Electrochemical Biosensor Based on Acetylcholinesterase and Indoxylacetate for Assay of Neurotoxic Compounds Represented by Paraoxon

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Acetylcholinesterase (AChE) is an enzyme terminating neurotransmission in cholinergic nervous system. It is targeted by several drugs and toxins. From a toxicological point of view, pesticides and nerve agents are probably the most toxic compounds inhibiting AChE activity. On the account of the enzyme inhibition, AChE is suitable for an in vitro assay of inhibitors and analytical devices such as biosensors are known to work on principle of recording of AChE residual activity. Here reported experiment is based on previously performed colorimetric dipsticks. Presently, it is further improved by an electrochemical assay. The dipsticks are prepared by an immobilization of electric eel AChE into gelatin membrane on cellulose matrix and assay of the enzyme activity by sticking zone with AChE and indoxylacetate as a chromogenic substrate. The zone with immobilized AChE was placed onto screen printed electrode and enzyme reaction was assessed electrochemically using square wave voltammetry. In a separate experiment, cyclic voltammetry and square wave voltammetry of indoxylacetate. We proved suitability of indoxylacetate for electrochemical assay as it is simply oxidized by an applied voltage at potential approximately 0.650 mV. When present in cellulose matrix, the oxidation peak occurs in a higher potential. Performance of dipsticks for assay of AChE inhibitor paraoxon provided limit of detection 29.1 nM in sample sized 40 µl. The assay is finished within one hour and the results proved electrochemically can be further confirmed by coloration of dipstick surface. Suitability of the dipsticks for practical performance is discussed.

Keywords: biosensor; acetylcholinesterase; pesticide; inhibitor; screen printed electrode; voltammetry

1. INTRODUCTION

Cholinergic nervous system is a target of many compounds being important due to their pharmacological or toxicological significance. In the cholinergic nervous system, enzyme acetylcholinesterase (AChE; EC 3.1.1.7) plays a prominent role as it participate in termination of acetylcholine based signal transmission through neurosynaptic cleft or neuromuscular junction [1,2]. Biochemical scheme of neuromuscular junction is shown in Fig. 1.
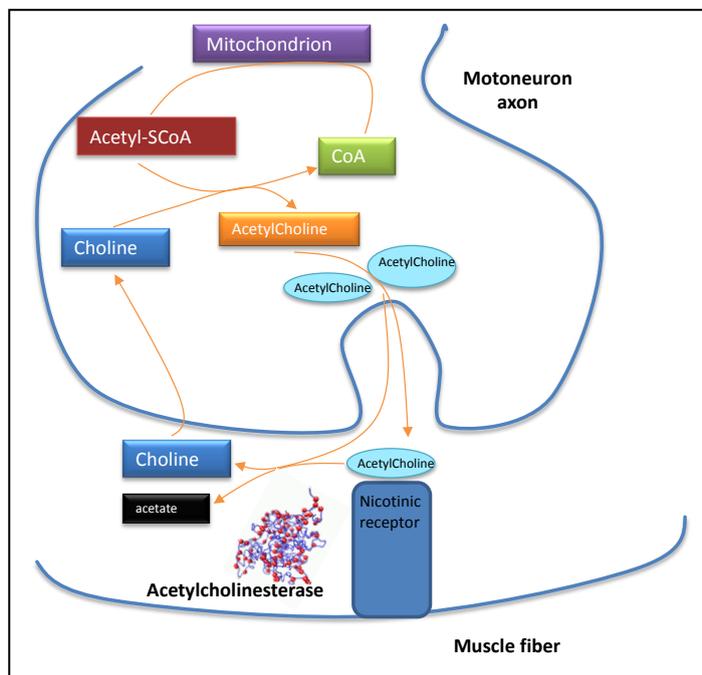
Figure 1. At the neuromuscular junction, acetylcholine is synthesized in the cytoplasm of the axon's terminal button, from acetyl coenzyme A and choline. Half of the released choline molecules are recaptured from the junction or cleft and used again. Many thousands of molecules of acetylcholine are thus stored in each pre-synaptic vesicle. As soon as the vesicles' contents are released into the synaptic gap or neuromuscular junction, they interact with acetylcholine receptors; however, hydrolysis of acetylcholine by an enzyme acetylcholinesterase also undergo. . Of course, all of this happens very quickly. Acetylcholinesterase can hydrolyze 4000 molecules of acetylcholine per site per second, so the half-life of the acetylcholine is estimated at short time 1 to 2 milliseconds. In fact, the distribution of the acetylcholinesterase perfectly matches that of the postsynaptic nicotinic or muscarinic acetylcholine receptors.

