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RESEARCH OPINIONS IN ANIMAL & VETERINARY SCIENCES

Comparison of an Alzheimer disease drug ability to bind acetylcholinesterase using both electrochemical and spectrophotometric assays

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Abstract

Acetylcholinesterase (AChE) is an enzyme playing important role in termination of neurotransmission within cholinergic system. The enzyme is targeted by many compounds including nerve agents used for chemical warfare, natural toxins and drugs for Alzheimer disease. Thousands of new inhibitors are prepared for pharmaceutical purposes over the year and necessity to characterize them is given for this reason. In this work, standard spectrophotometric test is compared to electrochemical test in order to assay AChE activity and measure its inhibition. The both test were performed for characterization of an Alzheimer disease drug galantamine. The spectrophotometric test was based on standard Ellman´s reaction. Electrochemical test used enzymatic hydrolysis to acetylthiocholine with consequent electrochemical oxidation. In the experiments, we proved that electrochemical test was able to reach better limit of detection for galantamine than the standard spectrophotometric test. Calculated median inhibitory concentration was same for electrochemical and spectrophotometric tests. Interference in the assay was tested as well. We proved that electrochemical test is reliable and more sensitive than the standard spectrophotometric test. The electrochemical test would be preferred when median inhibitory concentration is the assayed output.

Keywords: acetylcholinesterase; Alzheimer disease; myasthenia gravis; huperzine; galantamine; donepezil; biosensor

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Introduction

Enzyme acetylcholinesterase (AChE; E.C. 3.1.1.7) is involved in the regulation of cholinergic nerves where it terminates neurotransmission by hydrolysis of neurotransmitter acetylcholine (Wessler and Kirkpatrick, 2008; Pohanka, 2012a). The enzyme has a wide pharmacological and toxicological significance. Many compounds including nerve agents used as chemical warfare (sarin, soman, tabun, VX), secondary metabolites such as caffeine or aflatoxins, some drugs for Alzheimer disease (donepezil, rivastigmine, galantamine, huperzine), and drugs for myasthenia gravis (neostigmine, pyridostigmine) can be exampled as inhibitors of AChE (Holzgrabe et al., 2007; Pohanka, 2011, 2012b; da Silva et al., 2011; de los Rios, 2012).

Necessity to analyze newly prepared drugs is given in pharmacological research. In the drug selection, AChE activity is analyzed in the presence of the tested compound and the most potent inhibitors are given to the next stages of preclinical tests. Currently, use of acetylthiocholine as a substrate and 5,5´-dithiobis-(2-nitrobenzoic acid) (further abbreviated as DTNB) as a chromogen are used for spectrophotometric assay of

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AChE activity (Pohanka 2013c). The reaction is called as Ellman´s reaction in honour to team of doctor Ellman who firstly suggested the application (Ellman et al., 1961; Gorun et al., 1978; George and Abernethy, 1983; Runion et al., 1984). Though the assay is simple, sensitive and readily to use in the both laboratory and field conditions, there are some drawbacks. Beside instability of DTNB in water conditions, interference of nucleophilic substances including thiol, oxime and the other chemical groups can be expected (Pohanka, 2012b, 2013b; Pohanka et al., 2013; Pohanka et al., 2012).

In the present paper, the test based on electrochemical evaluation of AChE activity assay is proposed as an alternative to the standard spectrophotometric assay. It is believed that the new test can avoid the drawbacks typical for Ellman´s reaction. For the experiment purposes, standard drug galantamine, compound with proper chemical name (4a*S*,6*R*,8a*S*)-5,6,9,10,11,12- hexahydro- 3-methoxy- 11-methyl- 4a*H*-[1] benzofuro[3a,3,2-*ef*] [2] benzazepin- 6-ol, for Alzheimer disease was chosen as compound inhibiting AChE and being measured by the standard test.

