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RESEARCH ARTICLE

Evaluation of 2,6-dichlorophenolindophenol acetate as a substrate for acetylcholinesterase activity assay

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Abstract

Ellman's method is a standard protocol for the determination of cholinesterases activity. Though the method is ready for laboratory purposes, it has some drawbacks as well. In the current article, 2,6-dichloroindophenol acetate is performed as a chromogenic substrate suitable for acetylcholinesterase (AChE) activity examination. Michaelis constant and maximal velocity for 2,6-dichloroindophenol acetate were determined (38.0 μM and 244 pkat) and compared to the values for acetythiocholine (K_m 0.18 mM; V_{max} 5.1 nkat). Docking for 2,6-dichloroindophenol acetate and human AChE was done as well. In conclusion, 2,6-dichloroindophenol acetate seems to be suitable chromogenic substrate for AChE and spectrophotometry and based on this it can be easily performed whenever AChE activity should be tested.

Keywords

Acetylcholinesterase, assay, butyrylcholinesterase, chromogen, Ellman method, spectrophotometry, substrate

History

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Introduction

Acetylcholinesterase (AChE; EC 3.1.1.7) is an important enzyme responsible for termination of cholinergic neurotransmission. The enzyme is an object of interest in pharmacology research because inhibitors of AChE have wide use as drugs for, e.g. Alzheimer disease, myasthenia gravis and some other purposes[1]. In addition to the pharmacological applications, AChE can be targeted by many harmful substances such as nerve agents in chemical warfare and pesticides such as carbofuran[2]. Besides AChE, the second cholinesterase, butyrylcholinesterase (BChE; EC 3.1.1.8), can be found in the body. Physiological relevance of BChE is, however, lower compared to AChE and it is not unsubstitutable in regulatory processes as well[3–5].

Activity of AChE is frequently measured because of new drugs testing or in an assay of neurotoxic compounds in, for example, food or environment[6,7]. When activity of AChE assessed, Ellman's method is typically preferred as a standard protocol. The method was discovered by Ellman et al. and used without particular changes up today[8,9]. The method consists of two steps. In the first step, acetylthiocholine (or butyrylthiocholine when BChE assayed) is split by AChE into thiocholine and acetic acid. In the next step, thiocholine reacts with 5,5′-dithiobis(2-nitrobenzoic acid) providing yellow colored 5-thio-2-nitrobenzoic acid. The method has some drawbacks such as low stability of 5,5′-dithiobis(2-nitrobenzoic acid) and interference of hemoglobin[10], thiol moiety containing compounds such as cysteine, reduced glutathione[11] or oximes such as obidoxime, asoxime[12]. Because of the aforementioned disadvantages of the assay, there is a demand for a new, more reliable, assay suitable for a routine determination of cholinesterases activity[12,13].

The current article is devoted to setup of a new protocol suitable for AChE activity measurement. Here, a new substrate, 2,6-dichlorophenolindophenol acetate, is used as a chromogenic reagent. It is hypothesized that the new substrate would be an alternative to the standard Ellman's method and assay based on the new substrate will not have drawbacks of the Ellman's method. Pros and cons of the new and standard methods are discussed in the article.

Materials and methods

AChE

In the assay, human recombinant AChE expressed in HEK293 cells was purchased from Sigma Aldrich (Saint Louis, MI). The AChE had activity of 1500 μmol/min per milligram of protein and was received in lyophilized form. AChE was solubilized in phosphate buffered saline (pH 7.4) and activity was adjusted up 0.67 μmol/min/μl (0.40 nkat/μl) for 1 mM acetylthiocholine chloride.

Ellman's method

A total of 100 μl of either donepezil (Sigma Aldrich) solution or phosphate buffered saline, 200 μl of 1 mM of 5,5′-dithiobis(2-nitrobenzoic acid) in phosphate buffered saline, 100 μl of tested sample (e.g. interfering compound) or phosphate buffered saline, 490 μl of phosphate buffered saline and 10 μl of the aforementioned AChE solution was injected into disposable cuvette with maximal volume 2 ml and gently shaken. Finally, 100 μl of 10 mM acetylthiocholine chloride was given into the cuvette. Absorbance

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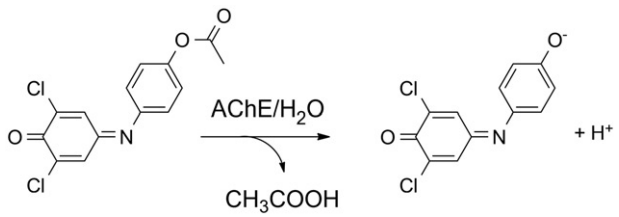

Figure 1. Assay of AChE activity using 2,6-dichloroindophenol acetate hydrolysis to 2,6-dichloroindophenol.

was measured at 412 nm from acetylthiocholine application for 2 min. Absorbance difference was calculated from the time interval. Enzyme activity was calculated using nitrothiobenzoate extinction coefficient $\varepsilon = 14\,150\,1 \times \text{mol}^{-1} \times \text{cm}^{-1}$ [14,15].

