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ORIGINAL PAPER

Determination of acetylcholinesterase and butyrylcholinesterase activity without dilution of biological samples

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Two cholinesterases: acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), are known. The enzymes are important in the body and alteration of their activity has significant use in the diagnosis of poisoning, liver function, etc. Currently available methods for the determination of cholinesterases have some major drawbacks including various interferences and the inability to be used for decreasing the enzyme activity in the presence of reversible inhibitors due to sample dilution; hence, a method for dilution free assay of cholinesterases is desired. Here, microplates were modified with indoxylacetate (100 μL of 10 mmol L^{-1} solution) and used for cholinesterases assay after drying at 37 °C. The fact that indoxylacetate remains stable in dry state and serves simultaneously as a chromogen and substrate provide good prerequisites for the method. The limit of detection for BChE was 0.71 U while that for AChE was 2.8 U per a 100 μL sample (solution of enzyme or plasma sample). The limit of detection is low enough to allow standard examination of cholinesterasemia. The two cholinesterases can be distinguished from each other using selective inhibitors such as donepezil and iso-OMPA. The new method was also successfully validated for the standard Ellman's assay using plasma samples with BChE activity adjusted by carbofuran. The new method based on indoxylacetate seems promising for routine tests.

Keywords: acetylcholinesterase, butyrylcholinesterase, cholinesterasemia, liver function test, indoxylacetate, Ellman's assay

Introduction

Acetylcholinesterase (AChE; EC 3.1.1.7) and butyrylcholinesterase (BChE; EC 3.1.1.8) are the two cholinesterases present in the human body. Despite broad similarities between the two enzymes, their biological significance is different. AChE has particular importance in the junction of nerves to affected cells, where it terminates the neurotransmission by the neurotransmitter, acetylcholine, hydrolysis and it can be considered as a physiologically irretrievable enzyme (Pohanka, 2011). Compared to AChE, BChE has no major endogenous substrate hence its biological role is hard to be understood. On the other hand, it does not mean that BChE is completely useless. It is able to degrade some toxic compounds of natural origin as well as drugs such as cocaine and succinylcholine (Duysen & Lockridge, 2011; Lejus et al., 2006; Pohanka, 2015).

Both cholinesterases can serve as a biochemical marker of some pathological processes. AChE is accessible in blood samples and immediate decrease of its activity can be interpreted as poisoning with some neurotoxic inhibitors such as nerve agents, some drugs approved for e.g. Alzheimer disease, myasthenia gravis, and/or pesticides such as carbofuran (Pohanka, 2012a; GhattyVenkataKrishna et al., 2013). BChE has wider diagnostic applications than AChE despite its lower physiological significance. Plasmatic activity of BChE, cholinesterasemia can be used as a liver function test because BChE is synthesized in liver and secreted into blood (Iwasaki et al., 2007; Pohanka, 2013a). BChE assay can be applied for e.g. acute hepatitis or liver cirrhosis diagnosis (Prellwitz et al., 1976; Kemkes-Matthes et al., 1987). Beside the

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diagnostic purposes, cholinesterases can be used in the construction of biosensors for neurotoxic compounds assay.

Disparate protocols for the determination of cholinesterases activity have been presented. In past, simple spectrophotometric tests based on 5,5′-dithiobis-(2-nitrobenzoic) acid as the chromogenic reagent and acetyl- (or butyryl-)thiocholine as the substrate (Ellman et al., 1961; George & Abernethy, 1983; Gorun et al., 1978), potentiometric tests with pH titration (Bazire et al., 2011), and electrochemical sensors and biosensors (Khaled et al., 2010; Pohanka, 2014) have been employed. The mentioned testes are based on an addition of reagents to the sample followed by measurements of the enzyme activity. In case of spectrophotometric tests, the addition of a chromogenic substance and a substrate are necessary. Methods such as pH titration can be applied directly with added substrate. On the other hand, the shift of pH or the necessity to add a neutralizing buffer are evident disadvantages of these methods.

