Talanta 106 (2013) 281–285

Contents lists available at SciVerse ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Spectrophotometric methods based on 2,6-dichloroindophenol acetate and indoxylacetate for butyrylcholinesterase activity assay in plasma

Miroslav Pohanka *, Lucie Drtinova

Faculty of Military Health Sciences, University of Defense, Trebesska 1575, 50001 Hradec Kralove, Czech Republic

ARTICLE INFO

Article history:
Received 24 August 2012
Received in revised form
22 October 2012
Accepted 29 October 2012
Available online 2 November 2012

Keywords:
Butyrylcholinesterase
Acetylcholinesterase
Liver function test
Ellman's method
Indoxylacetate
2,6-Dichloroindophenol acetate
Butyrylthiocholine
Dibucaine number

ABSTRACT

Butyrylcholinesterase (BChE) is an enzyme presented in quite high level in blood plasma where it participates in detoxification reactions. Due to fact that the enzyme is constituted in livers, it is a marker of liver parenchyma function. It can be used for diagnosis of poisoning for e.g., nerve agents or carbofuran and intoxication by some drugs such as rivastigmine. The present experiment is devoted for the creation of new spectrophotometric tests for assay of BChE activity in biological samples. Standard Ellman's method was compared with use of 2,6-dichloroindophenol acetate and indoxylacetate as chromogenic substrates. Maximal velocities and Michaelis constants were calculated for the substrates. Considering calibration, 2,6-dichloroindophenol acetate provided the lowest limit of detection: 1.20×10^{-9} kat and a long linear range. All methods were verified using pooled human plasma samples and tested for potential interferents. 2,6-dichloroindophenol acetate is recommended as suitable substrate for BChE assay in clinical diagnostics.

1. Introduction

Two cholinesterases occur in the human body. Both acetylcholinesterase (AChE; EC 3.1.1.7.) and butyrylcholinesterase (BChE; EC 3.1.1.8.) are known. The both enzymes are present in blood and nervous system [1]. In the past, BChE was called plasmatic cholinesterase due to fact that BChE is predominantly constituted in liver and excreted into plasma where the major part of its activity is [2]. In plasma, BChE is presented in a quite high level (5 mg/ml) with a half time of the molecule (12 days) [3]. Biological significance of BChE is not well known. In the past, detoxification of compounds influencing nervous system: procaine and succinylcholine (suxamethonium) were discovered [4,5]. In course of BChE constitution in liver, assay of BChE level in plasma can be used as a liver function test [6]. Concurrently, decrease of BChE activity can be caused by poisoning with some pesticides such as carbofuran or overdosing by drugs such as rivastigmine [7].

In a clinical point of view, BChE participates in degradation of myorelaxants succinylcholine and mivacurium. Two types of BChE subunits can be found in population: type A that hydrolyze the myorelaxants and type K with minimal ability to hydrolyze some esters including the myorelaxants. Patients with K variant of BChE have prolonged neuromuscular paralysis [8]. In order to precede the life treating events during anesthesia, sensitivity to the myorelaxants can be estimated by the investigation of BChE affinity to dibucaine (cinchocaine), fluoride or some other inhibitors using plasma samples and a standard biochemical test. While the normal A variant, of BChE is fully inhibited by dibucaine with percent of inhibition (called dibucaine number, simply indicated by DN in many sources without explanation of the abbreviation) above 70, K homozygotes and AK heterozygotes have the DN significantly lower [9].

Currently, both AChE presented in blood and BChE presented in plasma and serum are assayed using modified Ellman's method [10]. Principle of the method is based on the hydrolysis of thioesters such as butyrylthiocholine for BChE assay or acetylthiocholine for AChE assay [9]. The created thiocholine spontaneously reacts with 5,5′-dithiobis-(2-nitrobenzoic) acid providing yellow colored 2-nitro-5-thiobenzoic acid (see Fig. 1). The method has some drawbacks such as strong interference of hemoglobin, poor stability of the used reagents, and false positive assay of BChE activity when plasma contains some drugs containing thiole, oxime, or another nucleophilic group [11]. Poor stability of the 5,5′-dithiobis-(2-nitrobenzoic) acid is another drawback of the method.