Inhibitors of AChE are a wide group of structures such as acridinium derivatives, piperidine derivatives, organophosphorus and carbamate compounds. The compounds are used as pesticides, nerve agents, and drugs for treatment of Alzheimer´s disease (e.g. donepezil, rivastigmine, galantamine, in some countries huperzine), myasthenia gravis (pyridostigmine, neostigmine etc.) and some of them have multiple importances [3,4]. The fact that AChE can be inhibited by toxic compounds can be used for their detection as different dipsticks and biosensors containing AChE or

butyrylcholinesterase are available [5]. The enzyme activity serves as a manner how to express presence of the toxic compound. Several electrochemical and optical protocols were adopted for the assay purposes. Splitting of acetyl- or butyrylthiocholine by the enzyme and consequent electrochemical oxidation or interaction with chromogenic compound such as 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB) are suitable for analytical purposes [6].
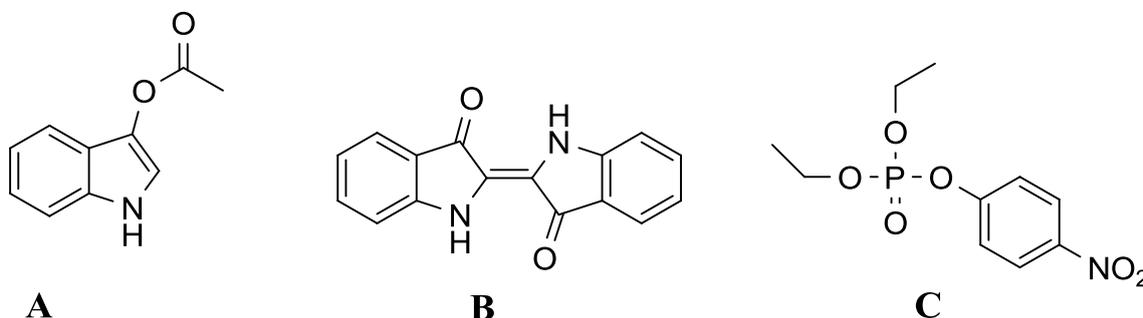
Figure 2. Chemical structure of indoxylacetatate (A), indigo (B) and paraoxon ethyl (C).

The present investigation is based on investigation of indoxylacetate as a substrate for electrochemical determination of AChE inhibitors (Fig. 2). The indoxylacetate undergo splitting of acetyl moiety and spontaneous oxidation up to blue indigo. In a biochemical point of view, the substrate suitability for AChE based assay was reported in our previous work [7]. Consequently, indoxylacetate based dipsticks were developed and used for simple but sensitive assay of neurotoxic compounds such as paraoxon ethyl with an evaluation by a naked eye [8]. Here, we introduce an electrochemical platform for the previously developed dipsticks performance. It is an improvement of the previous semiquantitative assay as it uses objective parameter instead of the originally used arbitrary units. The electrochemical assay is expected to be able to lower limit of detection and quantify level of inhibitor.

2. EXPERIMENTAL PART

2.1. Dipstick preparation

AChE from electric eel (*Electrophorus electricus*) was achieved from Sigma-Aldrich (Saint Louis, Missouri, USA). The enzyme had specific activity 16.7 μkat/mg protein. It was dissolved in phosphate buffered saline shortly before experiment and activity was adjusted up 5 U (83.4 nkat) in 20 μl for substrate acetylthiocholine chloride and standard Ellman´s protocol as described in the previous paper [9]. AChE solution was mixed with gelatin up to final level 2% (w/w) and 20 μl of the reagents was added on one edge of cellulose filter paper (Whatman, Kent, UK) cut into bands 50×5 mm. The wet bands were dried in a silica gel filled desiccator. The second edge of the band was imbued by 20 μl of 100 mM indoxylacetate in ethanol and let to dry in laboratory conditions.