All the described methods have a major drawback: dilution of samples by the used reagents. It can be a problem when cholinesterase activity in e.g. blood or serum is to be measured to diagnose poisoning by a reversible inhibitor. No or only limited decrease of the cholinesterase activity can be determined by the standard methods while the contrary is true. The necessity to use chromogenic compounds and substrates of cholinesterase resulting in spontaneous reactions providing colored products is another disadvantage of the standard methods. In the present paper, a new method suitable for the determination of cholinesterases activity in biological samples is established and validated. The new method is expected to be reliable and available enough to be applicable in diagnostic tests including diagnosis of overdosing by e.g. Alzheimer disease drugs huperzine, donepezil, and galantamine. The presented method is designed to be performed by a simple addition of the sample with no necessity of its pretreatment or addition of other reagents.

Experimental

In the experiment, human recombinant AChE (from HEK 293 cells, obtained as lyophilized powder $\geq$ 1500 U per mg of protein) and human recombinant BChE (expressed in goat, obtained as lyophilized powder with the enzyme activity $\geq$ 500 U per mg of protein) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Phosphate buffered saline pH 7.4 and carbofuran in analytical purity were bought from Sigma–Aldrich as well. Acetylthiocholine chloride, butyrylthiocholine chloride, 5,5′-dithiobis-(2-nitrobenzoic) acid, indoxylacetate, bovine serum albumin, ascorbic acid, donepezil, and tetraisopropyl pyrophosphoramide (iso-OMPA) were received from Litolab (Chudobin, Czech Republic). Ethanol and dimethyl sulfoxide were purchased from Penta-Chemicals (Prague, Czech Republic). Deionized water was prepared by an Aqua Osmotic system (Aquaosmotic, Tisnov, Czech Republic).

Mice BALB/c (Velaz, Unetice, Czech Republic) were used as the source of plasma samples. A group of 12 female mice were used for the purpose. The mice weighted (19 $\pm$ 1) g and they were two months old on the day of euthanasia. Prior to euthanasia, the mice were kept in standard conditions recommended for small rodents (temperature of (22 $\pm$ 2) °C, humidity of (50 $\pm$ 10) %, light/dark period each of 12 h, no limitations in access to chow and water). The experiment was approved as well as supervised by the ethical committee (Faculty of Military Health Sciences, University of Defence, Hradec Kralove, Czech Republic).

The mice were sacrificed under carbon dioxide anesthesia by cutting the jugular vein. Blood was collected into tubes with lithium heparin (Dialab, Prague, Czech Republic) and centrifuged at 1000g for 5 min. Finally, plasma was isolated by a micropipette.

Spectrophotometry was performed for AChE as well as for BChE solutions in phosphate buffered saline with pH 7.4. Standard disposable PS cuvettes with the optical length of 0.01 m and spectrophotometer adjusted to the wavelength of 412 nm were used. The cuvettes were filled with the following reagents:

i) 400 μL of 1 mmol L^{-1} 5,5′-dithiobis-(2-nitrobenzoic) acid;

ii) 100 μL of enzyme solution or plasma;

iii) 400 μL of phosphate buffered saline with pH 7.4;

iv) 100 μL of 10 mmol L^{-1} butyrylthiocholine chloride in case of the BChE assay or 10 mmol L^{-1} acetylcholine chloride for the AChE assay.

After the final substance addition, the absorbance was measured immediately and then after 5 min. Enzyme activity was calculated using the extinction coefficient $\varepsilon = 14.150$ L mol^{-1} cm^{-1} for pH 7.4 (Eyer et al., 2003).

Indoxylacetate was dissolved in ethanol or dimethyl sulfoxide up to the concentration of 10 mmol L^{-1} and 100 μL of the solution were injected per one well of a 96 well-microplate. The plate was left to dry for at least 12 h. In the total, 100 μL of the sample were applied per one well and the absorbance at 670 nm was measured immediately and then after 3 h. The wavelength was chosen as optimal in compliance with the referred works (Villatte et al., 2001; Pohanka, 2012b; de Melo et al., 2006). The principle of the indoxylacetate based reaction is depicted in Fig. 1.

