CHOLINESTERASE INHIBITOR BIOSENSORS

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Abstract

This paper describes the partial results of the development of a new biosensor of cholinesterase inhibitors based on biochemical enzymatic reaction with colorimetric evaluation. A biosensor consists of an indicator zone with immobilised enzyme (butyrylcholinesterase), an auxiliary zone with a substrate (butyrylthiocholine) and a chromogenic agent, as well as a zone with colour etalon. The presence of cholinesterase inhibitors is indicated by the fact that there is no discoloration of the chromogenic reagent. A biosensor is suitable for the rapid and simple detection of chemical warfare agents (nerve gases) or industrial pesticides (organophosphates, carbamates) in water and in the air.

Key words: *butyrylcholinesterase, 2,6-dichlorophenolindophenol, physostigmine, inhibitors*

1. INTRODUCTION

Compounds acting as cholinesterase inhibitors include organophosphorus and carbamate pesticides, but also extremely toxic and military significant nerve chemical warfare agents (Romano 2007). It is therefore understandable that continuous attention is dedicated to their analysis (Mesilaakso 2005) worldwide. Besides relatively exacting physical or physicochemical instrumental techniques, even simpler chemical techniques based on colour reactions with chromogenic or fluorogenic agents have still been in use (Royo 2007). However, because of the extreme toxicity of cholinesterase inhibitors, the most important method for their detection and determination is based on biochemical (cholinesterase) reaction (Halámek 2009).

The simplest means of technical analysis of cholinesterase inhibitors can be found among colorimetric detectors. They usually take the form of indication papers and strips, detection tubes and various detection kits or handheld laboratories for specific purposes and for specific conditions. Some of those means are in the area of the protection of troops and population used as personal colorimetric detectors, which are usually designed to protect individuals in particularly difficult conditions, especially when they have to rely only on themselves.

If simple detectors of cholinesterase inhibitors are based on the enzymatic reaction, we usually call them biosensors. An example of biosensors used to protect troops and the population against the effects of militarily significant cholinesterase inhibitors (i.e. nerve agents) includes DETEHIT (Czech Republic), NAVD (Anachemie, Canada), DET INDIV mle F1 (Giat, France), CDK (IIBR, Israel), the Akker Krutbruk detector (Sweden) and the detector from the M256A1 kit (USA), which even enables the determination of cholinesterase inhibitors, among other substances. Enzymatic (cholinesterase) reaction used in biosensors is based on the hydrolysis of a suitable substrate through cholinesterase, and on colour indication of its hydrolytic product. The degree of inhibition of the enzyme (colour change) is then proportional to the concentration of the pollutant. Conventional enzymatic methods with the acetylcholinesterase (AChE) or butyrylcholinesterase (BuChE) enzyme use a substrate acetylcholine or butyrylcholine, which are cleaved to choline and acetic acid or butyric acid, which can be detected by acid-base indicators. In practice, however, it is more advantageous to use acetylthiocholine or butyrylthiocholine substrates. These substrates are decomposed to form

thiocholine, which easily reduces certain substances with simultaneous changing of their colouration. Ellman's reagent (Ellman, 1961, Tusarova 1999), 2,6-dichlorophenolindophenol (Bissbort 2001) or triphenylmethane dyes (e.g. Guinea green, malachite green) (Halámek 2009), are typically used as the colour indicators. The colour effect is then not only dependent on the disposition of these substances to reducing reactions, but also on the enzyme – substrate system used. Another option is using the so-called chromogenic substrates that are hydrolyzed directly to the coloured product, e.g. 2,6-dichlorfenolindofenyl acetate (Barendsz 1979) or indoxyl acetate (Gelman 1962).

Although the known colorimetric detectors / biosensors generally excel in design simplicity and high sensitivity, they have some disadvantages and drawbacks, such as limited use in certain environments (e.g. atmosphere analysis only), less pronounced colour effect, or high production costs (e.g. limited availability of chromogenic substrates and their stability) and a shorter life. Some of these disadvantages are substantially removed by a newly developed cholinesterase inhibitor biosensor briefly described in this paper.

2. EXPERIMENTAL SECTION

2.1 Chemicals, materials and instruments

Butyrylcholinesterase (BuChE), substrate butyrylthiocholine iodide (BuTChI) redox chromogenic indicator 2,6-dichlorophenolindophenol – sodium salt, dextran (all Sigma-Aldrich, Czech Republic), non-ionic surfactant C12-14 alcohol 7EO, trade name Spolapon 247 (Enaspol, Czech Republic), absolute ethanol (Penta, Czech Republic) and redistilled water were used for the work. A buffer solution of pH 7.3 was prepared using Na2HPO4.H2O and KH2PO4 (both Sigma-Aldrich, Czech Republic). To verify the functionality of the biosensor physostigmine (Sigma-Aldrich, Czech Republic) was used.

