Short Communication

Rapid and sensitive detection of the inhibitory activities of acetyl- and butyryl-cholinesterases inhibitors by UPLC–ESI-MS/MS

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A B S T R A C T

Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) are legitimate therapeutic targets for Alzheimer’s disease. The classical approach for screening potential AChE/BChE inhibitors was developed by Ellman. However, the background color of compounds or plant extracts remained uncertain and frequently interfered with the detection of the secondary reaction, thereby easily yielding false positive or false negative results. Rapid, selective, and sensitive ultra-performance liquid chromatography combined with electrospray ionization tandem mass spectrometry method was developed and used for the detection of AChE and BChE inhibition by directly determining the common product, choline (Ch). Proper separation was achieved for choline and chloromequat (internal standard) within 1.2 min via isocratic elution (0.1% formic acid:methanol = 98:2) on an HSS T3 column following a simple precipitation of proteins for sample treatment. The relative standard deviations of the intra- and inter-day precisions were below 7.34 and 9.09%, respectively, whereas the mean accuracy for the quality control samples was 100.31 ± 10.93%. The method exhibited the advantages of small total reaction volume (100 μL), short analysis time (1.2 min), high sensitivity (LOQ of 0.036 μM for Ch), and low cost (little consumption enzymes of 0.0035 and 0.008 unit mL⁻¹ for AChE and BChE, and substrates of 5.505 and 7.152 μM for ACh and BCh in individual inhibition, respectively), and without matrix effect (90.00 – 105.03%). The developed method was successfully applied for detecting the AChE and BChE inhibitory activities for model drugs, including galanthamine, tacrine, neostigmine methylsulfate, ezerine, as well as β-carboline and quinazoline alkaloids from Peganum harmala.

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1. Introduction

Alzheimer’s disease (AD) is a fairly common age-related neurodegenerative disease with many cognitive and neuropsychiatric manifestations that result in progressive disability and eventual incapacitation. Recent studies have indicated that the main cause of the loss in cognitive functions in AD patients is the continuous decline in cholinergic neurotransmission in the cortical and other regions of the human brain [1]. Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) are hydrolytic enzymes that function on acetylcholine (ACh) to terminate its actions in the synaptic cleft by cleaving the neurotransmitter to choline (Ch) and acetate. Despite the unknown etiology of AD, these findings supported that the activity of cholinesterase (ChE) at different stages of AD progression needed to be controlled. One of the most effective treatment strategies that has been suggested is to restrain the cholinergic function and to elevate the ACh level by inhibiting AChE and BChE. Therefore, AChE and BChE inhibitors, such as tacrine, donepezil, rivastigmine, and galanthamine, have been approved for AD treatment [2]. Most AChE and BChE inhibitors have been isolated from natural plants or structural modification from natural compounds. Meanwhile, the identification of a candidate drug that can prevent or delay the progression of neurodegenerative pathology without side effects still remains the goal for medicinal chemists [3].

The most commonly used method for screening potential AChE/BChE inhibitors is based on a spectrometric/colormetric method developed by Ellman [4]. In this method, acetylthiocholine (ATCh)/butyrylthiocholine (BTC) rather than ACh, a natural transmitter in vivo, is used commonly as a substrate to form thiocholine...
and react with dithiobisnitrobenzoate for yellow color detection. However, the background color of compounds or plant extracts is uncertain, frequently interferes with the detection of the secondary reaction, and easily yields false positive or false negatives results [5]. To overcome these drawbacks, several methods, such as fluorimetric assay, thin layer chromatography, high performance liquid chromatography [6–8], capillary electrophoresis (CE), and mass spectrometry (MS) [9–11], have been developed for screening potential AChE/BChE inhibitors. Among these methods, CE has many advantages because of the small dimensions of the capillaries used for the reaction, high efficiency separations, short analysis times, and the ability to employ several detection techniques [9]. The MS method is a label-free, non-radioactive assay that offers greater flexibility in experiments. The MS method is used to monitor catalytic reactions by the simultaneous detection of the concentration changes of the reported molecules (substrate, product), thereby allowing the screening of the inhibitors of the enzyme [11]. Anna et al. developed the electrospay ionization ion trap mass spectrometry method running within 1.4 min without the use of an HPLC column to evaluate the potential AChE inhibition of a candidate compound [3]. However, the potential interference of the candidate compound or matrix contents did not provide enough consideration. Meanwhile, matrix-assisted laser desorption-ionization mass spectrometry (MALDI-MS) has also been proven to be an effective method for inhibiting the screening of AChE and BChE [12]. MALDI-MS is a newly developed mass spectrometric technique used for the analysis of large biomolecules; thus, its application is limited to a particular degree in small molecules and is not more general and useful to date than ESI-MS. UPLC–MS provides a rapid and accurate method for the determination of microamount compounds and is especially applicable for enzyme activity assay [13]. Therefore, the present study aims to establish a practical and convenient method by using UPLC–MS for the fast screening of exogenous AChE and BChE inhibitors from herbal medicines based on a direct method where the direct product (Ch) from the substrate (ACh/Bch) catalyzed by AChE or BChE is quantitated. The method involves the classic principle of AChE/BChE activity determination method in vitro for the fast and sensitive screening of inhibitors with best efforts to avoid interferences of the matrix effects.

