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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

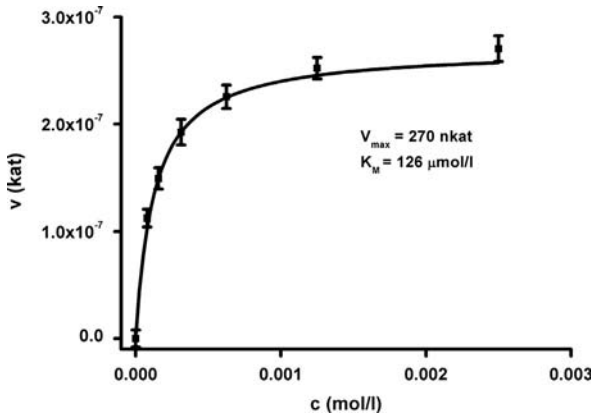

Fig. 1. Saturation curve for human BChE and butyrylthiocholine as a substrate. Error bars indicates standard deviation for $n=5$.

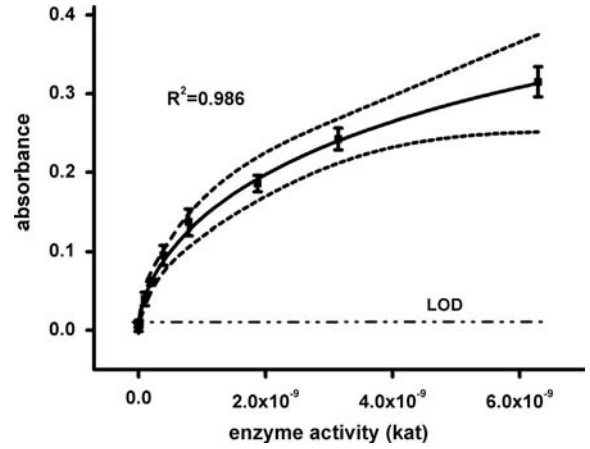

Fig. 2. Calibration curve for human BChE as a marker using spectrophotometry. Error bars indicates standard deviation for $n=5$. Confidence intervals at probability level $P=0.05$ is depicted by dashed lines. Limit of detection (LOD) level is shown using dash – dot line.

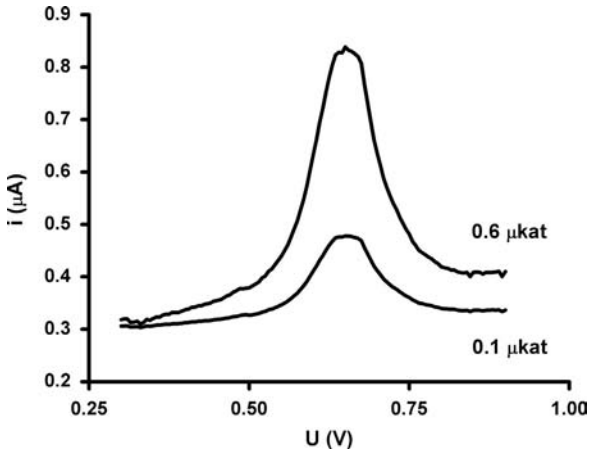

Fig. 3. Example of real curves from square wave voltammetry assay. Assayed activity of BChE is written beside the curve. Curves from assay of two activities were chosen.

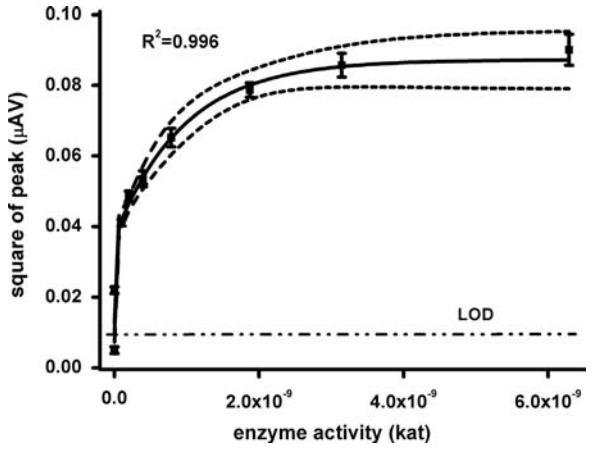

Fig. 4. Calibration curve for human BChE as a marker using electrochemistry. Error bars indicates standard deviation for $n=5$. Confidence intervals at probability level $P=0.05$ is depicted by dashed lines. Limit of detection (LOD) level is shown using dash – dot line.

detections. On the other hand, economical aspects and needs to operate a large number of samples have to be considered. Shortening of the time has its drawback as well. Beside worsening of limit of detection, error caused manipulation with samples and time measuring can take plece in the assay. The chosen time interval seems to be optimal in this point of view.