Materials and Methods

Experimental Section

Spectrophotometric assay

The spectrophotometric assay was based on the aforementioned application of acetylthiocholine chloride and DTNB as a chromogenic substance. Principle of the assay is depicted in Figure 3. For the assay purposes, lyophilized human recombinant AChE expressed in HEK293 cells with activity above 1,500 U per milligram of protein was purchased from Sigma Aldrich (Saint Louis, Missouri, USA). AChE was solved in phosphate buffered saline (PBS) and activity was adjusted up 0.5 U/µl for 1 mmol/l acetylthiocholine chloride. Reaction was performed using standard disposable PS cuvettes. The following reagents were subsequently given into the cuvette: 100 µl of the tested solution in PBS, 200 µl of 0.4 mg/ml DTNB in PBS, 590 µl of PBS and the solution was shaken and let to incubate for 10 minutes. After that, 100 µl of 10 mmol/l acetylthiocholine chloride was given into the cuvette, shaken and absorbance was measured at 412 nm immediately after splitting and then after two minutes. Mixture with acetylthiocholine replaced by pure PBS was used as a blank. Control measurement with sample replaced by PBS was done as well. Galantamine was used as a standard compound inhibiting AChE. The all aforementioned reagents and standards used for the assay purpose were achieved from Sigma-Aldrich.

Electrochemical assay

Electrochemical assay was done in similar conditions like the spectrophotometric one. Scheme of the assay is depicted as figure 4. Screen printed sensors were purchased

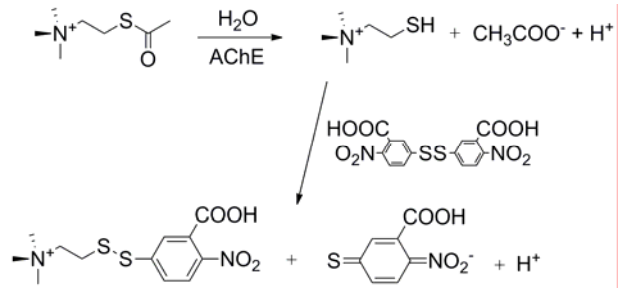

Fig. 1: Principle of assay based on Ellman´s reaction. The first step is catalyzed by acetylcholinesterase (AChE).

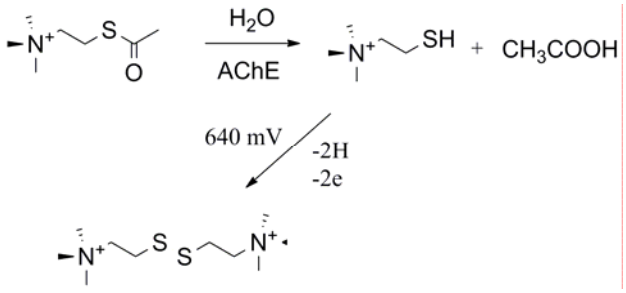

Fig. 2: Principle of electrochemical assay of acetylcholinesterase (AChE) activity.

from BVT Technologies (Brno, Czech Republic). The sensors were sized 25.4×7.3×0.6 mm and they consisted from platinum working (dot shaped with diameter 1 mm), silver covered with silver chloride reference, and platinum auxiliary electrodes. Reagents used for the electrochemical assay were the same like the used in spectrophotometric assay.

Prior to the assay, sensor was linked with PalmSens device (Palm Sens BV, Houten, Netherlands), inserted into a disposable plastic cuvette with maximal volume 2 ml and operated by a computer using PsLite 1.8 (PalmSens BV) software. Into the cuvette, the following reagents were added: 100 µl of the tested solution in PBS, 790 µl of PBS and the solution was shaken and let to incubate for 10 minutes. After that, 100 µl of 10 mmol/l acetylthiocholine chloride was injected. The mixture was shaken and current was measured chronoamperometrically after two minutes. Applied potential 640 mV was given in order to receive oxidation of the released thiocholine. The potential was chosen using experiences from the former experiment (Pohanka et al., 2013). In control measurement, sample was replaced by PBS.