White cellulosic fabric served as a carrier of the enzyme. The substrate and the chromogenic agent were applied to the filter paper. The individual working zones were mounted on a white backing made of plastic.

Portable tristimulus colorimeter LMG 173 (Dr. Lange, Germany) was used to objectively measure the colour change.

2.2 Preparation of biosensor

The biosensor whose structural diagram is shown in Figure 1, consisted of plastic backing on which a zone with immobilised enzyme was stuck, etalon and a zone impregnated with substrate and chromogenic agent. The enzyme zone was prepared so that the white cellulosic fabric was for 25 minutes immersed in a solution containing BuChE of specific activity of 5 to 50 nkat/ml, 3.5% (wt.) of dextran and 1.5% (wt.) of non-ionic surfactant in a buffer solution of pH 7.4. The impregnated fabric was dried at a temperature of 20 to 25°C for 24 hours. Etalon was made of white cellulose fabric, the same as the one used for enzyme immobilisation. The zone with the substrate and chromogenic agent was prepared so that the filtration paper was immersed for 5 minutes in a solution containing 0.1 to 10% buTChI and 0.05 to 1% of 2,6-dichlorophenolindophenol in 50% ethanol. The impregnated paper was dried at a temperature of 20 to 25°C for 6 hours.

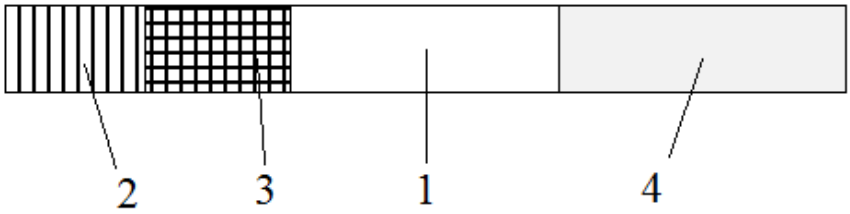

Figure 1. Schematic structure of biosensor (1 – plate, 2 – indication zone with the enzyme, 3 – etalon, 4 – zone with substrate and chromogenic agent)

2.3 Biosensor testing

The biosensor prepared in the above described manner was tested for a case of water analysis. Biosensor zones with immobilised enzyme and etalon were immersed for 60 seconds in the analysed sample and, when removed, incubated for 1-10 minutes. The plastic plate was then folded and the opposing carriers were pressed (for 60 seconds) to one another so that the zone with the substrate and the chromogenic agent overlapped two remaining zones, which were stained due to that contact. Once the zones separated, the discoloration rate of the zone with immobilised enzyme was observed in comparison to the etalon, the coloration of which remained stable.

2.4 Tristimulus colorimetry

To objectively measure the colour changes tristimulus colorimetry – reflex colorimetry (spectrophotometry) based on the CIE-L*a*b* colour system – was used. In this system, L* represents the neutral luminance axis, a* the chromatic green-red axis (+a* red, -a* green) and b* the chromatic blue-yellow axis (+b* yellow, -b* blue). In practice colour differentiation ΔE is also used, which is defined by the below equation:

$$\Delta E = \sqrt{\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2}},$$

where ΔL*, Δa* and Δb* are the differences between the individual values L*, a* and b* of the standard and controlled colour. In this work, the values on the b* axis were used as an analytical signal.

2.5 Determination of detection limit

The detection limit was determined visually as the inhibitor concentration corresponding to 25% inhibition efficiency (I) according to the below relation

$$I\ (\%) = (1 - T_0 / T) \times 100$$

where T_0 is the time (min) of the discolouration of the redox indicator with a blank sample (control time) and time T (min) its discolouration in the presence of the inhibitor.

3. RESULTS AND DISCUSSION

3.1 Staining characteristics

The studied method was based on the evaluation of the kinetics of the enzymatic reaction and the change in the colouration (discolouration) of the redox indicator. The scheme of enzymatic hydrolysis of the substrate and the reduction of 2,6-dichlorophenolindophenol by the formed thiocholine is shown in Figure 2. The original blue colouration (λ_{max} = 606 nm) and the discolouration of the indicator zone was homogeneous. Colour homogeneity of the indicator zone was achieved by optimising the concentrations of the individual analytical reagents and the reaction conditions. Figure 3 demonstrates the various phases of development of the colouration indicator zone in case of uncontaminated water (blank sample).