Measurement using 2,6-dichlorophenolindophenol acetate

In the assay, 2,6-dichloroindophenol acetate (Sigma-Aldrich, purity >97%; catalog number of the reagent 36190-10MG-F) is used as a chromogen providing blue 2,6-dichloroindophenolate as a reaction product. In physiological pH 7.4, the blue product absorbs at 606 nm with extinction coefficient $\varepsilon = 18\,800\,1 \times \text{mol}^{-1} \times \text{cm}^{-1}$ [16]. A disposable cuvette was consequently filled with following: 790 µl of phosphate buffered saline, 100 µl of sample solution (tested interfering compound) or phosphate buffered saline and 10 µl of the AChE solution. Finally, 100 µl (10 mM) of 2,6-dichloroindophenol acetate solution in 5% (v/v) ethanol was added; then the absorbance was measured for 60 min. Absorbance change was calculated from the time interval. Principle of the assay is depicted in Figure 1.

Computational docking

Docking studies were carried out using Autodock Vina (version 1.1.2, The Scripps Research Institute, La Jolla, CA) with default parameters[17,18]. The crystal structure of AChE was prepared using PDB structure 4YE7 as starting geometry. The ligand (donepezil) and the non-bonding water molecules were removed. Energy minimization was performed (no. of conjugate gradient steps: 100 Gradient: 0.02 RMS kcal/mol/A^2) using UCSF Chimera 1.8. Grid box (search space) was set to cover entire AChE cavity. The ligand structures were prepared using PRODRG, the hydrogens were added and energy minimized. All structures receptor and ligand were consequently modified via Autodock Tools 1.5.7rc1 in order to be used by Vina. 2,6-dichloroindophenol acetate was docked using the Lamarckian genetic algorithm. Exhaustiveness of the method was increased from default 8 to 64 in order to find minimum energy pose. Minimum electrostatic potential (41.82 kcal/mol) and maximum electrostatic potential (40.09 kcal/mol) were set up. At the end of calculation, Autodock Vina performed cluster analysis. Root-mean square deviation (RMSD) for best scored pose of donepezil after redocking was 1.05 Å (predicted free binding energy of −12.1 kcal/mol).

Experimental data processing

All measurements were made five times. Origin 8 (OriginLab Corporation, Northampton, MA) software was used for the experimental data processing and plots construction. Calculation of Michaelis constant (K_m) and maximal velocity (V_{max}) was based on non-linear curve fitting with Hill equation. Limit of detection was calculated from confidence interval (95%) in calibration plot. ANOVA with Bonferroni test was performed in order to judge significance of differences on probability levels $p = 0.05$ and 0.01. In this article, enzyme activity is further expressed in katal unit: 1 kat = 1 mol/s; 1 pkat = 10^{-12} mol/s; 1 nkat = 10^{-9} mol/s.

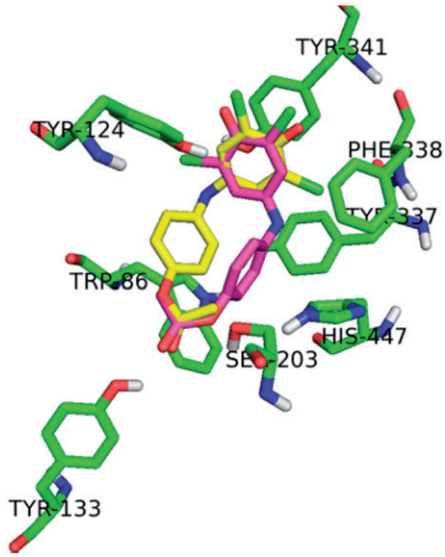

Figure 2. Localization of 2,6-dichloroindophenol acetate (top scored pose: violet = right position for uncolored printing; second top scored pose: yellow in the middle of the picture, left from the top scored pose) in active site of AChE.

Results and discussion

The top scored docked posse (predicted free-binding energy of −9.1 kcal/mol) shows the proximity of the ester bond of 2,6-dichloroindophenol acetate to the catalytic residues Ser203 and His447. It could be assumed that such orientation allows the ester bond cleave by AChE. This pose of 2,6-dichloroindophenol acetate within the active site is reached by interactions with hydrogen bonds Ser203 and His447 and ester oxygen (3.5 Å and 3.6 Å, respectively). Another very strong interaction is formed between Tyr133 and carbonyl oxygen in 2,6-dichloroindophenol acetate (2.2 Å). This binding orientation is stabilized by the strong hydrogen bond (2.9 Å) formed between carbonyl oxygen and Tyr124 hydroxyl. Further stabilization is facilitated by iminocyclohexanedienone ring stacking via π–π interactions with Tyr341 (3.4 Å).

