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Photocolorimetric Biosensor for Detection of Cholinergic Organophosphorus Compounds

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ABSTRACT

To detect nerve agents in practice, the analytical methods such as gas, liquid and thin-layer chromatography, mass spectrometry or capillary electrophoresis are usually used. Apart from these analytical methods, we developed an analytical device (tape photocolorimetric biosensor) based on the modified Ellman`s cholinesterase biochemical reaction for multidetection of cholinergic organophosphorus compounds. Enzyme butyrylcholinesterase was used as a biorecognizing component and its activity was evaluated by red, blue, green (RGB) sensor. This method eliminates errors in the evaluation and provides automatic data collection with their subsequent evaluation. The unique method of dosing allows appropriate dispensing of reagents in microlitres volumes and the whole system is simple to operate. Suitability of the constructed biosensors was evaluated using the six organophosphates (Tabun, sarin, Soman, cyclosin, VX and R33 compound). Biosensor showed the ability to measure substances at concentrations ranging between ~ 1×10^{-8} mg/l - 1×10^{-6} mg/l in the air, according to their inhibition effect.

Keywords : Nerve agent, Ellman´s reaction, tape biosensor, colorimetric detection

1. INTRODUCTION

Nerve agents are the most toxic synthetic compounds that were selected for combat use[1]. Many non-toxic organophosphorus compounds with the same basic structure are used in industry as softeners, hydraulic fluids, plasticizers, etc.[2]. The wide-reaching application of these substances is in agriculture and forestry as pesticides[3]. The main toxic effect of nerve agents is interference with nervous system. Nerve agents affect the transmission of nerve impulses by inhibiting acetylcholinesterase (AChE), which ensures neurotransmitters acetylcholine (ACh) degradation. Acetylcholine is responsible for the transmission of nerve impulses. Acetylcholine is decomposed in normal cholinergic transmission immediately after transmission of nervous impulses by the catalytic action of the enzyme acetylcholinesterase[4,5]. Under optimal conditions, a molecule of the enzyme hydrolyzes about 2,000 molecules of ACh per second[5]. Nerve agents inhibit the active hydrolytic centre of AChE and irreversible enzyme-inhibitor complex is formed. This process is known as ageing. Enzyme inactivation is irreversible and recovery mainly depends on synthesis of new enzyme. The half-time ageing (time for half of involved cholinesterase to age) varies from 2 min for Soman, to >40 hours for VX and Tabun[6].

To detect nerve agents in practice, the analytical methods such as gas, liquid and thin-layer chromatography, mass spectrometry or capillary electrophoresis are usually used[1,7].

The detectors operating on the ion mobility spectrometry principle (RAID - 1, Raid M, detector Dräger X-AM, ChemPro detector) are widely used to detect of nerve agents in the air. The detectors using flame spectrophotometer (AP2C, AP4C) and the detectors using surface acoustic wave technology (JACD ChemSentry detector) are used, too. These miniaturized detectors compare measured data with the database stored in the memory. Difficult determination of less known toxic derivatives (i.e. the false negative detection) and higher acquisition prices are their main disadvantages. Its detection limit is 10^{-5} mg/l in the air.

The above mentioned shortcomings can be eliminated by using biosensors. Biosensors are portable, fast and highly sensitive bioanalytical devices. The biosensors based on cholinesterase inhibition achieve extremely high sensitivity[8]. Generally, these biosensors use two types of cholinesterase: acetylcholinesterase and butyrylcholinesterase (BuChE)[9]. Many AChE-based biosensors have been proposed on electrochemiluminescence[10,11], fluorescence[12,13], voltametric[14] and amperometric methods[15-18].

This study describes the results achieved during the testing of the laboratory sample of the tape photocolorimetric portable biosensor using utilizing cholinesterase reaction (CHR) for detection of nerve agents.

CHR is a colorimetric method based on hydrolysis of

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the substrate (usually an ester of choline, or thiocholine) by cholinesterase (acetyl-or butyrylcholinesterase). This catalyzed hydrolysis produces acid and choline (thiocholine). Thiocholine produced by the CHR can be determined by Ellman´s method. The principle of this method is based on the reaction of thiocholine with chromogenic thiol reagent, 5,5 '-dithiobis-(2-nitro) benzoic acid (DTNB). 5-sulphanyl-2-nitrobenzoic acid (yellow colour) is formed during the CHR, and measured at 412 nm (as shown in Fig. 1). In the presence of nerve agent, the reaction is blocked (no colour change)[19,20].

