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Voltammetric assay of butyrylcholinesterase in plasma samples and its comparison to the standard spectrophotometric test

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ABSTRACT

Butyrylcholinesterase (BChE) is an enzyme abundantly constituted in the livers and released into blood where it is soluble. It may be found in the both plasma and serum. BChE can serve as a biochemical marker. BChE activity is typically measured by spectrophotometric Ellman's method. In the present work, voltammetric assay of cholinesterasemia is proposed as a simple and reliable method. In the experiments described here, limits of detections 4.57 pkat for the spectrofotometric test and 1.14 pkat for the voltammetric assay were determined. Interference caused by acetylcholinesterase (AChE) and organic solvents was characterized and counter measurement to the AChE caused interference was proposed. Finally, the both methods were correlated one to each other using mouse plasma spiked with carbofuran resulting in a promising coefficient of determination. In a conclusion, the voltammetric assay seems to be reliable and suitable for routine performance.

1. Introduction

In the present, two cholinesterases are known in the human body. Acetylcholinesterase (AChE; EC 3.1.1.7.) is an enzyme hydrolyzing neurotransmitter acetylcholine and butyrylcholinestrase (BChE; EC 3.1.1.8.), which is an enzyme without known natural substrate [1–3]. AChE is localized in central nervous system and peripheral nerves including the sympathetic nerves, parasympathetic nerves and neuro-muscular junctions. The aforementioned complex can be named as cholinergic nervous system. Neurotransmitter acetylcholine initiates response by interaction with muscarinic and nicotinic acetylcholine receptors and AChE terminates the neurotransmission [4,5]. In the blood, AChE is located on erythrocytes where it hydrolyzes acetylcholine released from nerve terminations into the blood stream. Comparing to AChE, BChE is dominantly constituted in the livers and released into blood where it remains solved in blood plasma [2,6,7]. In some works, AChE was called as a blood chlolinesterase while BChE as a plasma or serum cholinesterase.

BChE can serve as an outstanding biochemical marker suitable for diagnosis of some pathological processes in the body. Firstly, the enzyme is dominantly constituted in the livers so plasmatic level of BChE can be used as a liver function test [8,9]. Decreased level of BChE in the plasma (hypocholinesterasemia) can indicate liver pathologies such as chronic liver diseases, acute hepatitis or liver cirrhosis [10,11]. Diagnosed hypocholinesterasemia well responds to the other liver markers such as albumin and transaminases [10–12]. In the liver pathologies, BChE level in plasma is affected without any change in AChE activity. Hypocholinesterasemia combined with decrease of blood AChE activity can diagnose decrease poisoning with an inhibitor of cholinesterases including nerve agents such as sarin or VX or pesticides such as carbofuran [2,3,13]. Abundant activity of BChE in plasma, hypercholinesterasemia, can be diagnosed as well. This finding is quite rare and it can appoint at hepatocellular carcinoma [14].

Several protocols were proposed for assay of cholinesterases in the past. Use of acetylcholine or butyrylcholine is the easiest way how to assay the activity. There can be either assayed the released acid using pH metry or oxidized choline into betaine using choline oxidase [15–18]. Though the methods provide good results in biosensors comprised from cholinesterase, they have limitation to be used for BChE activity assay in biological samples [9,19]. Both AChE and BChE can be assayed using Ellman's method. The method is based on two steps. Acetylthiocholine (for AChE assay) or butyrylthiocholine (for BChE assay) is converted by the enzymes into the acid and thiocholine. In the next, spontaneous, step, thiocholine reacts with 5,5′-dithiobis-(2-nitrobenzoic) acid providing yellow 5-thio-2-nitrobenzoic acid [20–22]. The reaction have some drawbacks including poor stability of the

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5,5′-dithiobis-(2-nitrobenzoic) acid. Hemoglobin has similar absorption maximum like the 5-thio-2-nitrobenzoic acid which is another disadvantage as well. Voltammetry is another option for assay of cholinesterases activity. Voltammetric methods based on acetylthiocholine or butyrylthiocholine are quite common for biosensors construction [23,24]. However, no extensive effort to use voltammetry for BChE activity measurement in biological samples has been made. In the present work, voltammetric method is performed as a tool for a fast and reliable assay of BChE activity and critical comparison to the standard Ellman's method is done.

2. Material and methods

2.1. Chemicals

Human recombinant BChE (expressed in goat, enzyme activity ≥ 500 U/mg of protein), human recombinant AChE (expressed in HEK 293 cells, lyophilized powder ≥ 1500 U/mg of protein), (−) huperzine A, carbofuran, 5,5′-dithiobis-(2-nitrobenzoic) acid, butyrylthiocholine chloride and acetylthiocholine chloride were received from Sigma-Aldrich (Saint Louis, Missouri, USA). Phosphate buffered saline pH 7.4 (Penta-Chemicals, Prague, Czech Republic) was used for solving of BChE, AChE, 5,5′-dithiobis-(2-nitrobenzoic) acid, acetylthiocholine chloride, butyrylthiocholine chloride and huperzine A. Carbofuran was dissolved in isopropanol (Penta-Chemicals). Ethanol and dimethyl sulfoxide were received also from Penta-Chemicals.