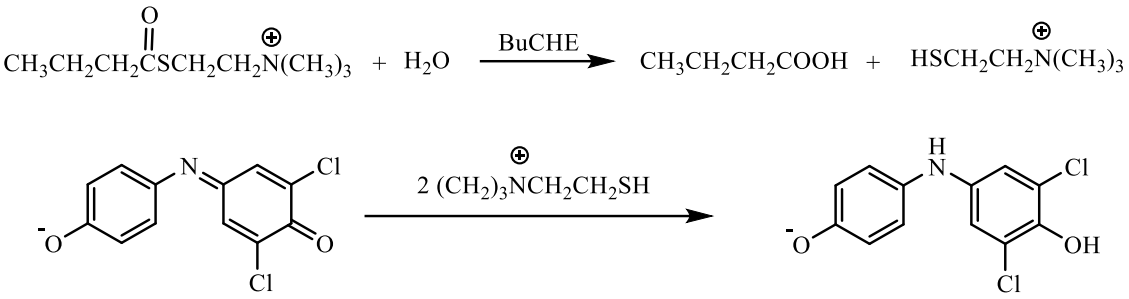

Figure 2. Reaction scheme

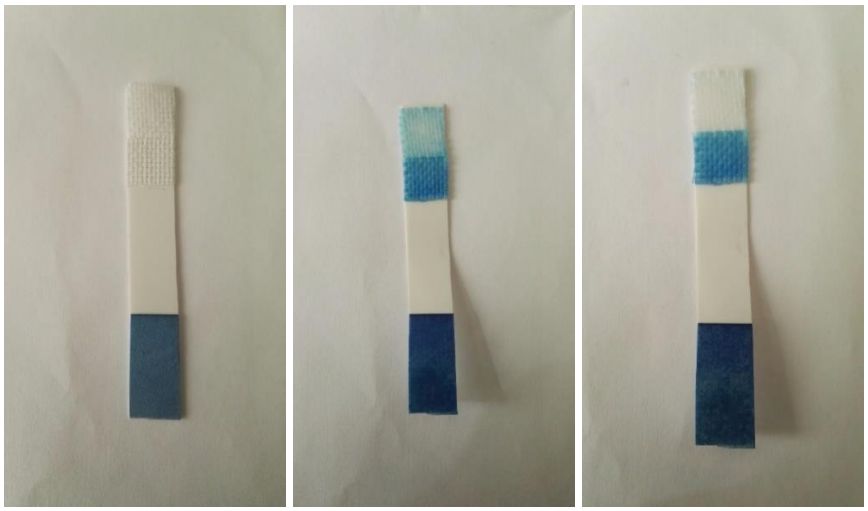

Figure 3. Biosensor before using (left), after exposure to a blank sample (middle), and after the complete discolouration of indication zone (right)

3.2 Effect of the reaction conditions

The paper investigates the analytical effect of BuChE activity in a solution used for impregnating the sheet carrier. The dependence of change in the colouration (discolouration) of the 2,6-dichlorophenolindophenol redox indicator on the activity of BuChE in the impregnating solution was observed up to the value of 60 nkat/ml; further activity increase of the enzyme had virtually no effect. It was determined that for an ideal course of detection the concentration of the substrate to the redox indicator must be in excess of at least 6:1, ideally then 10:1. A lower proportion of the substrate resulted in reduction of the discolouration rate (Figure 4) and a less prominent colour response. Based on the study of the relation between the rate of discolouration of the blank sample (control time) and

the sample with inhibitor physostigmine, the selected ideal redox indicator concentration in the impregnating solution was 0.5%. This paper further observes the dependence of the analytical system on pH. An analytically optimum environment had a pH range between 7 and 9. The dynamics of the redox indicator discolouration (colouration intensity dependence on time) is shown in Figure 5.