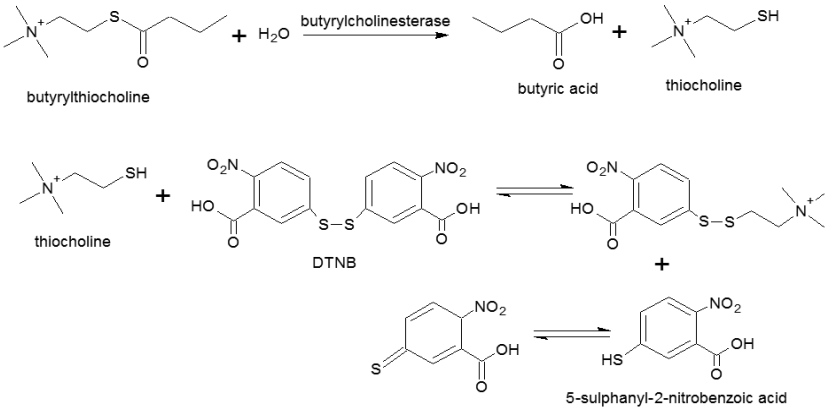

Figure 1. The course of CHR with Ellman´s reagent.

2. EXPERIMENTAL WORK

2.1 Chemicals and Materials

O-ethyl N,N-dimethylphosphoroamidocyanidate (Tabun; GA - purity 97.24 %), O-isopropyl methylphosphono-fluoridate (Sarin; GB - purity 93.61 %), O-pinakolyl methylphosphonofluoridate (Soman; GD - purity 99.00 %) O-cyclohexyl methylfosfonofluoridate (Cyclosarin; GF - purity 98.01 %) O-ethyl S-[2-(diisopropylamino)ethyl] methylphosphonothioate (VX - purity 90.76 %) and O-isobutyl S-[2-(diethylamino)ethyl] methylphosphonothioate (R33 - purity 30.30 %) were manufactured in the Slovak Republic (Zemianske Kostol'any). 5,5´-dithiobis-2-nitrobenzoic acid (DTNB - purity: > 97.5 %), butyrylthiocholin iodide (BTCHJ - purity: ≥ 98.0 %), sodium bicarbonate ($NaHCO_3$ - purity 99.0-100.0 %), sodium hydrogen phosphate ($Na_2HPO_4.2H_2O$ - purity min. 99.5 %) and potassium dihydrogen phosphate (KH_2PO_4 - purity ≥ 99.0 %) were supplied by Sigma Aldrich. Sodium hydroxide (*NaOH* - purity min. 98.0 %) and boric acid (H_3BO_3 - purity min. 99.5 %) were supplied by Lach-Ner. Methanol (purity 99.8 %) was supplied by Chromservis. Butyrylcholinesterase (BChE, EC 3.1.1.8., specific activity 150 μkat/l) was supplied by Bioveta.

2.2 Solutions

- Buffers: phosphate (pH 7; 0.1 M), borate (pH 7; 0.05 M)
- Substrate: butyrylthiocholin iodide, 0.075 M (23.77 mg were dissolved in 1 ml distilled water).
- Reagent: 5,5'-dithiobis-2-nitrobenzoic acid, 0.01 M (39.6 mg were dissolved in 10 ml phosphate buffer and 15 mg of sodium bicarbonate was added)
- Mixture: 0.1 ml substrate and 0.5 ml of reagent were dispersed into 13.3 ml of borate buffer
- Enzyme: 50 mg of butyrylcholinesterase was dissolved in 2 ml of borate buffer.