2. Experimental

2.1. Chemicals and reagents

AChE from Electrophorus electricus, BChE from equine serum, Ach chloride, BCh chloride, Ch chloride, cloromqueat (internal standard, IS), galanthamine, tacrine, neoestigmine methylsulfate, eserine, harmaline, harmine, and harmaline were purchased from Sigma–Aldrich (St. Louis, MO, USA). Harmalol hydrochloride and harmol were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, NA, Japan). Vasicine, vasicinone, deoxyvasicinone, and deoxyvasicine were isolated from seeds of Peganum harmala in our laboratory and identified using NMR (Bruker NMR AV 400, Bruker BioSpin GmbH, Rheinstetten, Germany) and mass spectrometry data [6] with purity of more than 98% and assayed based on the peak area normalization via HPLC. HPLC-grade methanol was obtained from Fisher Co. (Santa Clara, CA, USA). Formic acid was obtained from Tedia Inc. (Fairfield, OH, USA). Water was produced using a Milli-Q Academic System (Millipore Corp., Billerica, MA, USA). The other reagents were of analytical grade.

2.2. Standard stock solutions and quality control samples

Stock solutions of Ch and IS, with concentrations of 5.386 and 2.012 mM, were prepared by dissolving a proper amount of each standard substance in 25 mL of methanol, respectively. The working solutions of the desired concentrations for the calibration standards and quality control (QC) was obtained and serially diluted with the initial mobile phase. An IS working solution (1.899 µM) was prepared by diluting the stock solutions with methanol. All solutions were stored at 4 °C and brought to room temperature before use.

2.3. Sample preparation procedure

The AChE and BChE enzymes were dissolved in 20 mM sodium phosphate buffer (pH 7.6) to make stock solution with a concentration of 3.470 unit mL–1, and the solutions were stored at −80 °C before use. The stock solutions of the test compounds were prepared by dissolving an adequate quantity of each compound in 0.2% DMSO to obtain 20 mM solutions. The substrates ACh and BCh were dissolved in 20 mM sodium phosphate buffer (pH 7.6) to achieve concentrations of 11.011 and 14.305 µM, respectively. All the stock solutions were diluted to a series of concentrations with 20 mM sodium phosphate buffer solution (pH 7.6) before each experiment.

2.4. UPLC–ESI-MS/MS analysis

Separation was performed on a Waters-ACQUITY™ UPLC system (Waters Corp., Milford, MA, USA) using an ACQUITY UPLC HSS T3 column (100 mm × 2.1 mm, 1.8 µm particle size) maintained at 40 °C. The mobile phase consisted of A (0.1% formic acid) and B (methanol) at a flow rate of 0.3 mL min−1 with isotropic elution of 98% A and 2% B. The injection volume was 5 µL, and the injection was performed using a partial loop with a needle overfill mode. A Micromass Quattro Premier XE tandem quadruple mass spectrometer equipped with an electrospray ionization (ESI) interface (Waters Corp., Manchester, UK) was used for analytic detection. The mass spectrometer was operated in the positive ionization mode by using multiple reaction monitoring (MRM). The main working parameters were set as follows: capillary voltage, 3.000 kV; extractor voltage, 3.00 V; source temperature, 120 °C; desolvation temperature, 400 °C; desolvation gas flow, 550 Lh−1 (N2); and cone gas flow, 50 Lh−1. Nitrogen (99.99% purity) and argon (99.999% purity) were used as cone and collision gases, respectively. The inter-channel and the inter-scan delays were both set at 0.1 s. A mass scan method was used to test the exact molecular weight of the product, and a daughter scan of the [M+H]+ ion of the product was used to obtain its MS/MS spectrum. Data acquisition was carried out using the MassLynx 4.1 software.