Data processing and statistics

All samples were assayed in pentaplicate and the both mean and standard deviation were calculated for each group of measurements. From the experimental data, percent of inhibition I was calculated using following equation 1.

$$I = \left(\frac{S_C - S_I}{S_C} \right) \times 100 \quad \text{(Equation 1)}$$

Where S_C is a signal received by either spectrophotometric (absorbance) or electrochemical (current) assay for the control measurement. S_I is a signal found for assay of the tested compound in given concentration.

Software Origin 8 (OriginLab Corporation, Northampton, MA, USA) was used for data processing and statistics evaluation. Limit of detection was calculated from confidence interval (95 %) in calibration plot.

Results and Discussion

Galantamine was chosen as a representative compound used for Alzheimer disease therapy. It is well tolerated by patients and it has good efficacy in the therapy (Bond et al., 2012). It was discovered by Mashkovsky and Kruglikova-Lvova as a secondary metabolite in Caucasian snowdrops *Galanthus* sp. in early 1950s (Heinrich and Teoh, 2004). Currently, galantamine is sold over the world under trade names Razadyne™, Razadyne™ER, Reminyl™ER, and Reminyl®, and it remains as one of the most relevant compounds used for the therapy.

Calibration for galantamine was done by the both spectrophotometry and electrochemistry in concentration range 10^{-9} – 10^{-2} mol/l (final concentration in the cuvette). PBS was used for control measurement. Calibration is depicted as figure 1 for the spectrophotometry and figure 2 for the electrochemistry. When searched limit of detection, electrochemical assay was able to detect as low as 5×10^{-9} mol/l of galantamine. Comparing to it, spectrophotometric assay have limit of detection 2×10^{-9} mol/l for galantamine. When comparing the two limits of detection, the electrochemical method was able to detect 3.7 times lower amount of galantamine. However, the lower limit of detection does not privilege any method if accuracy to estimate median inhibitory concentration is not reached.

For the above mentioned reason, the both methods were fitted by Boltzmann function and median inhibitory concentration was calculated. The median inhibitory concentration for galantamine was 2.19×10^{-6} mol/l when used spectrophotometry and 2.09×10^{-6} mol/l when used electrochemistry. The data are insignificant one to each other when used analysis of variance and probability level 0.05. The two methods were correlated one to each other. Coefficient of determination R^2 was 0.997. Slope for the correlation was 1.04. Considering the calculated median inhibitory concentration, we can claim that the electrochemical assay is reliable and very close in the results to spectrophotometry.

The reached limits of detection are quite good for characterization of drugs. On the other hand, AChE was not immobilized for the assay purposes. Decision not to use immobilization was initiated by decision to establish method close to standard spectrophotometric test. Immobilization of AChE would lead to preparation of a biosensor and further improvement to limits of detection. However, comparison of such experimental data and data from spectrophotometric test would be complicated. Limits of detection known from literature about AChE based biosensors are slightly lower than the here described device. In an example, AChE based electrochemical biosensor using Co phtalocyanine and carbon electrodes was used for assay of organophosphate pesticides with limit of detection 2 ppb for malaoxon (Ivanov et al., 2011). In another paper, biosensor containing AChE immobilized on carbon nanotubes was used to detect chosen pesticides and nerve agents (Liu and Lin, 2006). Limit of detect for organophosphate paraoxon was 0.4 pmol/l.