2.3 Principle of Detection with Photocolorimetric Detector

The proposed biosensor described in this article uses Ellman´s modification of CHR. This reaction goes on the white cotton tape 15 mm wide and 0.35 mm thickness. This is an ordinary tape biosensor with improving elements. In particular, this is a new principle of evaluating the results of this reaction using as photocolorimetric sensor. Photocolorimetric sensor can evaluate the colour change of a reaction. The method of dosing, which allows the appropriate dispensing reagents in microlitres volumes, is also an advantage. The whole system consists of three basic parts as shown in Fig. 2. The first part is pneumatic and provides sampling across the tape by sampling pump, flow stabilization and the measurement of its value, using a flow meter. The second part is the dosage system that ensures each reagent dosing pump and dispenser containment vessels with the pressure relief system. The last part serves as the evaluation part and is described below.

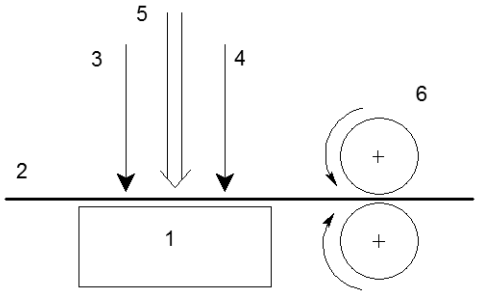

Figure 2. Scheme of the detector.

Note: 1- digital RGB sensor, 2 - cotton tape, 3 - spraying of enzyme, 4 - spraying of reagents, 5 - sampling samples, and 6 - tape shifting mechanism.

2.4 Description of the Measurement Process

The algorithm of the measuring procedure is as follows:

(a) Insertion of the cotton tape for detection and its mechanical shift
(b) Spraying a defined volume of enzyme solution BuChE (the enzyme is applied at ambient temperature and at pressure not higher than 0.15 bar)
(c) Sampling samples across the tape
(d) Spraying of a defined volume of the mixture of substrate butyrylthiocholin iodide (BTChI) and Ellman´s reagent
(e) Scanning an optical signal
(f) Data evaluation

The whole measurement process takes 5 min (2 min for sampling a sample, 1 min for incubation, and 2 min for scanning an optical signal). In case of VX compound, a part of the sampling device was heated to 40 °C during the whole measurement. Limit of detection (LOD - is the lowest analyte concentration likely to be reliably distinguished from the blank

and at which detection is feasible)[21] and limit of quantification (LOQ - is the lowest concentration at which the analyte can not only be reliably detected but at which some predefined goals for bias and imprecision are met)[21] were obtained for each substance from statistical processing of measured data (depending on the rate constant of the absolute amount of nerve agent):

$$LOD = k + 3 \times s_y,$$

$$LOQ = k + 10 \times s_y,$$

where s_y is the residual standard deviation of measurement, and k (the rate constant) is the value on the y axis of the regression line (mg/l).

3. RESULTS AND DISCUSSION

3.1 Design of Evaluation

The course of a reaction was evaluated using a digital colour sensor which is sensitive to red, blue and green spectrum. Each colour is given by the intensity of these three primary colours. Ellman´s modification of CHR is based on the colour transition yellow-white. For this transition, there is critical representation of blue colour, i.e. the value of the signal B. The changes of the values of the signal B depending on time were measured for each concentration of nerve agents as shown in Fig. 3. Methanol was used for the preparation of the calibration solutions (its presence does not affect the activity and stability enzyme during detection). Its presence also improved sample evaporation during the spraying. The rate constant k was obtained from the linear regression of the equation (a polynomial approximation of the measured data): $y = (k \times x) + q$.

The time for reading of the rate constants was optimized on the values of determination coefficients R^2 (it´s main purpose is the prediction of future outcomes on the basis of other related information) for Soman as shown in Table 1. Table 1 shows the values of the determination coefficients of the regression equations for different time intervals during the measurement of Soman (time counted from spraying a mixture of substrate and DTNB).

As the optimal time, 90-100 seconds after spraying the mixture of substrate and reagents were chosen. From the calculated rate constants, it can be determined whether or not the sample contains nerve agents.

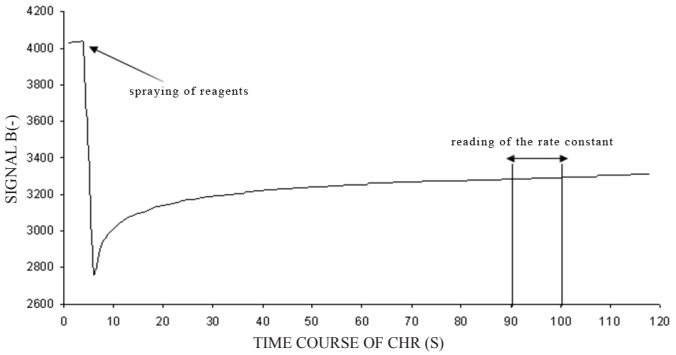

Figure 3. Example of the curve obtained from measurement.