2.2. Dipstick performance

Paraoxon ethyl (Sigma-Aldrich) was used throughout for dipstick performance. It was solved in a calibration scale 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} mol/l. Each paraoxon solution or blank contained 5% isopropanol in deionized water. The assay was done by application of sample in a volume 40 µl to AChE containing edge of dipstick and incubated for 15 minutes. After that, the dipstick was folded in the middle and the opposite edges were pressed one to each other for 30 minutes. Finally, the edge containing immobilized AChE was pressed with electrochemical sensor and SWV was recorded.

2.3. Electrochemical device, its setting and performance

Screen printed sensors (BVT, Brno, Czech Republic) were sized 25.4×7.3×0.6 mm and contained dot shaped platinum working electrode with diameter 1 mm, circle shaped platinum auxiliary, and circle shaped argent chloride reference electrode. The assay was done using an electrochemical device PalmSens (PalmSens BV, Houten, Netherlands) connected with a computer, and processed by PSLite 1.8 (PalmSens BV) software. Setting for cyclic voltammetry (CV) was following. Scanning range 0 – 1 V, potential step 0.005 V, and scan rate 0.005 V/s. Electrochemical strip was covered with 20 µl of 10 mM indoxylacetate in phosphate buffered saline and every sample was scanned by three cycles. Measurement was repeated five times. Square wave voltammetry (SWV) was performed in a range 0 – 1.1 V. The added voltage alternated with frequency 1 Hz and amplitude 0.01 V. Step of scanning was 0.005 V. Every experimental point was made in pentaplicate for both SWV and CV.

3. RESULTS AND DISCUSSION

3.1. Indoxylacetate voltammetry

The cyclic voltammetry of indoxylacetate proved oxidation peak at 0.645±0.023 V for the every first voltammetry (Fig. 3). Indoxylacetate was exhausted by applied voltage so the oxidation peak was not found in the second or third cycle. During depolarization, another peak is formed. It is positioned at 0.210±0.036 V and it re-appears in the second and third cycles unlike to the oxidation peak. The peak respond to reversible polarization of indole moiety and it has not analytical meaning. In point of analytical view, indoxylacetate can be assayed electrochemically and the molecule is suitably for construction of analytical method or device using the molecule as a reagent. The fact that indoxylacetate can be oxidized is not surprising as the basic indole ring structure is common with low molecular weight antioxidant melatonin [10] and structures like melatonin can be simply assay by a CV method [11]. Beside CV, we assayed indoxylacetate again using standard SWV. In this assay, one peak appeared at potential 0.653±0.008 V. It well corresponded with the oxidation peak proved in CV. No other peak was recognized. When indoxylacetate soaked into cellulose filter paper with gelatin and

SWV was performed, peak at 0.880±0.047 V appeared. The increase of peak position can be caused by interaction of indoxylacetate oxidation products with the matrix and slow down distribution of indoxylacetate in the matrix. The curves shape is obvious from Fig. 4.
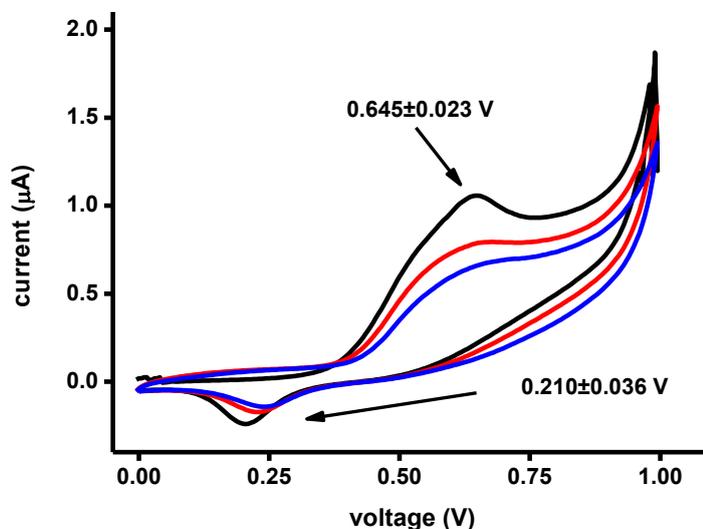
Figure 3. CV of 10 mM indoxylacetate. Black curve: first, red curve: second, and blue curve: third cycle.