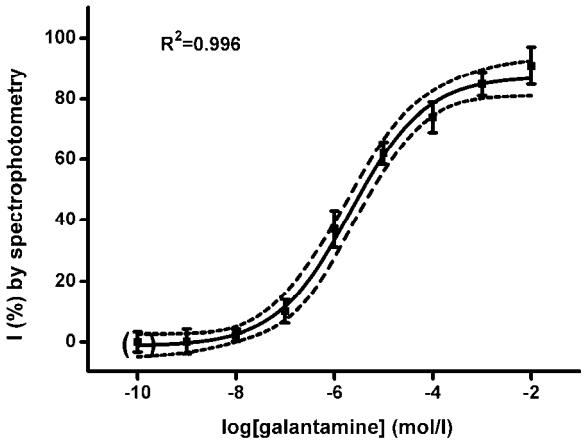

Fig. 3: Spectrophotometric assay of galantamine. Error bars indicate standard deviation for n=5. The data were fitted (full line) and confidence interval for probability level 0.05

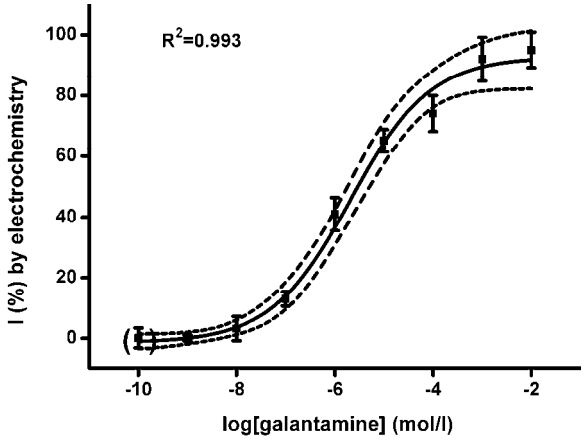

Fig. 4: Electrochemical assay of galantamine. Description is the same as in figure 1.

Table 1: Interference in the assays in percent of control signal

	Spectrophotometry (%)	Electrochemistry (%)
Acetone oxime	+109	not detected
Cysteine	+112	+104
Ethanol	-35.5	-36.7

Interference was measured using acetone oxime, cysteine, and ethanol. The interference is resumed in table 1. The compounds were given into the cuvette up to final concentration 0.1 mmol/l for acetone oxime and cysteine. Ethanol was added up to concentration 10% (*v*/*v*). For purpose of this part of experiment, volume of applied PBS described above was decreased for 100 µl and the tested compounds were applied just in volume 100 µl.

As seen in the table, cysteine was oxidized by applied voltage and caused interference more than 100 % when applied final concentration 0.1 mmol/l in the electrochemical assay. The same interference was found in spectrophotometric assay. The fact that free thiol can interfere is not surprising, because it is well known for Ellman´s reaction (Prokofieva et al., 2012). It is frequently used for the both electrochemical and spectrophotometric assay of thiol containing antioxidants (Pohanka, 2012c; Tipple and Rogers, 2012; Squellerio et al., 2012). Interference of an oxime group containing compound is not surprising as well. This kind of compounds causes oximolysis. The oximolysis leads to spontaneous reaction with DTNB (Worek et al., 2012; Pohanka, 2012a, 2013a). Electrochemical assay is not sensitive to the interference because oxime is not redox active during the measurement. Ethanol influences the assay in course of AChE inhibition. The inhibitory effect is not specific and it can be anticipated for other organic solvents as well (Turdean and Turdean, 2008; Pietsch et al., 2009). Ethanol is a typical example of such solvents (Fekonja et al., 2007). When considered the mentioned results. We can claim that the main advantage of the electrochemical assay lays in ability to avoid interference by some nucleophilic substances. On the other hand, compounds inhibiting AChE and free thiol containing compounds will cause the same interference in the spectrophotometrical like in the electrochemical assay.

Conclusions

Electrochemical assay was introduced as a tool for a fast and reliable testing of drugs inhibiting enzyme AChE. The new assay was found to be more sensitive than the traditional spectrophotometric test. Moreover, the electrochemical assay is less sensitive to interference caused by nucleophilic substances. On the other hand, electrochemical assay is not advantageous if there is a demand to calculate enzyme activity in katals. Electrochemical assay should be privileged above the spectrophotometric test when median inhibitory concentration is an outputting value.

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