used to give reproducibility data ($n=3$). The negative peak areas of liensinine, isoliensinine, and neferine showed larger RSDs in the biochemical detection (3.79%, 4.65%, and 4.48%, respectively), but the data were still reliable for later calculation. Besides, methanol was injected as a blank control during the *on-line* analysis, from which little effects on the signal intensity (baseline) of the activity profile had been detected. This result suggested that methanol could be a good solvent for sample detection.

Different concentrations of tacrine (see Fig. 8C) were subjected to analysis by the HPLC-MS-BChEBCD method, for which the detection limit of this compound is 1.0×10^{-9} mol. A functional equation for tacrine was generated in terms of amount of substance (X, μmol) and activity peak area (Y). The regression equation was $Y=-95226778X^2+56518554\,X-121222$; $R^2=0.9931$.

The activities of the three alkaloids were demonstrated by using tacrine as a positive control. The IC_{50} of tacrine calculated by the 96-well plate method was 0.98×10^{-8} M. If tacrine (1 μmol) was taken to represent a potency unit, the potencies of the respective enzyme inhibitors in 1 g of *Plumula nelambinis* sample could be obtained by means of the functional equation, and the relative total activity of 1 g of sample should be equal to the sum of the potencies of the three inhibitors. The BChE inhibition potencies of liensinine, isoliensinine, and neferine in the sample are shown in Table 2. Tacrine equivalence (μmol) is expressed as mean values ± standard deviation ($n=3$).

As shown in Table 2, the ratio of contributions of liensinine, isoliensinine, and neferine to the whole sample was 3.5:1.0:2.8; the ratio between their HPLC areas was 1.0:3.2:15.6, therefore, the relative potency ratio of them would be 19.5:1.7:1.0. The results indicated that liensinine apparently possesses the strongest BChE inhibition activity, followed by isoliensinine and neferine. However, this finding needs to be verified by further experiments on the BChE activities of these compounds after separation and purification, and study of other BChE active compounds in *Plumula nelambinis* is already in process.

4. Conclusions

The reaction system of butyrylcholinesterase inhibition activity was firstly developed into an *on-line* biochemical detection method. The established HPLC-UV-ESI-MS-BChEBCD method enabled the simultaneous separation, identification, and activity evaluation of BChE inhibitors from a *Plumula nelambinis* sample in one run. This new method has proved to be rapid, precise, and sensitive for screening of BChE inhibitors; the detection limit for tacrine in the *on-line* detection was 1.0×10^{-9} mol. Five alkaloids, including two new compounds, norisoliensinine and 6-hydroxynorisoliensinine, have been identified by ESI-IT-TOF-MS detection. And liensinine, isoliensinine, and neferine, for the first time, have been demonstrated to possess strong BChE inhibition activity by the *on-line* method. The BChE inhibition activity of 1 g of the *Plumula nelambinis* sample was calculated to be equivalent to that of 127.88 μmol of tacrine. In summary, *Plumula nelambinis* might represent a potential medicinal source of