The second best scored binding mode should also be taken into consideration. The free-binding energy is slightly lower compared to top scored binding mode (−9.0 kcal/mol). However, the less favorable orientation of ester with respect to catalytic triad residues suggests that cleavage of ester bond in this binding conformation is not plausible. Moreover, the phenyl part of 2,6-dichloroindophenol acetate stacks with Trp86 via π–π interactions similar to donepezil. Such binding mechanism is probably cause of 2,6-dichloroindophenol acetate mild inhibitory properties and may contribute to slower hydrolysis velocity. Localization of 2,6-dichloroindophenol acetate in active site of AChE can be learned from Figure 2.

When tested acetylthiocholine as a substrate (Figure 3), $K_m = 0.18$ mM and $V_{max} = 5.1$ nkat were found. The constant were reached when concentration of acetylthiocholine up to 1 mM taken into consideration. Acetylthiocholine caused inhibition of AChE when presented in excessive concentration. The fact is known from quoted papers[19,20]. In this experiment with acetylthiocholine, inhibition of AChE by excess of substrate was perceived when it reached concentration 5 mM. The finding is not surprising because it is known from literature[21–23]. The K_m

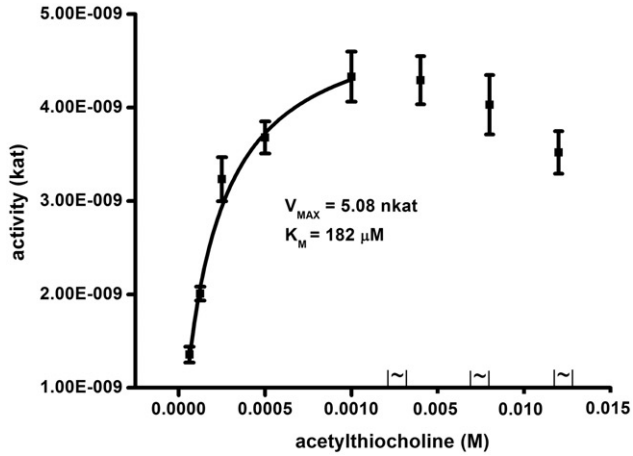

Figure 3. Saturation for human AChE and acetylthiocholine as a substrate. Error bars indicate standard deviations for $n = 5$.

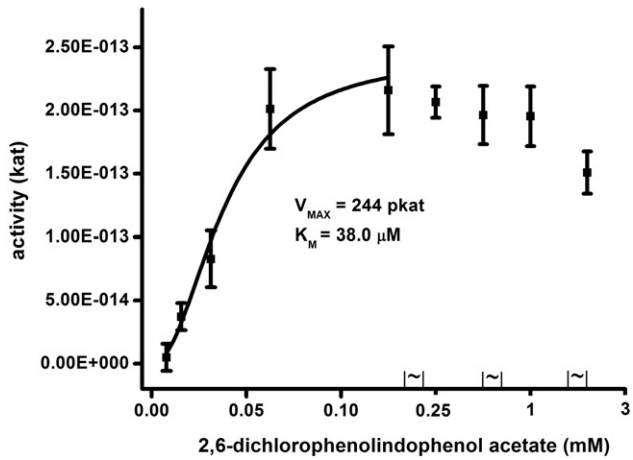

Figure 4. Saturation for human AChE and 2,6-dichlorophenolindophenol acetate as a substrate. Error bars indicate standard deviations for $n = 5$.

and V_{max} values are in a good correspondence with literature as well[24–26].

Saturation curve for 2,6-dichlorophenolindophenol acetate is depicted as Figure 4. 2,6-dichlorophenolindophenol acetate caused inhibition by excess of substrate like the acetylthiocholine do. The inhibition was obvious when 2,6-dichlorophenolindophenol acetate was presented in concentration 0.25 mM or higher. When concentration of 2,6-dichlorophenolindophenol acetate up to 0.13 mM was taken into consideration, K_m 38.0 μM and V_{max} 244 pkat were found. The found values and the finding about inhibition by substrate are original one. On the other hand, the fact that 2,6-dichlorophenolindophenol acetate can serve as a chromogenic substrate was mentioned by other scientist as well[27].