The present experiment is devoted for the introduction of new methods suitable to substitution of the Ellman's method in assay of BChE in biological samples such as plasma. 2,6-dichloroindophenol acetate and indoxylacetate were chosen as substrates being able to provide contrast coloring [9,12]. Optimization,

* Corresponding author. Tel./fax: +420 973253091.
E-mail address: miroslav.pohanka@gmail.com (M. Pohanka).

0039-9140/$ - see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.talanta.2012.10.085

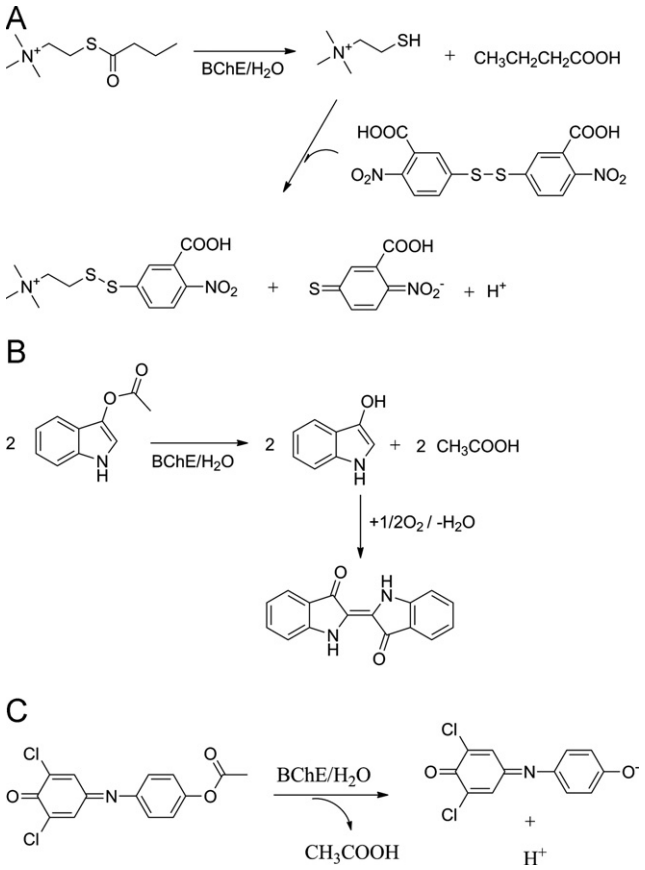

Fig. 1. Principle of BChE activity assays used in the experiments. (A) represents usage of 5,5'-dithiobis-(2-nitrobenzoic) acid as a chromogen and butyrylthiocholine as a substrate, (B) indoxylacetate, and (C) 2,6-dichloroindophenol acetate.

characterization of the methods and validation using human plasma samples were done. Pros and cons for the new methods are discussed in comparison with the standard Ellman's method.

2. Methods

2.1. Samples

BChE from human plasma was used as a standard enzyme (product code B4186-1VL; Sigma-Aldrich, Saint Louis, Missouri, USA; specific activity ≥ 50 U/mg). Pooled plasma from three adult human volunteers was used for comparison of the tested methods. Blood was collected from the brachial vein and kept in heparinized tubes. Plasma was prepared by centrifugation of the blood at 3000 × g for 15 min.