Table 1. Determination coefficients of the regression equations for different time intervals

Time course of CHR (s)	Coefficient of determination R^2 (-)
10-20	0.521
20-30	0.787
30-40	0.909
40-50	0.924
50-60	0.937
60-70	0.937
70-80	0.957
80-90	0.982
90-100	0.986
100-110	0.977
110-120	0.968

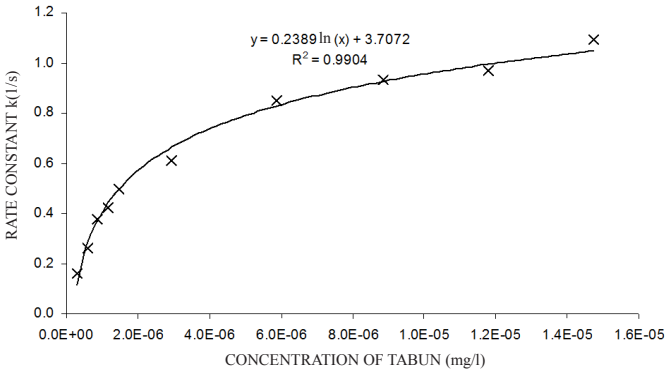

Figure 4. The detector response to GA in the air.

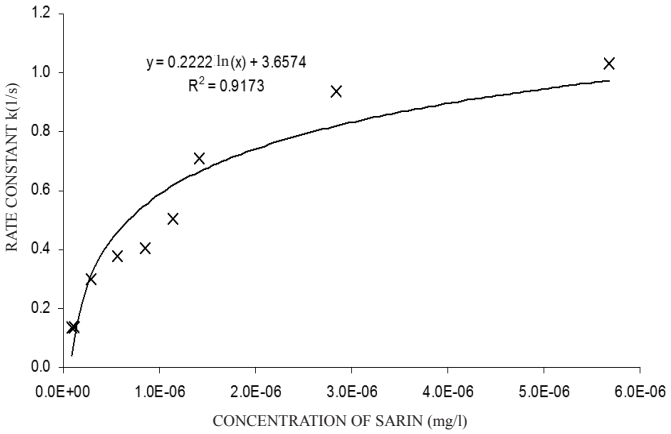

Figure 5. The detector response to GB in the air.

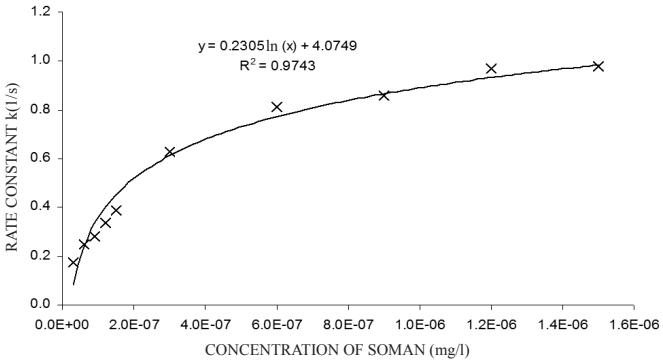

Figure 6. The detector response to GD in the air.

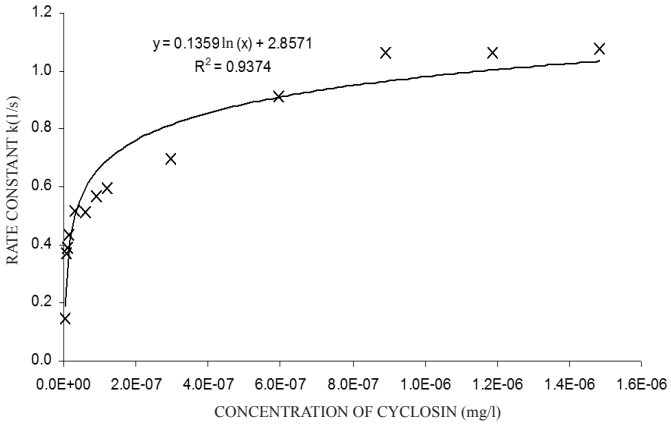

Figure 7. The detector response to GF in the air.