2.5. In vitro anticholinesterase assays

The AChE and BChE activity assays were carried out using ACh and BCh as substrates, respectively, with the optimal incubation condition. Compounds including galanthamine, neoestigmine methylsulfate, tacrine, serine, harmaline, harmine, harmalol, harmaline, vasicine, vasicinone, deoxyvasicinone, and deoxyvasicine were tested in concentration ranges between 0.01 and 1 mM. A 100 µL incubation system composed of 10 µL of the test compound solution and 40 µL of the enzyme solutions (with final concentrations: 0.0035 unit mL−1 for AChE, or 0.008 unit mL−1 for BChE) were mixed and pre-incubated for 15 min. Afterward, 50 µL of the substrate solutions (final concentrations 5.50 µM for ACh, or 7.15 µM for BCh) was added and incubated for 20 min at 25 °C. The reaction was terminated by immediately adding 300 µL of ice acetonitrite solution (0 °C) dissolved with 1.899 µM IS. The solution was then centrifuged (15,000 × g, 10 min), and the supernatant was used for the analysis. The inhibitory IC50 values of the individual model compound on AChE and BChE were calculated using the Prism software (GraphPad Software Inc., San Diego, CA).
The kinetic characterization of AChE and BChE was performed via the UPLC–ESI MS/MS method by performing triplicate analysis. In the incubation system, 10 μL of the phosphate buffer was used to substitute the inhibitors, and the varying concentrations of the substrate from 2.08 μM to 1063.83 μM (ACh) and 2.79 μM to 1430.48 μM (BCh) were investigated. The kinetic parameters for the maximum reaction rate ($V_{\text{max}}$) and Michaelis–Menten constant ($K_m$) of AChE/BChE were extrapolated from the individual Eadie–Hofstee plots of $V$ versus $V/S$.

2.6. Method validation

2.6.1. Linearity validation

The linear relationship of the method was evaluated by preparing seven different concentrations of Ch in a buffer solution range of 0.036–7.162 μM. The calibration curves were established by plotting the peak area ratios of the product to the IS versus the respective true standard concentrations. The limit of detection (LOD) and limit of quantification (LOQ) were defined as the lowest concentration giving a signal-to-noise ratio of at least 3-fold and 10-fold, respectively.

2.6.2. Precision

Precisions were assessed by performing replicate analysis of the quality control (QC) samples (n = 6) at three levels (0.036, 0.358, and 7.162 μM). The intra-day precision was evaluated by repeating the analysis of the QC samples three times a day, whereas the inter-day precision was determined by repeating the analysis on three consecutive days. The relative standard deviation (RSD) was calculated from the observed concentrations ($C_{\text{obs}}$) by using the following equation: %RSD = [standard deviation (SD) / $C_{\text{obs}}$] × 100.

2.6.3. Accuracy

The accuracy of the method was expressed by the recovery of the QC samples at three levels (0.036, 0.358, and 7.162 μM; n = 6). The apparent concentrations of these samples were calculated using the calibration curves. The recovery rates (%) were calculated from the mean value of the observed concentrations ($C_{\text{obs}}$) and the theoretical concentrations ($C_{\text{theo}}$) by using the following equation: % = ($C_{\text{obs}}/C_{\text{theo}}$) × 100.

2.6.4. Stability

Stability studies were also conducted at three levels of the QC samples previously described (n = 6), which were stored for 24 h at ambient temperature (25 ± 2 °C) and three days at 4 °C.

2.6.5. Matrix effect

The matrix effects were determined by comparing the MRM peak responses of the standard QC samples (A, n = 6) in enzymes (pretreatment with acetonitrile) devitalized to those of the same analyte presented in the initial mobile phase (B, n = 6). The mean value (A)/(B) × 100% was considered as the matrix effect, which is acceptable when the ratio is less than 85% or more than 115%.