3.2. Dipstick coloration

The dipstick processing provided contrast blue color when AChE remained intact by paraoxon. The mechanism of coloration is described in references [7,8]. The color changes are depicted as table 1. The matrices of dipsticks were colored by creating indigo. The color was quite intensive and well observable as it is not only absorbing but also fluorescent in visible light wavelength [12]. Coloration for the control dipstick and dipstick covered with 10 nM paraoxon was very intensive. Compared to it, higher level of paraoxon caused decrease of coloration. Estimated limit of detection was approximately 1 µM for paraoxon when coloration evaluated by a naked eye. The coloration intensity was equal to the previously published paper and the other devices using similar coloration principle [8,13].

Table 1. Calibration for paraoxon using colorimetric dipsticks.

paraoxon (mM)						
10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	0

3.3. SWV using the colored dipsticks and calibration

When indoxylacetate based dipsticks performed by SWV, typical peaks were formed as clearly demonstrated in Fig. 4. The peaks are increasing when AChE in the dipsticks become inhibited by paraoxon. We judge that electrochemistry act as a competing process for substrate reaction. When AChE fully active, the substrate undergo into oxidation process and indoxylacetate level for electrochemical oxidation is decreased. In the opposite phenomenon, inhibited AChE cannot split indoxylacetate so the electrochemically assayed reaction has maximal response. The proportionality between peak height (area) and inhibitor level is advantageous for simple understanding and better option for pertinent commercialization of device.
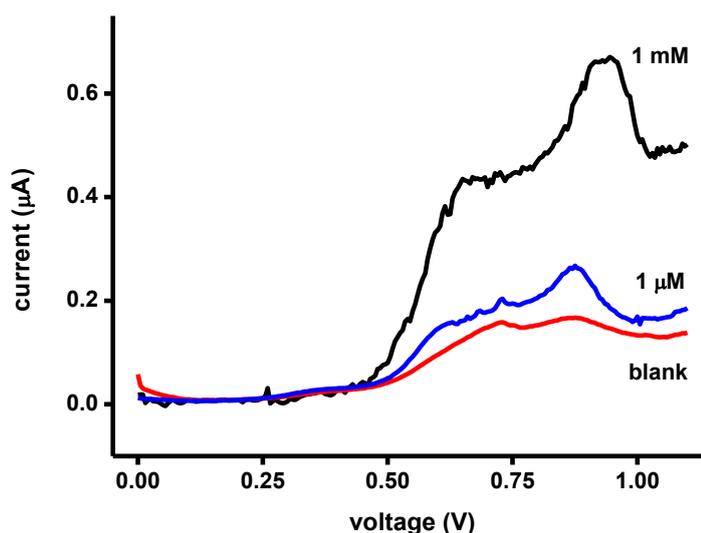
Figure 4. SWV for colored dipsticks with bound AChE. Black and blue curves: assay of 1 mM and 1 µM paraoxon, red curve: assay of 5% isopropanol only (blank).