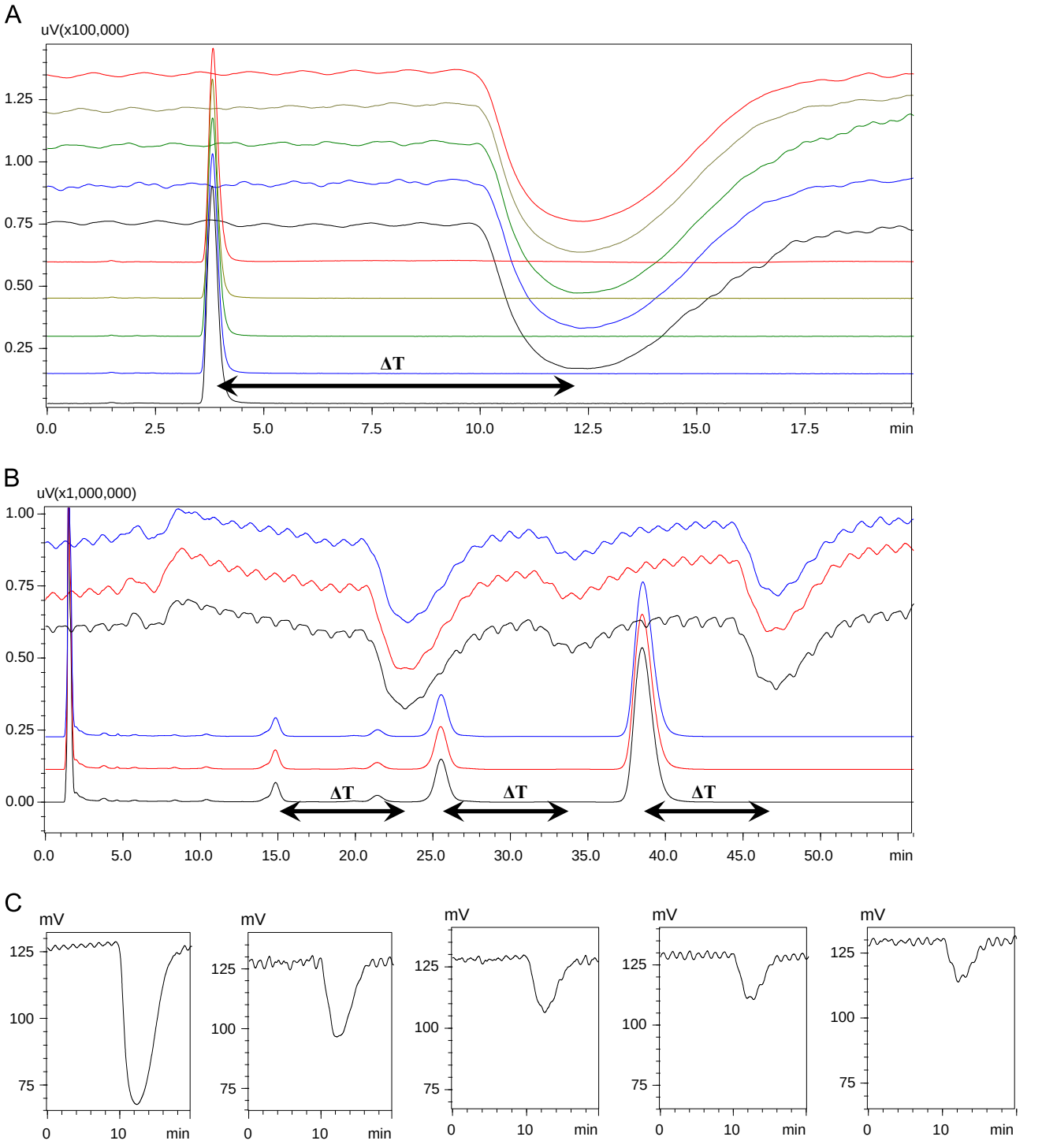

Fig. 8. Method validation of the *on-line* detection. *On-line* detection of tacrine (A) and *Plumula nelambinis* sample (B), activity profiles of tacrine at different concentrations (C) (from left to right: 0.1 mM, 0.02 mM, 0.01 mM, 0.008 mM, and 0.006 mM).

Table 1
Retention times and peak areas in the *on-line* HPLC-MS-BCD method.

Compound	Retention times (min)			Time delays (min)		Peak areas	
	HPLC	BCD	MS	BCD-HPLC	BCD-MS	HPLC	BCD
Tacrine	3.816 ± 0.006	12.347 ± 0.089	3.923 ± 0.008	8.531 ± 0.098	8.424 ± 0.087	108217 ± 2187	16578632 ± 395620
Liensinine	14.834 ± 0.013	23.321 ± 0.076	14.917 ± 0.022	8.488 ± 0.063	8.405 ± 0.054	2884186 ± 15713	4317837 ± 163680
Isoliensinine	25.513 ± 0.021	34.034 ± 0.177	25.620 ± 0.036	8.521 ± 0.156	8.389 ± 0.177	9119535 ± 1549	1332365 ± 62052
Neferine	38.516 ± 0.030	47.154 ± 0.006	38.601 ± 0.006	8.642 ± 0.030	8.554 ± 0.012	45102108 ± 39749	3575829 ± 160326

Table 2
BChE inhibition activities of alkaloids in the *Plumula nelambinis* sample.

Compound	Liensinine	Isoliensinine	Neferine	Total
Tacrine equivalence of sample (μmol/g)	58.25 ± 2.16	16.81 ± 0.77	46.81 ± 2.09	127.88

cholinesterase inhibitors for the treatment of Alzheimer's disease; the proposed method offers a practical and efficient approach for identifying new BChE inhibitors in natural medicines and other complex samples.

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