2.2. Assay based on butyrylthiocholine and 5,5-dithiobis-(2-nitrobenzoic) acid

The first assay was done as a standard protocol. It used Ellman's method based on 5,5-dithiobis-(2-nitrobenzoic) acid as chromogen and butyrylthiocholine chloride as a substrate. Principle of the assay is depicted in Fig. 1. The reagents were purchased from Sigma-Aldrich. In the cuvette assay, 0.4 ml of 5,5-dithiobis-(2-nitrobenzoic) acid 0.4 mg/ml, 100 µl of BChE solution (2.3×10^{-9} kat/µl for 1 mmol/l butyrylthiocholine) in phosphate buffered saline (PBS; composition 137 mmol/l NaCl, 2.7 mmol/l KCl, 10 mmol/l Na_2HPO_4, 0.24 mmol/l KH_2PO_4, pH 7.4) and 400 µl of PBS was poured. The reaction was started by addition of butyrylthiocholine chloride (100 µl; 10 mmol/l unless reported other value). Absorbance was measured at 412 nm 5 min after pouring and enzyme activity was calculated using extinction coefficient $\varepsilon = 14{,}150\ \mathrm{l\ mol^{-1}\ cm^{-1}}$. The coefficient was taken from literature for SATP conditions and pH 7.4 [13]. Optical density (O.D.) was assessed using standard polystyrene 96 wells microplates and a multichannel reader. 10 µl (2.3×10^{-8} kat/µl for 1 mmol/l butyrylthiocholine) of BChE standard solution or plasma sample was poured with 10 µl of 5,5-dithiobis-(2-nitrobenzoic) acid 10 mg/ml and 60 µl of PBS. The reaction was started by addition of butyrylthiocholine (20 µl) and O.D. was assessed after 5 min. The time of the assay and the subsequent assays was chosen as an interval long enough allowing assay in a range where changes in absorbance are responding to Lambert Beer law (O.D. to approx. 0.6 for the used device). The time upper limit was adjusted up 1 h for indoxylacetate (see below) as BChE has low affinity to the substrate and enlarging of time interval above the interval is not purposeful in clinical tests.

2.3. Assay based on indoxylacetate

Indoxylacetate (received from Sigma-Aldrich) was used as both substrate and chromogen. Principle of the reaction is depicted as part B of Fig. 1. The indoxylacetate is converted into indole and acetic acid by BChE during the assay. Indole is spontaneously oxidized to indigo blue providing contrast coloration. Peak absorbance is found at 670 nm with extinction coefficient $\varepsilon = 3900\ \mathrm{l \times mol^{-1} \times cm^{-1}}$ [14]. Cuvette based assay was done in following way: 800 µl of PBS was mixed with 100 µl of BChE solution (2.3×10^{-9} kat/µl for 1 mmol/l butyrylthiocholine) in PBS and 100 µl of indoxylacetate solution (10 mmo/l) in 5% ethanol. Assay based on 96 well microplates was performed by pouring of 10 µl of BChE (2.3×10^{-8} kat/µl for 1 mmol/l butyrylthiocholine), 10 µl of the indoxylacetate solution and 80 µl of PBS. Absorbance was measured 1 h after injection of the indoxylacetate solution.

2.4. Assay based on 2,6-dichloroindophenol acetate

The last assay used 2,6-dichloroindophenol acetate as a chromogenic substrate. The reaction is shown as (C) in Fig. 1. 2,6-dichloroindophenol is a reaction product. The compound is blue in neutral and basic pH strongly absorbing at 606 nm with extinction coefficient $\varepsilon = 18{,}800\ \mathrm{l\ mol^{-1}\ cm^{-1}}$ at physiological pH 7.4 [15]. BChE activity assay in cuvettes was done in following way: 800 µl of PBS was mixed with 100 µl of BChE solution (2.3×10^{-9} kat/µl for 1 mmol/l butyrylthiocholine) in PBS and 100 µl (10 mmo/l) of 2,6-dichloroindophenol acetate solution in 5% (v/v) ethanol and absorbance was measured after 5 min. Assay based on 96 well microplates was performed by pouring 10 µl of BChE (2.3×10^{-8} kat/µl for 1 mmol/l butyrylthiocholine), 10 µl of the 2,6-dichloroindophenol acetate solution and 80 µl of PBS. O.D. was measured after 5 min.