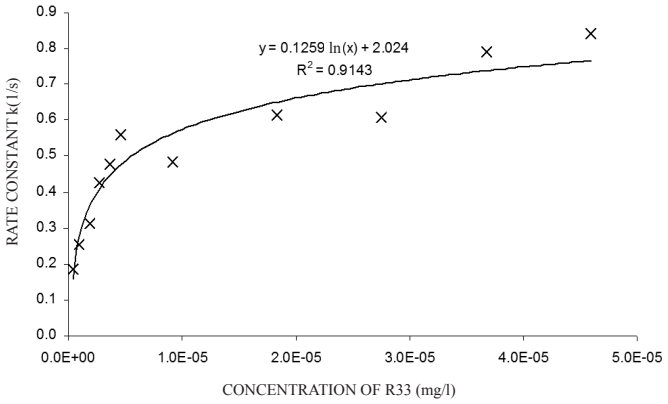

Figure 9. The detector response to R33 in the air.

3.2 Calibration Curves of Nerve Agents in the Air

Dependencies of rate constants on the concentration of nerve agents for Tabun, Sarin, Soman, Cyclosin, VX compound, and R 33 are shown in Figs. 4, 5, 6, 7, 8, and 9 respectively, were measured by the proposed tape photocolorimetric detector. The point of each concentration level was measured 10 times and each curve was constructed from their average values. The Figs. 4-9 shows that the highest inhibitory effect (according to the lowest nerve agent amount giving the positive signal of detector) was reached for Cyclosin and VX substance, while Tabun and R33 showed the lowest inhibitory activity. This fact correlates with the inhibition effect and thus the toxicity of individual organophosphorus inhibitors.

Limit of detection (LOD) and limit of quantification (LOQ) for proposed photocolorimetric biosensor were obtained by statistical processing of linear parts of the measured dependencies of rate constants on the nerve agents concentration as shown in the Table 3.

Table 3. LOD and LOQ for nerve agents in the air

Nerve agents	LOD (mg/l)	LOQ (mg/l)
GA	7.66×10^{-7}	2.55×10^{-6}
GB	7.16×10^{-7}	2.39×10^{-6}
GD	1.32×10^{-7}	4.41×10^{-7}
GF	1.69×10^{-7}	5.62×10^{-7}
VX	1.12×10^{-6}	3.73×10^{-6}
R33	3.08×10^{-6}	1.03×10^{-5}

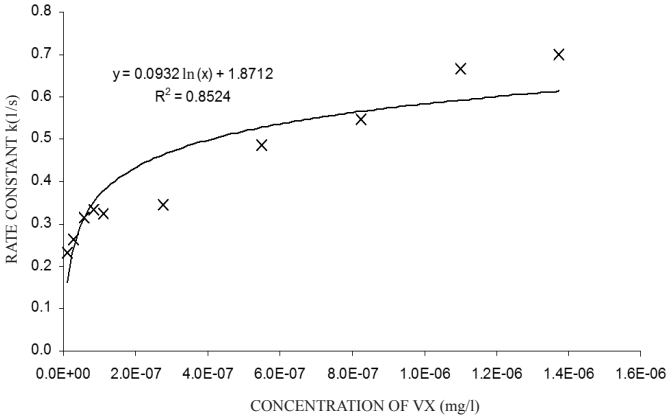

Figure 8. The detector response to VX in the air.

4. CONCLUSION

In summary, the simple and sensitive portable means for detection of nerve agents has been demonstrated. It is characterized by high robustness and reliability. The evaluation of the results is not dependent on subjective evaluation and allows an operator to work under difficult visual conditions. The whole analysis needs only 5 min and is simple to operate without sophisticated instrument. The method of dosing allows to dose the appropriate dispensing reagents in microlitres volumes. On the basis of the measurement it is clear, that this photocolorimetric biosensor can be used in future to detect all type of organophosphorus cholinesterase inhibitors in environment.

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