All measurements were made in five replicates and the mean as well as the standard deviation were calculated. The saturation curve was constructed by non-linear curve fitting using the Michaelis–Menten equation and the data were processed in Origin 9.1 (OriginLab Corporation, Northampton, MA, USA). Enzyme activity was expressed in U where 1 U re-

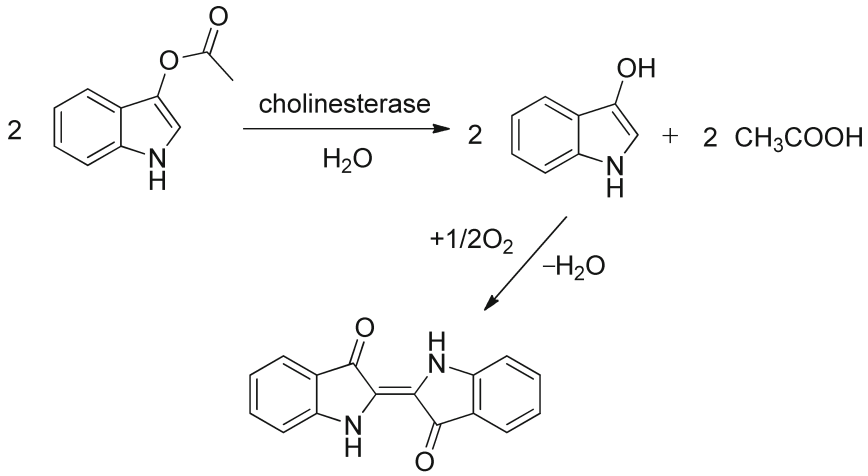

Fig. 1. Principle of the reaction based on the indoxylacetate hydrolysis.

sponds to 1 μmol of acetylthiocholine (butyrylthiocholine in BChE case) converted per 1 min in the given solution. Signal vs. noise equal to three criterion ($S/N = 3$) was applied as the limit of detection calculation.

Results and discussion

The microplates were prepared as described in the Experimental. A solution of indoxylacetate in ethanol and/or dimethyl sulfoxide was used. Microplates were dried in either laboratory conditions or in an incubatory box adjusted to 37 °C. The experiment was aimed at the selection of an optimal protocol to get a thin and homogenous layer of indoxylacetate. Dispersion of the obtained values was assessed. The use of ethanol as a solvent and drying in an incubatory box provided the best results. While the combination of ethanol and drying at 37 °C provided optical density of wells in quite a narrow interval of values: 0.033 ± 0.006, lower temperature resulted in a broader interval: 0.034 ± 0.011. Results obtained when using dimethyl sulfoxide were worse compared to those when using ethanol: 0.037 ± 0.014 for the higher and 0.035 ± 0.017 for the lower temperature. The findings correspond with visual observation when the wells treated with the ethanol solution and dried at 37 °C appeared to have the finest homogeneity. This processing of the wells was chosen for the following assays.

Concentration of indoxylacetate underwent optimization and the saturation curve was constructed from the experimental data (Fig. 2). Indoxylacate in the given concentration was applied on plates in the amount of 100 μL per one well and left to dry at 37 °C for 12 h. The Michaelis constant, K_m, was equal to (1.36 ± 0.17) mmol L^{-1} for BChE and (4.36 ± 0.53) mmol L^{-1} for AChE. In the following assays, the indoxylacetate concentration of 10 mmol L^{-1} was used as an optimum because the assay is not limited by the access to substrate but it is directed by the enzyme activity. Higher indoxylacetate concentrations are not reasonable because the layer on the well surface is too strong and typically not optically homogenous.