2.5. Experimental data processing

Software Origin 8 (OriginLab Corporation, Northampton, MA, USA) was used for experimental data processing. Significance testing was done using Scheffé's method on probability levels $P = 0.05$ and $P = 0.01$. Michaelis constant (K_M) and maximal velocity (V_{MAX}) were calculated in a standard way using nonlinear curve fitting with Hill equation for Hill coefficient adjusted at $n = 1$. Limit of detection was calculated from confidence interval (95%) in calibration plot. All samples were measured in tetraplicate.

3. Results and discussion

Affinity of BChE toward the substrates was investigated in the first part of experiment. It was done using standard cuvettes in order to calculate activities of enzymes in katals. Affinity toward butyrylthiocholine as a substrate is summarized in Fig. 2. K_M value for BChE was 1.39 mmol/l and V_{MAX} was calculated to be 3.93×10^{-7} kat. The found K_M value corresponds well with the reported papers where K_M for butyrylthiocholine was 1.87 mmol/l for *Equus cabalus* BChE [16], 1.7 mmol/l for *Oryctolagus cuniculus* [17] and 0.4–6 mmol/l for *Homo sapiens* [18]. BChE has a slightly higher affinity to indoxylacetate than butyrylthiocholine as K_M and V_{MAX} for the substrate were 0.403 mmol/l and 5.53×10^{-8} kat (see Fig. 2). In the last of the affinity experiments, BChE was proved to have higher K_M and V_{MAX} values for 2,6-dichloroindophenol acetate comparing to indoxylacetate (Fig. 2). For 2,6-dichloroindophenol acetate, K_M value was 3.64 mmol/l and V_{MAX} was 2.48×10^{-7} kat. Owing to the K_M values, indoxylacetate can be designated as the main substrate. Surprisingly, V_{MAX} was the lowest one from the three tested substrates. It can be caused by lower solubility and related lower distribution into active site of the enzyme when presented in higher concentration [19]. Advantages of indoxylacetate use were discussed by some authors concluding their work by description of beneficial contrast in coloring [20]. The three substrates have not been simultaneously investigated and compared each other yet. Moreover, available data about 2,6-dichloroindophenol acetate as a substrate for BChE are incomplete though some researchers experimented with it in order to establish an analytical method [12,21]. Application of the substrate for plasmatic BChE assay is an original idea. Beside 2,6-dichloroindophenol acetate, 2,6-dichlorophenol was proved to be reliable chromogenic reagent in the cholinesterase assay. However, 2,6-dichlorophenol is not converted by esterases as it is not ester but it can react with thiocholine [22].

Calibrations for the tested activities of BChE are depicted as Figs. 3–5. Commercially available pure human BChE was used for the calibration purposes and the assay was done on 96-wells microplates. The microplates based assay was chosen as the best platform for pertinent clinical application as it is suitable for a simultaneous assay of higher number of samples. Coefficients of determination R^2 for the tested methods were favorable. Calibration using butyrylthiocholine and indoxylacetate provided exponential plot type. Contrary to it, 2,6-dichloroindophenol acetate provided linear calibration. The coefficients of determination