Spectrophotometric assay of BChE activity was done using a multichannel spectrophotometer. Calibration plot is depicted as Fig. 2. Limit of detection was 4.57 pkat and coefficient of determination for the assay was 0.986. Suitability of the Ellman's method for assay of cholinesterases is not surprising. In the past, the method was successfully used in assays where either AChE or BChE took place [30–33].

In the electrochemical assay, square wave voltammetry was chosen as a suitable method for the determining of thiocholine accumulation during the reaction. The method is the sensitive and selective and unwanted contribution of capacitative charging of the electrodes is minimized due to changing potential [34,35]. Calibration using electrochemical sensor was done using the same calibration scale of BChE as in the case of spectrophotometric assay. Real curves are depicted in Fig. 3. Peak of thiocholine oxidation was observed at 651 ± 45 mV. Position of the peak is not surprising because it well correspond with data from literature where electrochemical biosensors using oxidation of thiocholine and platinum working electrodes were described [36–39]. Calibration plot for the voltammetric assay is depicted as Fig. 4. Limit of detection for the assay was 1.14 pkat. Coefficient of determination for the assay was equal to 0.996. When the two calibration plots depicted in Figs. 2 and 4, spectrophotometric and voltammetric, are correlated one to each other, coefficient of determination was equal to 0.970. The finding appoint at fact that the voltammetry provides results comparable to the standard spectrophotometric test. Limits of detection are quite low for the both methods. Limit of detection is slightly better for the voltammetry. It should be emphasized that the limits of detection was low enough to assay activity of BChE in plasma samples. Reference values for BChE in human population is approximately 5.33 nkat/ml for adults and 4.67 nkat/ml for children [40]. In another paper, reference range for BChE activity in plasma was stated to be 131–304 pkat/ml [32] or 150 pkat/ml as a mean of health population [41].

Owing to known mechanism of butyrylthiocholine hydrolysis, no serious interference can be expected [3]. Cholinesterases are sensitive to organic solvents causing depression of their activity [42–44]. The problem is serious if a biosensor with cholinesterase as biorecognition element is constructed. A sample with not known composition would cause inhibition of the enzyme indicating presence of neurotoxic compound but sample consisting from a pure organic solvent would be true as well. Though the organic solvents are not expected to be interferents in biochemical examination of plasma, a test with the organic solvents was made to judge their effect. In the test, 100 μl of either dimethylsulfoxide

or ethanol were used instead 100 μl of phosphate buffered saline and final concentration of organic solvent was 10% (v/v). The both electrochemical assay and spectrophotometry were performed. The reached experimental data are summarized in Table 1. It can be seen that organic solvents cause decrease of assayed signal. There is no significant difference in the inhibition between spectrophotometric and voltammetric assays. This result was expected as the organic solvents do not interfere in the terminal part of the assay reaction but they causes inhibition of BChE only.

BChE has higher affinity toward butyrylthiocholine than AChE has [45,46]. However, AChE can act as an interfering compound because the assayed plasma can be polluted by fragments from erythrocytes. The fragments can contain AChE as well and when AChE becomes solved in the plasma, it cannot be separated by centrifugation like the whole erythrocytes. In this test, addition of AChE into the assayed BChE was done in order to estimate interference caused by AChE. 100 μl of a solution containing 1 nkat BChE (activity expressed for butyrylthiocholine) and either 100 μl of a solution containing 1 nkat AChE (activity expressed for acetylthiocholine) or 100 μl of phosphate buffered saline was used. Huperzine was chosen as a selective inhibitor of AChE [3,47]. It was applied in an amount 100 μl containing 1 nmol/l of huperzine A in phosphate buffered saline. The further conditions of assay including total volume of mixture were identical to conditions described in the experimental part. Experimental data are summoned in Table 2. It can be seen that the applied AChE caused approximately 20% interference in the assay of BChE. The increase of signal when compared the assay of BChE and the assay of BChE with addition of AChE was significant on probability level 0.05. However, the interference can be suppressed by huperzine resulting in not significant interference of AChE. Huperzine does not significantly influence assay of BChE when no AChE is applied. Considering the aforementioned, huperzine can be used as a reagent suppressing interference of AChE without influencing measurement of BChE activity.