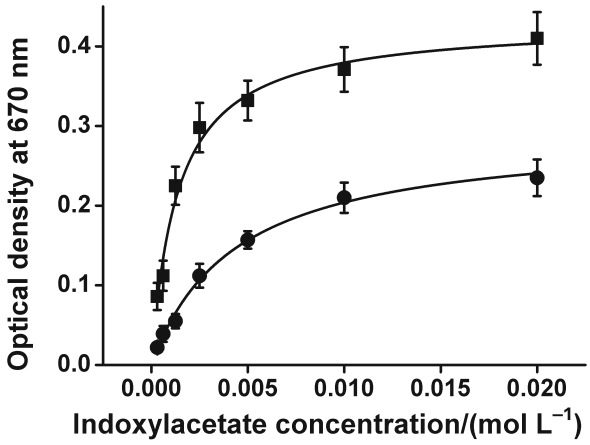

Fig. 2. Determination of the Michaelis constant for indoxylacetate as the substrate and AChE (●) or BChE (■) as the enzymes. Non-linear curve fitting (Michaelis–Menten kinetics) was applied in order to determine the constant. Error bars indicate standard deviation for $n = 5$.

Calibration for standard commercial AChE and BChE when using the prepared microplates can be seen in Fig. 3. Limits of detection for the tested cholinesterases were calculated from the calibration plots and they are valid for a sample with the volume of 100 μL. BChE was assayed with the limit of detection of 0.71 U (or 12 nkat) per 100 μL of the sample. AChE was assayed with a limit of detection higher compared to that of BChE (2.8 U; 47 nkat). The obtained limits of detection were low enough to enable the determination of cholinesterases in biological samples. In one example, cholinesterasemia was reported

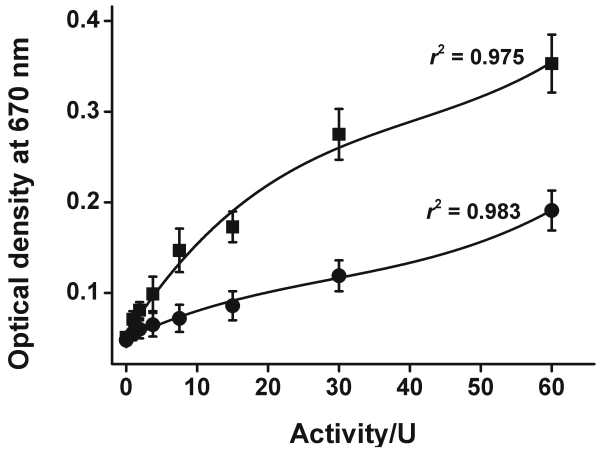

Fig. 3. Calibration for AChE (●) and BChE (■) solutions using microplates with indoxylacetate. Activity expressed for a sample of 100 μL. Error bars indicate the standard deviation for $n = 5$.

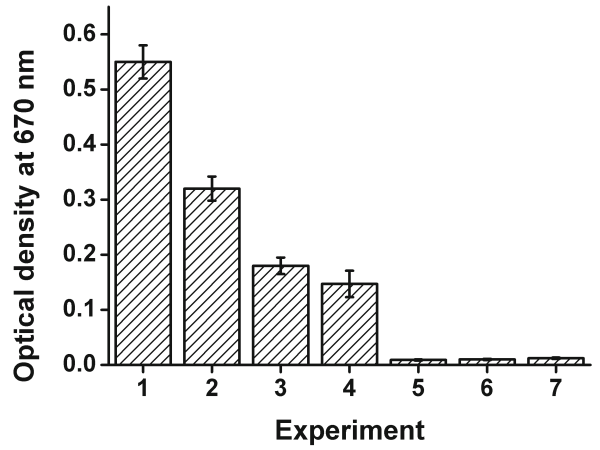

Fig. 4. Interference testing: 1 – 60 U of AChE and 60 U of BChE for sample of 100 μL; 2 – 60 U of AChE and 60 U of BChE with an addition of donepezil, 1 mmol L^{-1}; 3 – 60 U of AChE and 60 U of BChE with addition of iso-OMPA, 1 mmol L^{-1}; 4 – 6 U of BChE; 5 – 1 mmol L^{-1} of ascorbic acid; 6 – 1 mmol L^{-1} of reduced glutathione; 7 – 1 mg mL^{-1} of albumin. Error bars indicate standard deviation for $n = 5$.

to be approximately 8 U mL^{-1} (Rastogi et al., 2008) or 18–67 U mL^{-1} in another source (Guemei et al., 2001).