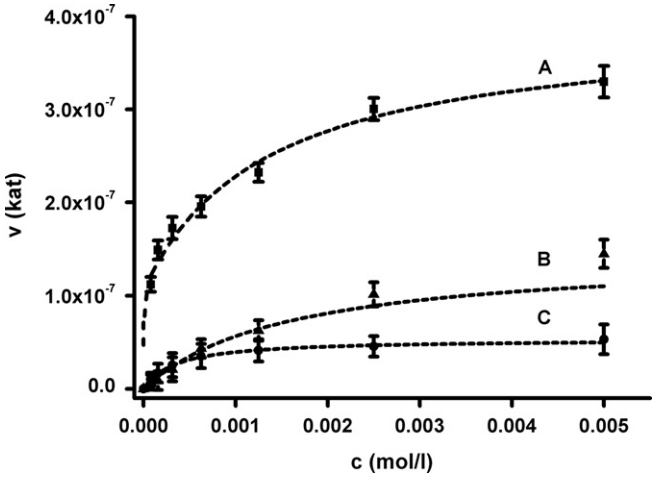

Fig. 2. Affinity of BChE to substrates: butyrylthiocholine chloride (A), 2,6-dichloroindophenol acetate (B), and indoxylacetate (C). The assay was performed on standard cuvettes.

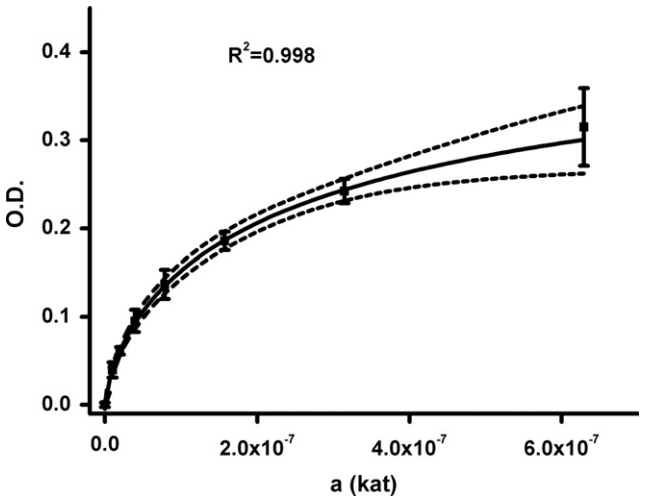

Fig. 3. Calibration of BChE using butyrylthiocholine. The assay was performed on standard 96 wells microplates. Dashed lines indicate confidence interval for $P=0.05$.

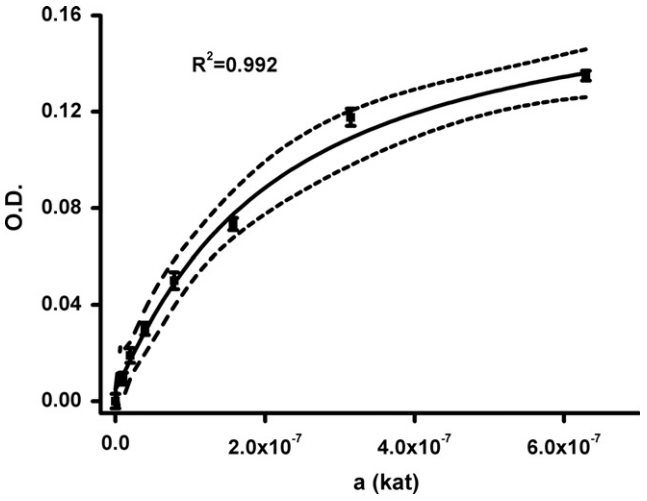

Fig. 4. Calibration of BChE using indoxylacetate. The assay was performed on standard 96 wells microplates. Dashed lines indicate confidence interval for $P=0.05$.

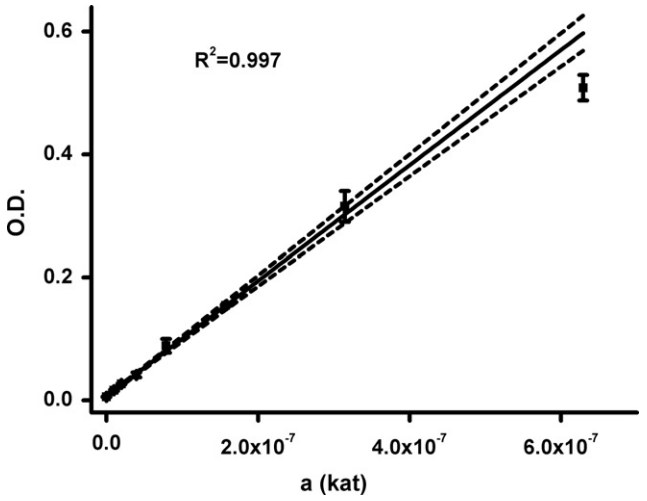

Fig. 5. Calibration of BChE using 2,6-dichloroindophenol acetate. The assay was performed on standard 96 wells microplates. Dashed lines indicate confidence interval for $P=0.05$.