We were encouraged by the preliminary experimental data described previously and decide to examine dipsticks for paraoxon assay. Semi-log calibration plot (negative logarithm of paraoxon concentration versus area of peak in µAV) is depicted as Fig. 4. The calibration was fitted by Boltzman equation (OriginPro 8, Origin Lab Corp, Northampton, MA, USA) and limit of detection was calculated as a point on the curve responding to triplicate of blank assay standard deviation (signal to noise equal to 3). The median inhibitory concentration for paraoxon was calculated to be 5.93 µM and limit of detection 29.1 nM for a sample sized 40 µl. The coefficient of determination R^2 for the calibration plot was 0.981. Limit of detection decreased due to application of electrochemical output approximately 34 times compared to the evaluation by a naked eye. Owing to the literature search, the limit of detection for the dipsticks is similar to limit of detection reached by a disposable electrochemical device containing AChE on gold chips: 36 nM for paraoxon [14]. Limit of detection

for a biosensor containing polypyrrole immobilized AChE was even nearly seven times higher (200 nM) as reported by Sulak [15,16].

Organic solvents were examined as typically interfering compounds in AChE based assays [17,18]. Ethanol, isopropanol and dimethylsulfoxide (DMSO) were adjusted up final concentration 1, 2, 5, 10, and 20% (w/w). The solutions were assayed in the same way like samples with paraoxon. We did not prove any significant inhibition for ethanol and isopropanol. DMSO did not inhibit up to concentration 10%. The highest level of DMSO (20%) caused 5% decrease of detected area under peak which approximately responds to paraoxon in concentration slightly above limit of detection. The low interference of organic solvents is quite surprising regarding to the previously reported paper, where electrochemical biosensor with immobilized AChE was strongly influenced by solvents even in a low level [19]. In this paper, DMSO caused 74% inhibition of AChE immobilized on electrochemical sensor. Here reported dipstick is significantly more stable. It is probably caused by better interception of AChE in cellulose matrix. Organic solvents can be also preferably depot within cellulose matrix resulting in lower influencing of AChE.
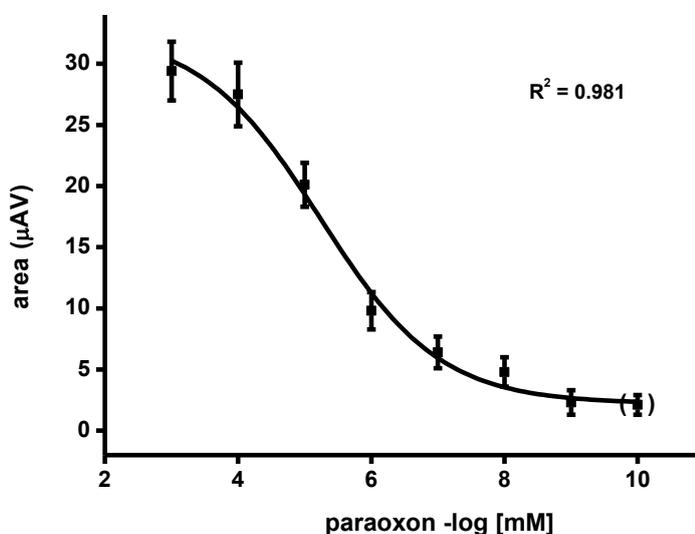
Figure 5. Calibration for paraoxon. Error bars indicate standard error of mean for n = 5. Point in brackets was achieved by assay of blank (5% isopropanol).

The dipsticks are suitable for dual assay of neurotoxic compounds such as pesticides or nerve agents using scoring by a naked eye or by electrochemical device for more sensitive assay. The reported limit of detection is good when considered toxicity of organophosphorus compounds. The limit of detection is below concentration when it causes cytotoxicity effect: 36 μM [20]. Median lethal dose for paraoxon administered subcutaneously into rats is 0.9 mg/kg (3.45 μmol/kg) as reported by Petrikovics et al. [21]. Using the dipsticks and electrochemical device, paraoxon presence can be evidently proved even in solutions that do not represent any health risk.

4. CONCLUSIONS

Previously developed colorimetric dipsticks were successfully adopted for electrochemical assay. The dipsticks are convenient for dual performance based on evaluation of coloration by a naked eye and by electrochemical assay by performance of screen printed electrodes. The principle of assay is quite intriguing as performance of electrochemical device is not compulsory but optional in the assay protocol. The dipsticks seem to be well approachable for field assay of neurotoxic pesticides as proved on the paraoxon.

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