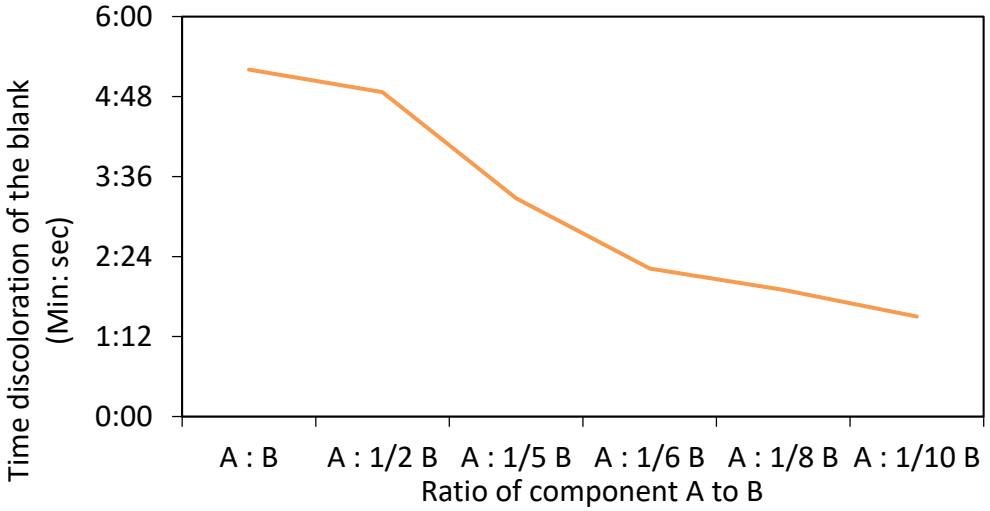

Figure 4. Discoloration time depending on the ratio of the substrate (A) and the chromogenic agent (B)

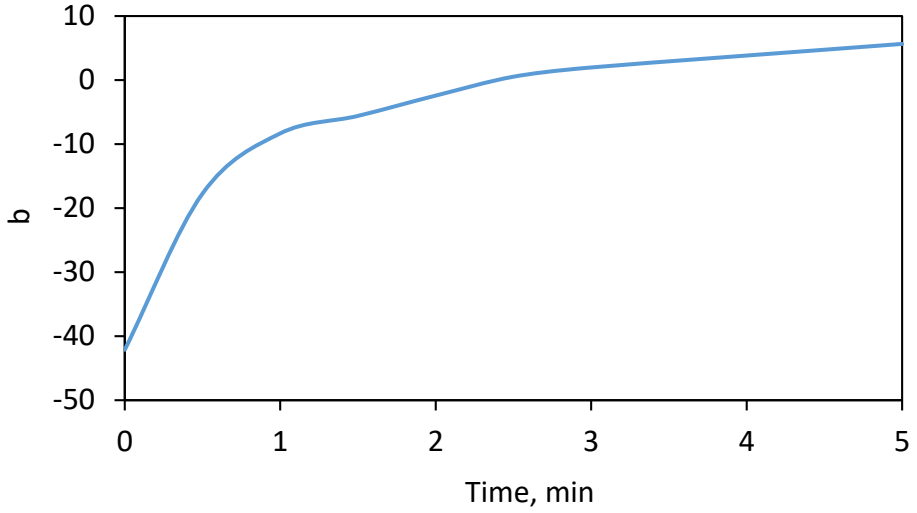

Figure 5. The dependence of value b (CIE-L* a* b*; three-dimensional colorimetry) on time

3.3 Analytical data

The function of the designed biosensor of cholinesterase inhibitors was tested in a solution of physostigmine in a concentration range of 0.001-100 mg/ml. The evaluation of the change in colouration (discoloration) of the redox indicator was performed visually (with the naked eye) and complementarily using the tristimulus colorimetry method. On the basis of the obtained results, the detection limit of 0.01 micrograms/ml was determined (optimum enzyme activity in the impregnating solution 15 nkat/ml, incubation time 5 minutes). The repeatability (precision) of the measurement was tested by repeating the analysis of the physostigmine control sample during 5 consecutive days. The measurement error (discolouration rate) was ± 30%.

3.4 Interference and stability

The detection is disturbed by reducing agent (sulphides, sulphites, thiols, cyanides) and strong oxidizing agents (chlorine, nitrites). The presence of denaturing active organic solvents, e.g. N, N-dimethylformamide, dimethylsulphoxide, high content of ethanol and methanol (more than 30%), acetone, benzene and the like is also undesirable. Carrier specimens with immobilised enzyme were stored under normal laboratory conditions. The activity of the enzyme on the carrier during the storage tests over eight months practically did not change. Also confirmed was the high resistance of the immobilised enzyme, substrate and redox indicator to temperatures up to 45°C.

4. CONCLUSION

For quick and simple enzymatic method of cholinesterase inhibitors detection a new biosensor was designed with immobilised butyrylcholinesterase, substrate butyrylthiocholine and chromogenic indicator 2,6-dichlorophenolindophenol. The function of the biosensor was tested on physostigmine in water with a detection limit of 0.01 μg/ml (visually with the naked eye). The construction of the biosensor allows detection of the presence of cholinesterase inhibitors in the air as vapours or aerosols (it is sufficient to moisten the zone with immobilised enzyme and etalon prior to the exposure with water). The designed agent is suitable for use in the area of protection of troops and population in times of extraordinary security measures, or for field monitoring of the compliance with the principles of hygiene and safety of work with toxic pesticides.

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