were 0.998 for butyrylthiocholine, 0.992 for indoxylacetate, and 0.997 for 2,6-dichloroindophenol acetate. Linearity of the analytical method is an advantage parameter in assays [23]. 2,6-dichloroindophenol acetate as a substrate can be favored for the reason. Limit of detection is another significant parameter as BChE level can decrease due to disparate pathologies or toxic exposures to minimal values [24]. In humans, the BChE level is quite stable and decrease in course of some pathologies [25,26]. As reported by Dimov and coworkers, normal population has BChE activity 9010 ± 2041 U/l ($(1.50 \pm 0.34) \times 10^{-4}$ kat/l; the value covers 94.1% of population); however, it may be reduced up to approximately 1600 U/l (2.67×10^{-4} kat/l) in some individuals [27]. Using a method for BChE assay with low limits of detection is rational. From the three substrates, the best limit of detection was achieved for 2,6-dichloroindophenol acetate: 1.20×10^{-9} kat (0.0720 U) followed by butyrylthiocholine: 3.41×10^{-9} kat (0.205 U). The worst limit of detection was found when indoxylacetate used: 9.22×10^{-9} kat (0.553 U). The limits of detection are low enough to investigate BChE activities in real samples when considered the aforementioned limits of detection.

From the tested methods, use of butyrylthiocholine and 5,5′-dithiobis-(2-nitrobenzoic) acid as a chromogen is a standard protocol for assessment of BChE in plasma [28,29]. We correlated the 2,6-dichloroindophenol acetate and indoxylacetate based methods just to the method based on butyrylthiocholine and 5,5′-dithiobis-(2-nitrobenzoic) acid. The correlations are depicted as Fig. 6. The correlation coefficients were quite good: 0.992 for 2,6-dichloroindophenol acetate and 0.961 for indoxylacetate. Correlation with 2,6-dichloroindophenol acetate proved higher slope for the substrate. On the other hand, indoxylacetate provided linear correlation to butyrylthiocholine. The findings indicate that BChE has higher affinity to the 2,6-dichloroindophenol acetate than to the other two substrates when they are presented in a upper concentration. The fact present another advantage of the 2,6-dichloroindophenol use.

Interference in the assays did not limit the methods to be used for BChE assay. We tested interference of ascorbic acid, glutathione, and obidoxime up to 10 mmol/l, and albumin up to 5 mg/ml. No interference in the tested methods occurred when zero value was reset prior to measurement. On the other hand, glutathione, albumin and obidoxime caused spontaneous yellowing of the mixture butyrylthiocholine and 5,5′-dithiobis-(2-nitrobenzoic) acid. It is caused due to chemical reaction between the 5,5′-dithiobis-(2-nitrobenzoic) acid and the compounds. It is not a surprising fact as the interference was reported for oximes [11] and reaction of thiols with 5,5′-dithiobis-(2-nitrobenzoic) acid serves for their assay in biological samples [30,31]. Stability of 2,6-dichloroindophenol acetate and indoxylacetate in presence of potential interferents was expected and proved in an independent experiment for indoxylacetate on a thin layer [32].