Interferences were also examined. Experimental data from the interference tests are summarized in Fig. 4. In compliance with the expectation from the calibration plot, the obtained signal is the highest when AChE and BChE are both presented in the sample. From this point of view, cholinesterases can be considered as interferents of each other. In order to distinguish the activity of AChE and BChE, specific inhibitors can be used. Donepezil has been chosen as a compound inhibiting AChE but not BChE (Colović et al., 2013; Darreh-Shori & Soininen, 2010; Sochocka et al., 2008). Compared to donepezil, iso-OMPA is a specific inhibitor of BChE but not AChE (Pohanka, 2013b; Harel et al., 1992). The inhibitors were added to the indoxylacetate solution up to the concentration of 1 mmol L^{-1} and left to dry. As seen in Fig. 4, the use of inhibitors initiated the decrease of the AChE activity in case of donepezil application and of the BChE activity in case of iso-OMPA application. Activities obtained in measurements No. 2 and 3 corresponded well with those obtained for the mixture in measurement No. 1 (Fig. 4). Donepezil had no effect on the assayed BChE activity and iso-OMPA had no effect on the AChE activity assay (data not shown). Ascorbic acid, glutathione, and albumin had no potential to interfere in the assay. It is an advantage of the assay because both glutathione and albumin are quite strong interferents in cholinesterase determination when using the Ellman's assay (Giustarini et al., 2013; Pohanka, 2012b; Prokofieva et al., 2012). Indoxylacetate is not chemically reactive with such compounds; hence, an assay based on indoxylacetate is not sensitive to the interference. From this point of view, it should be emphasized that indoxylacetate is quite stable and less reactive when compared to 5,5′-dithiobis-(2-nitrobenzoic) acid applied in the Ellman's assay.

The method was validated using plasma samples from the BALB/c mice. For the experiment purposes it was necessary to achieve samples with gradually increasing enzyme activity. The obtained plasma samples were mixed together and then divided into five groups: in the first, no treatment was applied; in the other four, carbofuran was added up to the concentration of 1 μmol L^{-1}, 5 μmol L^{-1}, 25 μmol L^{-1}, and 125 μmol L^{-1}, respectively. BChE activity in the plasma samples was determined using the calibration presented in Fig. 3 and the results are depicted in Fig. 5. It is clearly visible that the method based on indoxylacetate well correlates with the standard Ellman's assay as confirmed by the quite high coefficient of determination of 0.976.

Conclusions

The method described here seems to be promising for a routine assay of both AChE and BChE. The use of inhibitors such as donepezil and/or iso-OMPA can further improve the assay efficiency and specificity. The possibility to employ an assay without sample dilution is the major advantage. The absence of glutathione or albumin interference is another advantage of the presented assay. The assay can be easily used for the determination of poisoning by a reversible inhibitor of cholinesterases or for in vitro characterization of drugs. Overall simplicity and low cost of the assay support its application.

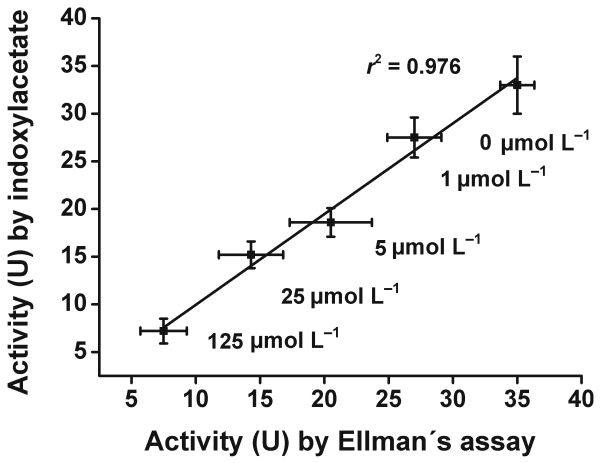

Fig. 5. Validation of the method based on indoxylacetate with the standard Ellman's assay using murine plasma samples. Concentration at each point corresponds to the final level of carbofuran concentration in the plasma sample. Error bars indicate standard deviation for $n = 5$.

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