We tested interference for the assay as well. First, compounds typically interfering in Ellman's method: reduced glutathione and albumin were chosen and interference was assessed. The substances did not cause any significant alteration in absorbance in a 100-μl solution taken in a cuvette in concentration 5 g/l for albumin and 5 μM reduced glutathione. The values covered expected range in a plasma sample[28–30]. Principle of the reaction is depicted as Figure 5. It should be emphasized that the assay was developed for physiological pH. In acidic pH, phenolic moiety in 2,6-dichlorophenolindophenol is not dissociated and coloration is different[27]. Ascorbic acid can act as another interfering compound

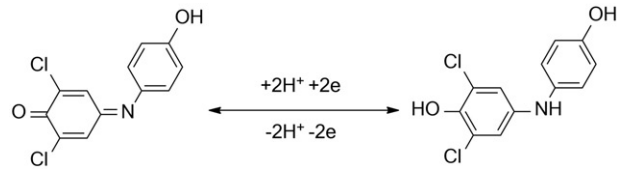

Figure 5. Principle of redox change of 2,6-dichlorphenoindophenol color: blue color (form in the left part) is changed to colorless form because of reduction by, for example, ascorbic acid.

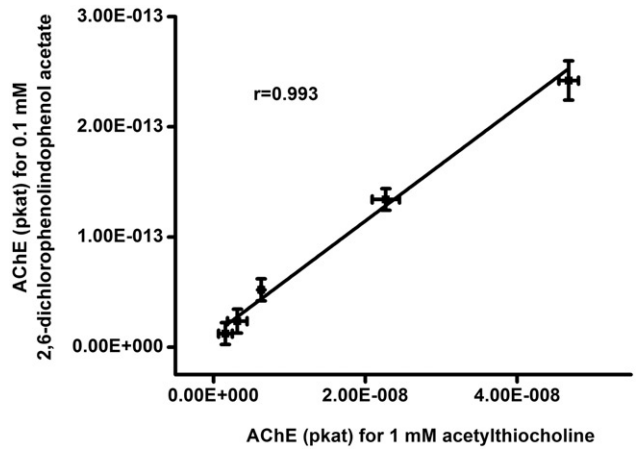

Figure 6. Correlation of AChE activity examination using 2,6-dichlorophenolindophenol acetate and Ellman's method. Error bars indicate standard deviations for $n = 5$.

because it can react with created 2,6-dichlorophenolindophenol. In the reaction, 2,6-dichlorophenolindophenol is reduced to colorless form[31]. Concentration of ascorbic acid in human plasma up to level 100 μM can be expected[32]. A 100-μl sized sample containing just ascorbic acid 100 μM was chosen for the reason. No significant interference was found when the sample was analyzed, which can be a result of the fact that pH of the reaction mixture was slightly alkaline (7.4).

When compared with acetylcholine and 2,6-dichlorophenolindophenol acetate as substrates for AChE activity measurement, some pros and cons were discovered. While the standard Ellman's method can be easily done because of high turnover rate, 2,6-dichlorophenolindophenol acetate has much lower turnover rate. In this experiment, the assay lasted 1 h which is much more than for the Ellman's method and it represent some disadvantage. On the other hand, assay based on 2,6-dichlorophenolindophenol acetate is less sensitive to interferences and the color change is more contrasts (intensive blue) when compared to the Ellman's method (light yellow). Because of higher wavelength, assay based on 2,6-dichlorophenolindophenol acetate does not overlap with compounds such as hemoglobin. It is similar to Ellman's assay, where absorbance peak has similar position like peak of hemoglobin[10]. The both methods correlates well one to each other as can be learned from Figure 6. In this measurement, samples of AChE were assayed by the both methods and correlation coefficient $r = 0.993$ was found.

When compared with 2,6-dichlorophenolindophenol acetate, the product of hydrolysis, 2,6-dichlorophenolindophenol, is used as an redox indicator. It can be, for example, used for determining antioxidant capacity[33] or study of electron transfer during metabolism[34]. Hence, interference in this way can be expected. However, it did not arise in this experiment probably because of the 2,6-dichlorophenolindophenol low concentration.

Conclusions

In this article, 2,6-dichlorophenolindophenol acetate was performed as an chromogenic substrate for AChE and compared to the standard Ellman's method. The finding presented here encourage authors to claim that 2,6-dichlorophenolindophenol acetate is suitable for routine assay where AChE activity should be performed. Assay based on 2,6-dichlorophenolindophenol acetate is not sensitive to interferences under the conditions tested. Moreover, the assay uses long wavelength for absorbance measurement, hence typical interferents such as hemoglobin do not overlap with the assay comparing to the Ellman's method. Lower turnover rate when compared to acetylthiocholine is major disadvantage of the method and it is necessary to plan long time intervals for coloration arising.

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Declaration of interest

The authors declare no conflicts of interests. The authors alone are responsible for the content and writing of this article.

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