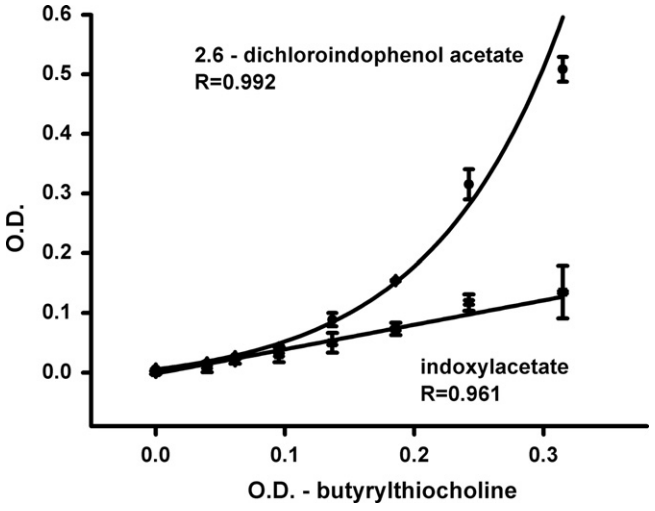

Fig. 6. Correlation of the methods on standard 96 wells microplates.

When considered the above discussed physiological levels of BChE in plasma, all of the three methods are suitable for BChE activity assay when used plasma from standard health population. However, the homozygote K type of BChE and other decreased plasma levels of BChE have too low activity to be simply assayed using indoxylacetate based assay as physiological BChE can be under limit of detection for the method [27]. The last two methods, assay based on butyrylthiocholine and 5,5′-dithiobis-(2-nitrobenzoic) acid and 2,6-dichloroindophenol acetate have limits of detections low enough to assay all of the expected BChE activities in plasma. Pooled human serum was used for verification of the methods based on 2,6-dichloroindophenol acetate and indoxylacetate. Using butyrylthiocholine, BChE activity in the pooled plasma was $(7.53 \pm 1.09) \times 10^{-9}$ kat per 10 μl sized sample. Using the above reported calibration plots, BChE activity was assessed to be $(7.35 \pm 0.95) \times 10^{-9}$ kat for indoxylacetate and $(7.61 \pm 0.98) \times 10^{-9}$ kat for 2,6-dichloroindophenol acetate. The results were not significantly different to each other when tested significances by the Scheffé's method.

4. Conclusions

From the three tested methods, we recommend the use of 2,6-dichloroindophenol acetate as a substrate for BChE assay. The substrate is favorable in both limit of detection and a long linear calibration plot. Moreover, it is not sensitive to spontaneous reaction to potential interferents like 5,5′-dithiobis-(2-nitrobenzoic) acid. Despite the lowest K_M from the tested compounds, indoxylacetate has many drawbacks including low extinction coefficient. We infer that 2,6-dichloroindophenol acetate would take place in routine examination of BChE activity.

Acknowledgments

A long-term organization development plan 1011 (Faculty of Military Health Sciences, University of Defense, Czech Republic) is gratefully acknowledged.

References

[1] E.G. Duysen, B. Li, O. Lockridge, Expert Opin. Drug Metab. Toxicol. 5 (2009) 523–528.
[2] T. Iwasaky, M. Yoneda, A. Nakajima, Y. Terauchi, Intern. Med. 46 (2007) 1633–1639.
[3] D. Ostergaard, J. Viby-Moogensen, H.K. Hanel, L.T. Skovgaard, Acta Anaesthesiol. Scand. 32 (1988) 266–269.
[4] J. Yuan, J. Yin, E. Wang, J. Chromatogr. A 1154 (2007) 368–372.
[5] Y. Hashim, D. Shepherd, S. Wiltshire, R. Holman, J.C. Levy, A. Clark, C.A. Cull, Diabetologia 44 (2001) 2227–2230.
[6] R. Boopathy, R.V. Rajesh, S. Darvesh, P.G. Layer, Clin. Chim. Acta 380 (2007) 151–156.
[7] M. Pohanka, Expert Opin. Ther. Pat. 22 (2012) 871–886.
[8] S.E. Kaufman, R.W. Donnell, D.C. Aiken, C. Magee, Ann. Pharmacother. 45 (2011) e21.
[9] M. Pohanka, Biomedical papers of the Medical Faculty of the University Palacky, Olomouc, Czechoslovakia, 155, 2011, pp. 219–229.
[10] G.L. Ellman, K.D. Courtney, V. Andres Jr., R.M. Feather-Stone, Biochem. Pharmacol. 7 (1961) 88–95.
[11] G. Sinko, M. Calic, A. Bosak, Z. Kovarik, Anal. Biochem. 370 (2007) 223–227.
[12] Y. Miao, N. He, J.J. Zhu, Chem. Rev. 110 (2010) 5216–5234.
[13] P. Eyer, F. Worek, D. Kiderlen, G. Sinko, A. Stuglin, V. Simeon-Rudolf, E. Reiner, Anal. Biochem. 312 (2003) 224–227.
[14] Z.L. Wu, M.L.M. Podust, F.P. Guengerich, J. Biol. Chem. 280 (2005) 41090–41100.