In the last experiment, mouse plasma samples were spiked with carbofuran. The application of carbofuran into the plasma samples post mortem rather than poisoning viable animals was chosen in order to precede treating which is painful for the life-forms. Carbofuran was selected as a model compound because of its high toxicity, environmental relevance and the fact that it is still used in some countries while in other misused as illegal pesticide and poison bait [2,48–50]. Pooled mouse plasma was divided into 35 samples and spiked by carbofuran up to final concentration $0–10^{-9}–10^{-8}–10^{-7}–10^{-6}–10^{-5}–10^{-4}$ mol/l. In order to reveal minor changes in detected signal after BChE inhibition by carbofuran, time of BChE incubation with butyrylthiocholine was increased five times comparing to the previous part of experiments. Experimental data are depicted as Fig. 5. The assays well correlated as the coefficient of determination reached 0.996 value. Considering the correlation, the both methods can be mentioned as a reliable assay for determining of BChE in biological samples.

Table 1
Effect of organic solvents on detected signal. Decrease of signal when considered of an assay without the solvent is expressed in relative (percent) scale. Every measurement was made in pentaplicate.

	Voltammetry (percent of inhibition)	Spectrophotometry (percent of inhibition)
Dimethylsulfoxide	23 ± 3%	25 ± 4%
Ethanol	13 ± 3%	12 ± 3%

Table 2
Interference of AChE in the assay of BChE and suppression of the interference by huperzine. Every measurement was made in pentaplicate.

Assayed mixture contains	Voltammetry (square nAV)	Spectrophotometry (absorbance)
BChE	71 ± 5	0.15 ± 0.01
AChE	12 ± 2	0.021 ± 0.008
AChE + BChE	89 ± 6	0.17 ± 0.01
BChE + huperzine	70 ± 3	0.15 ± 0.01
AChE + huperzine	1.3 ± 0.1	0.005 ± 0.003
AChE + BChE + huperzine	72 ± 6	0.15 ± 0.01

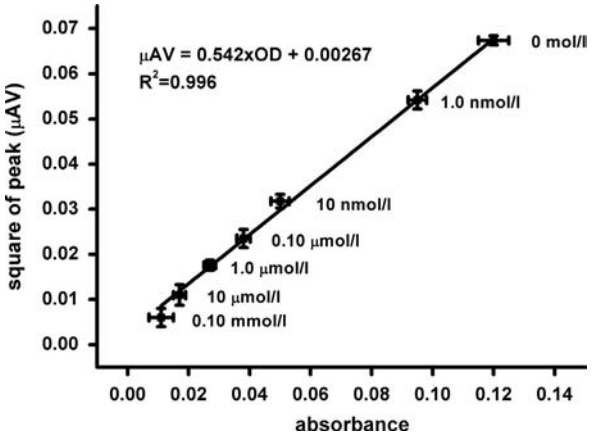

Fig. 5. Correlation of spectrophotometry and voltammetry using plasma samples spiked with carbofuran. Error bars indicates standard deviation for $n=5$.

4. Conclusions

Voltammetry seems to be reliable tool for assay of BChE in biological samples. The assay provides similar parameters like the standard spectrophotometry. However, unstable chromogenic reagent 5,5′-dithiobis-(2-nitrobenzoic) acid is avoided in the voltammetry. That fact represents an advantage. The lower limit of detection is another advantage. Voltammetry is readily to be miniaturized which make the voltammetric test suitable for the next development of a portable device designed. Personal glucometer based on a biosensor with glucoseoxidase known by diabetics can serve as an example how the device would look like. In a practical application, square wave voltammetry can be replaced by a simpler method such as chronoamperometry.

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