2.7. Data analysis

The assays were conducted in triplicate, and all tabulated results were expressed as means ± SD. The IC50 values were calculated from the concentration-response curves via nonlinear regression analysis using the Prism software.

3. Results and discussion

3.1. UPLC–MS/MS conditions

At the aforementioned UPLC conditions, the baseline separation of Ch and IS was achieved on a HSS T3 column, and both were eluted at 0.86 and 1.02 min, respectively (Fig. 1A and B). The MS parameters were optimized by injecting the Ch and IS standard solution with the MRM mode being used to obtain better selectivity. The ion transition of Ch from $m/z$ 103.6–59.6 at an optimized cone voltage of 35 V and a collision voltage 15 V and IS from $m/z$ 121.6–57.6 at an optimized cone voltage of 40 V and a collision voltage 15 V were used to quantify the analytes, respectively (Fig. 1C and D). To obtain an optimum response, three commonly used polar solvents (water, acetonitrile, and methanol) and three kinds of mobile phase modifiers that are compatible with MS (ammonium acetate, acetic acid, and formic acid) were examined. Finally, 0.1% formic acid and methanol were employed as the mobile phase to enhance the separation and MS sensitivity of Ch and IS. An optimal isocratic elution with a flow rate of 0.3 mL min–1 provided better peak shape of Ch and IS with the column set at 40 °C. The total analysis time for a single run was finished within 1.2 min (Fig. 1 A and B). The results suggested that the method was especially suitable for high-throughput screening.

3.2. Method validation

The calibration curves showed a good linear behavior over the Ch concentration range of 0.036–7.162 μM ($y = 1.942x + 0.154$, $R^2 = 0.9964$). The LOD ($S/N = 3$) of Ch was 0.011 μM, and the LOQ

![Fig. 1. UPLC–ESI MS/MS chromatograms of the product choline (Ch) (A) and chlormequat (internal standard, IS) (B), and product ion mass spectra of choline (C) and internal standard (IS) (D).](image-url)
(S/N = 10) was 0.036 μM for the product. The LOQ is appropriate for the quantitative detection of Ch in the enzymatic studies.

The results of intra- and inter-day precisions, accuracy, matrix effect, and stabilities of the method are summarized in Table 1. The RSD values of intra-day and inter-day precisions ranged from 1.34 to 7.34% and 1.38 to 9.09%, respectively. The average recovery of Ch at three concentration levels was 100.31 ± 10.93%. The matrix effects ranged from 90.00 to 105.03%. Ch was stable after being placed at room temperature for 24 h and kept in the autosampler or refrigerator (4 °C) for three days.

3.3. Influence of temperature on the enzymatic reaction

The effect of temperature on the enzymatic reaction was investigated by varying the temperature in the range of 20–40 °C (Fig. 2A and B). No obvious influence of temperature from 25 to 40 °C was observed on AChE and 20–30 °C on BChE reaction. Therefore, the temperature for both enzymatic reactions was selected at 25 °C.

3.4. Optimization of AChE/BChE, substrate concentrations, and reaction time

To ensure the utilization of the initial rate velocities for the calculation of enzyme constants according to steady-state kinetics, the optimum reaction time, substrate concentration, and AChE/BChE concentration should be within the linear region of the formation of the product. Three reaction functions were generated by monitoring the product as functions of reaction time, substrate concentration, and enzyme concentration. The reaction time was set as 20 min for the AChE and BChE assays (Fig. 2C and D). The optimal ACh and BCh substrate concentrations were selected at 5.505 and 7.152 μM with linear equations of \( y = 0.431x + 0.238 \) \((R^2 = 0.9992)\) and \( y = 0.224x + 0.210 \) \((R^2 = 0.9834)\) for AChE and BChE assays, respectively. The enzyme concentrations were selected at 0.0035 and 0.008 unit ml⁻¹ with linear equations of \( y = 496.750x + 0.962 \) \((R^2 = 0.9965)\) and \( y = 229.49x + 0.111 \) \((R^2 = 0.991)\) for AChE and BChE, respectively. The product Ch can be quantified sensitively, and even 99% of AChE/BChE enzyme activity was inhibited.