2.2. Plasma samples

In a total 9 female laboratory mice BALB/c (Velaz, Unetice, Czech Republic) were chosen for plasma collection. The mice weighted 20 ± 1 g and they were 2 months old in the experiment beginning. The mice were kept in an animal house with stable temperature 22 ± 2 °C, humidity 50 ± 10% and light/dark period each 12 h. For the whole time of animals keeping, there was no obstacle in access to food and water. The experiment was approved and supervised by the ethical committee at Faculty of Military Health Sciences (Hradec Kralove, Czech Republic). The sacrifice was done using CO_2 anesthesia for at least 1 min prior to decapitation and blood collection into tubes with lithium heparin (Dialab, Prague, Czech Republic). The freshly collected blood was centrifuged at 1000*g* for 5 min and plasma was separated and kept frozen at −80 °C until the laboratory examination.

2.3. Spectrophotometry using Ellman's method

The standard spectrophotometric test was based on the aforementioned Ellman's method. In the assay, phosphate buffered saline was used for solving of BChE and the suspension was adjusted up final activity 10^{-13}–10^{-8} kat/μl for 1 mmol/l butyrylthiocholine chloride. Standard disposable PS cuvettes with volume 1 ml or standard 96 well microplates were used for the assay. Absorbance was measured using standard spectrophotometer or microplate reader adjusted up wavelength 412 nm. Cuvette was consequently filled with: 400 μl of 5,5′-dithiobis-(2-nitrobenzoic) acid 1 mmol/l, 100 μl of BChE solution, 100 μl of tested compound or phosphate buffered saline and 300 μl of phosphate buffered saline. Reaction was started by adding 100 μl of 10 mmol/l butyrylthiocholine and absorbance was measured after 15 s and then after 315 s. Difference of the absorbances was used further. If enzyme activity calculated, extinction coefficient $\varepsilon = 14{,}150\ \mathrm{l} \times \mathrm{mol}^{-1} \times \mathrm{cm}^{-1}$ was considered. The coefficient was taken from literature for standard ambivalent temperature and pressure conditions and pH 7.4 [25]. Identical protocol was made for the microplates where difference of absorbances was used as an outputting value. Assay of AChE was performed in the same way like assay of BChE; however, butyrylthiocholine in the reaction was displaced using acetylthiocholine.

2.4. Electrochemistry

Screen printed sensors sized 25.4 × 7.3 × 0.6 mm^3 were bought from BVT Technologies (Brno, Czech Republic). Three integrated electrodes including platinum working (circle shaped, 1 mm diameter), silver covered with silver chloride reference and platinum auxiliary (the both ring shaped) were present in the sensor. The sensor was washed by ethanol prior to use and let to dry. It was used as a disposable device and ever experimental point was made by a new sensor. The sensor was linked to PalmSens analyzer (Palm Sens BV, Houten, Netherlands) and fixed into a disposable microtube with maximal volume 1500 μl. Assay was controlled from a computer using PsLite 1.8 (PalmSens BV) software. The microtube was consequently filled with 700 μl of phosphate buffered saline, 100 μl of BChE solution, 100 μl of tested compound or phosphate buffered saline and square wave voltammetry was done in order to oxidize potentially interfering compounds. Reaction catalyzed by the enzyme was started by adding 100 μl of 10 mmol/l butyrylthiocholine and square wave was measured after an incubation lasting 5 min. Conditions for the voltammetry were following: range 0–1.1 V, voltage alteration 1 Hz, amplitude 0.01 V. Step of scanning was 0.005 V.

2.5. Statistics

The assays were five times repeated in order to calculate confidence intervals and standard deviations. Software Origin 8 (OriginLab Corporation, Northampton, MA, USA) was used for calibration curves construction, confidence intervals estimation and significance testing. The calibration curves were fitted by Hill equation. In the equation, coefficient of cooperativity was adjusted to be equal to number one. Square of peaks and their position was calculated using aforementioned PsLite 1.8. Limit of detection was calculated as signal to noise equal to three ($S/N = 3$). Significance of difference between two sets of measurements was calculated using analysis of variance (ANOVA) tests on the both P 0.05 and P 0.01 levels. The enzyme activities are expressed in katals (kat) which respond to mols per second.

3. Results and discussion

In the first experiment, optimal concentration of butyrylthiocholine was chosen. For the purpose, saturation curve was done (Fig. 1). Calculated maximal velocity of reaction V_{max} was 270 nkat and Michaelis constant K_M was equal to 126 μmol/l. The found Michaelis constant well corresponds with the constants found in literature for butyrythiocholine. E.g. BChE from rats *Rattus novergicus* had K_M 0.094–0.134 mmol/l [26], human BChE was reported to have K_M 0.1 mmol/l [27,28], and BChE from squid *Berryteuthis magister* has K_M 0.15 mmol/l [29]. In this experiment, stock concentration of butyrylthiocholine was chosen to be 10 mmol/l. It means that the final concentration of butyrylthiocholine in the cuvette was 1 mmol/l. The chosen concentration was approximately eight times higher than value of Michaelis constant. The excess of substrate was high enough to prevent from substrate limitation in the assay.

Time of incubation was the second parameter necessary for the assay. Here, 300 s lasting interval of incubation was chosen. The time interval cannot be optimized as a simple physical condition. Enlarging of the time would improve the limit of