[15] S.H. Bissbort, W.J.H. Vermaak, J. Elias, M.J. Bester, G.S. Dhatt, J.K.W. Pum, Clin. Chim. Acta 303 (2001) 139–145.
[16] K. Kovacs, B. Szajani, L. Boross, J. Appl. Biochem. 4 (1982) 11–18.
[17] R.S. Rush, A.R. Main, S.K. Miller, B.F. Kilpatrick, J. Biol. Chem. 255 (1980) 7155–7160.
[18] P. Masson, W. Xie, M.T. Froment, O. Lockridge, Biochim. Biophys. Acta 1544 (2001) 166–176.
[19] H.Y. No, Y.A. Kim, Y.T. Lee, H.S. Lee, Anal. Chim. Acta 594 (2007) 37–43.
[20] F. Villatte, T.T. Bachman, A.S. Hussein, R.D. Schmid, Biotechniques 30 (2001) 81–86.
[21] D. Tingfa, Z. Shiguang, T. Mousheng, Enivron. Pollut. 57 (1989) 217–2222.
[22] J.X. Qiu, J. Chen, Q.Q. Ma, Y.Q. Miao, Chemosphere 77 (2009) 129–132.
[23] A. Switaj-Zawadka, P. Konieczka, E. Przyk, J. Namiesnik, Anal. Lett. 38 (2005) 353–376.
[24] K. Siskova, F. Bilka, A. Adameova, A. Balazova, M. Mydla, I. Paulikova, Pharmazie 67 (2012) 345–350.
[25] W.D. Lu, T. Hada, K. Fukui, H. Imanishi, N. Matsuoka, A. Iwasaki, K. Higashino, Intern. Med. 36 (1997) 9–13.
[26] E. Duran-Ferreras, F. Diaz-Narvaez, M. Raffo-Marquez, Gastroenterol. Hepatol. 34 (2011) 401–405.
[27] D. Dimov, K. Kanev, I. Dimova, Acta Biochim. Pol. 59 (2012) 313–316.
[28] A.A. Guemei, J. Cottrell, R. Band, H. Hehman, M. Prudhomme, M.V. Pavlov, J.L. Grem, A.S. Ismail, D. Bowen, R.E. Taylor, C.H. Takimoto, Cancer Chemother. Pharmacol. 47 (2001) 283–290.
[29] F. Dorandeu, A. Foquin, R. Briot, C. Delacour, J. Denis, A. Alonso, M.T. Froment, F. Renault, G. Lallement, P. Masson, Toxicology 248 (2008) 151–157.
[30] H. Khan, M.F. Khan, S.U. Jan, N. Ullah, Pak. J. Pharm. Sci. 24 (2011) 13–18.
[31] D.S. Prokofieva, R.O. Jenkins, N.V. Goncharov, Anal. Biochem. 424 (2012) 108–113.
[32] M. Pohanka, Anal. Lett. 45 (2012) 367–374.