3.5. Michaelis constant \((K_m)\) of AChE/BChE

Fig. 3 shows that the linear Eadie–Hofstee plots (II) exhibited Michaelis–Menten kinetics for both AChE- and BChE-catalyzed enzyme reaction. The values of \( K_m \) and \( V_{\text{max}} \) for AChE were 39.56 ± 3.41 μM and 81.92 μM min⁻¹ unit⁻¹, respectively. The \( K_m \) and \( V_{\text{max}} \) for BChE were 73.93 ± 10.03 μM and 29.83 μM min⁻¹ unit⁻¹, respectively. These values were in good agreement with those reported in literature [14]. However, these values were lower than the \( K_m \) of 130.56 ± 7.25 and 122.21 ± 3.98 μM for AChE and BChE, respectively, as reported by Ucar [15]. This phenomenon is normal in enzymatic research, which may be attributed to the inherent activity differences of AChE and BChE used in different laboratories.

![Fig. 2](image1.png) Optimization of reaction temperature and time in anti-cholinesterase assay. Influence of temperature on the production of choline converted from acetylcholine (A) and butyrocholine (B). Influence of incubation time on production of choline converted from acetylcholine (C) and butyrocholine (D).

### Table 1

<table>
<thead>
<tr>
<th>Concentration (μM)</th>
<th>Accuracy (%)</th>
<th>Mean accuracy (%)</th>
<th>Precision (RSD%)</th>
<th>Matrix effect (%)</th>
<th>Stability (RSD%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Added</td>
<td>Measured</td>
<td></td>
<td>Intra-day</td>
<td>Inter-day</td>
<td>(RT)</td>
</tr>
<tr>
<td>0.036</td>
<td>0.040 ± 0.0</td>
<td>111.17 ± 10.76</td>
<td>7.34</td>
<td>9.09</td>
<td>7.67</td>
</tr>
<tr>
<td>0.358</td>
<td>0.360 ± 0.02</td>
<td>100.45 ± 4.40</td>
<td>4.85</td>
<td>4.80</td>
<td>105.03</td>
</tr>
<tr>
<td>7.162</td>
<td>6.397 ± 0.09</td>
<td>89.32 ± 1.26</td>
<td>1.34</td>
<td>1.38</td>
<td>101.32</td>
</tr>
</tbody>
</table>

RT: Room temperature.
Table 2

Inhibitory activity (IC\textsubscript{50}) of the compounds investigated against AChE and BChE.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>\textbf{IC\textsubscript{50} (\mu M ± SD) for AChE}</th>
<th>\textbf{IC\textsubscript{50} (\mu M ± SD) for BChE}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>UPLC–ESI MS/MS</td>
<td>Ellman’s</td>
</tr>
<tr>
<td></td>
<td></td>
<td>UPLC–ESI MS/MS</td>
</tr>
<tr>
<td>Galanthamine</td>
<td>0.05 ± 0.0</td>
<td>0.80 ± 0.0 [16]</td>
</tr>
<tr>
<td>Neostigmine</td>
<td>1.00 ± 0.0</td>
<td>1.00 ± 0.0 [17]</td>
</tr>
<tr>
<td>methylsulfate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tacrine</td>
<td>0.01 ± 0.0</td>
<td>0.04 ± 0.01 [19]</td>
</tr>
<tr>
<td>Eserine</td>
<td>0.02 ± 0.0</td>
<td>0.11 ± 0.01 [16]</td>
</tr>
<tr>
<td>Harmaline</td>
<td>1.21 ± 0.04</td>
<td>9.05 ± 1.08 [16]</td>
</tr>
<tr>
<td>Harmine</td>
<td>1.95 ± 0.08</td>
<td>10.58 ± 2.01 [16]</td>
</tr>
<tr>
<td>Harmol</td>
<td>1.90 ± 0.02</td>
<td>21.58 ± 1.39 [16]</td>
</tr>
<tr>
<td>Harmol</td>
<td>3.45 ± 0.08</td>
<td>27.88 ± 1.13 [16]</td>
</tr>
<tr>
<td>Harmane</td>
<td>3.64 ± 0.19</td>
<td>7.11 ± 2.00 [16]</td>
</tr>
<tr>
<td>Deoxyvasicine</td>
<td>2.36 ± 0.40</td>
<td>&gt;1000 [16]</td>
</tr>
<tr>
<td>Vasicine</td>
<td>3.24 ± 0.08</td>
<td>13.68 ± 1.25 [16]</td>
</tr>
<tr>
<td>Deoxyvascinecinone</td>
<td>35.16 ± 1.34</td>
<td>294.44 ± 1.47 [16]</td>
</tr>
<tr>
<td>Vasicinone</td>
<td>76.60 ± 8.46</td>
<td>370.78 ± 1.07 [16]</td>
</tr>
</tbody>
</table>

3.6. Detection of inhibitory activity of model compounds against enzymes

The inhibitory activities of the model drugs, including galanthamine, tacrine, neostigmine methylsulfate, and eserine, as well as β-carboline and quinazoline alkaloids (harmaline, harmine, harmalol, harmol, harmane, vasicine, vasicinone, deoxyvascinecinone, and deoxyvascine) on AChE and BChE, were evaluated in vitro and summarized in Table 2. Galanthamine, neostigmine methylsulfate, tacrine, and eserine exhibited exceedingly strong inhibition on AChE and different potential inhibitions on BChE. However, the IC\textsubscript{50} values determined via the present method were significantly lower than the reported data in literature [16–20]. β-carboline and quinazoline alkaloids also exhibited different potential inhibition activities on AChE/BChE. All the IC\textsubscript{50} values were also significantly lower than the previous data determined via Ellman’s method [16].

Indeed, the IC\textsubscript{50} values obtained from different laboratories are difficult to compare. Table 2 shows that a significant variance
was acquired in different laboratories, although the variance was detected via the common Ellman’s method (for example, the IC₅₀ of the AChE of galanthamine was from 0.10 to 1.40 μM). These findings can be ascribed to the variance on the experimental and technical conditions. For instance, ACh was used as substrate in Ellman’s method, whereas the natural substrate (ACh/BCh) was used in the UPLC–ESI MS/MS method. In addition, the concentrations of the enzymes and the substrate used in Ellman’s method were several folds higher than that of the present method.

To interpret the correlation of the two methods, a bivariate correlation analysis for the IC₅₀ values obtained from the two methods was investigated (Fig. 4). The IC₅₀ values of the reference compounds, such as galanthamine, neostigmine methylsulfate, tacrine, eserine, β-carboline, and quinazoline alkaloids, were introduced for the correlation analysis, in which deoxyvasinicine, vasinicine, and deoxyvasinicinone did not participate in the correlation analysis because the IC₅₀ value was not obtained in previous studies by Ellman’s method [16]. The values exhibited a significantly linear correlation between UPLC–ESI MS/MS and Ellman’s method with correlation factors (r) of 0.964 (p < 0.001) and 0.882 (p < 0.01), respectively. The linear equations were y = 5.342x + 6.366 \( (R^2 = 0.9285) \) and y = 20.236x + 6.374 \( (R^2 = 0.7776) \) in the AChE and the BChE assay, respectively. The UPLC–ESI MS/MS method is reliable and is similar to that of Ellman’s method. Given that the detected IC₅₀ values by the UPLC–ESI MS/MS method is significantly lower than that by Ellman’s method, the UPLC–ESI MS/MS method was more sensitive. Notably, the spectrophotometry method (Ellman’s) did have an inaccurate risk in the determination of the IC₅₀ values of harmine, harmaline, and harmame because of their light yellow color in the buffer solution. In our previous study, deoxyvasinicine and deoxyvasinicinone were determined to have no inhibitory effects against AChE and BChE via Ellman’s method [16]. Deoxyvasinicine and deoxyvasinicinone exhibited significant inhibitory activity against AChE and BChE. Deoxyvasinicine exhibited stronger inhibitory activity against BChE than that of galanthamine, neostigmine methylsulfate, and eserine. Therefore, this method is more sensitive and reliable and can be used to avoid false negative results.

4. Conclusions

A UPLC–MS/MS method for the determination of the inhibitory activity of AChE/BChE inhibitors was developed using ACh/BCh as the substrate. The method achieved a proper separation for Ch and IS within 1.2 min by isocratic elution on an efficient HSS T3 column without any matrix effect. The method is fast, sensitive, accurate, and repeatable. The small total reaction volume, short analysis time, high sensitivity, and low cost are the advantages of the new method compared with the conventional methods. Thus, this method is suitable for the high-throughput screening of potential AChE/BChE inhibitors from natural medicinal plants.

Acknowledgments

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