Automated Detection of Organophosphate Warfare Gases (Nerve Agents) in Air Based on Micro-SIA – lab-on-valve System.

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Summary:

Equipment for fast and accurate detection of organophosphate nerve agents is presented. This method is based on the spectrophotometric monitoring of enzyme activity of butyrylcholinesterase after its contact with air in a special absorption unit (scrubber) developed. The scrubber was made from a glass tube filled with glass beads (diam. 3 mm) and filled with approx. 5 ml of butyrylcholinesterase in phosphate buffer of pH 7.4. The air sample was bubbled through this solution for 20 s at a flow rate of 80 l $hour^{-1}$. Thereafter 8µl of the enzyme solution were aspirated into the microSIA – LOV analyzer and the activity of the enzymes were evaluated by using Ellman's reagent, i.e. 2.5 mmol l^{-1} butyrylthiocholine iodide and 0.25 mmol 5,5'-dithiobis (2-nitrobenzoic acid). The absorbance of the coloured reaction product was measured at 412 nm after the reaction time of 60 s. The residue of the absorption liquid was washed away from the absorber and the system was washed with enzyme solution prior to next analysis. The contaminated air caused partial inhibition of the enzyme activity of absorption liquid. Activity of the contaminated sample was compared with the activity of the unaffected enzyme (blank measurement). The analysis was controlled by two PCs.

The effect of the concentration of analyte in the absorption liquid on the enzyme activity was tested for 10^{-5} –

10^{-9} mol l^{-1} sarin. A single analysis (including the absorption step) took <130s.

Key words:

Sequential injection analysis; lab-on-valve; organophosphate; cholinesterase; nerve agent; sarin

1. INTRODUCTION

Organophosphate-based warfare gases (nerve agents such as sarin, soman, tabun and VX that interfere with the central nervous system through inhibition of cholinesterases) are potentially fatal chemicals that are banned by the Chemical Weapons Convention (CWC) (OPCW, 1974). Relative simplicity of their illegal production at minimal expense, release of sarin vapors in the Tokyo subway system in 1995 and several other events in the past decade have raised concerns that they could be possibly used for large-scale terrorist attacks against civilians. Therefore relatively inexpensive automated analytical devices capable of rapid on-spot sensing of nerve agents (NAGs) in air and giving early warning to civilian responders or military personnel are of interest. A number of sophisticated but costly analytical methods (such as ion-mobility MS, GC-MS, Surface Acoustic Wave Sensing and FT-IR) were approved by the CWC as official ones for routine detection and monitoring of CWC chemicals (Mesillakso, 2005).

Mechanism of OP induced inhibition of cholinesterases is similar for the OP agents. Esteratic centre of cholinesterase is phosphorylated or phosphonylated. This complex can undergo spontaneous hydrolysis, resulting in active form of enzyme. Half-life of this complex ranges from hours to days. When dealkylation of phosphorylated/phosphonylated complex takes place, cholinesterase remains inactivated permanently and cannot be reactivated (this process is named “ageing”) (Bajgar, 2005). Spontaneous reactivation is affected by enzyme source, temperature, pH, ionic strength and structure of the acyl attached (Patočka et al., 2004).

The scheme of organophosphate- induced inhibition of cholinesterase (Patočka et al., 2004):

$$E + PX \underset{}{\overset{K_d}{\leftrightarrow}} EPX \overset{k_2}{\rightarrow} EP \overset{k_3}{\rightarrow} E + P$$

E…enzyme, PX…organophosphate, EPX…intermediate complex, EP…phosphorylated/phosphonylated enzyme, K_d…dissociation constant, k_2…reaction rate of phosphorylation/phosphonylation, k_3…reaction rate of defosforylation/defosfonylation (Patočka et al., 2004).

Both AChE and BuChE are target structures of organophosphate-based nerve agents. Since the mechanism of inhibition is assumed to be the same, both enzymes have been widely used in the estimation of the presence of

OP inhibitors (Ždárová Karasová, 2010). While AChE is more sensitive, BuChE is readily available and less expensive, that makes it convenient for routine sreening method. On the other hand, physiological function in living organisms, substrate specificity and sensitivity to inhibitors are different.

In the present paper we attempted to utilize the inhibitory effect of the NAGs on butyrylcholinesterase (BuChE) for their quantitative assay through spectrophotometric measurement of the decreased activity of BuChE (exposed to an NAG) by Ellman's reaction (Ellman et al., 1961). This indicator reaction involves interaction of the BuChE with butyrylthiocholine (BTCh) that is hydrolysed to thiocholine; thiocholine reacts with Ellman's reagent (5,5´-dithio-bis(2-nitrobenzoic) acid) (DTNBA) to produce yellow thionitrobenzoate exhibiting maximum absorption at 412 nm (proportionally related to the activity of the BuChE). Method has been widely used for cholinesterase activity assays in biological fluids, for cholinesterase activity assays in presence of inhibitors (both organophosphate- and carbamate-based) and reactivators. Limitations in the use of Ellman's method were discussed in literature (interaction of DTNBA with –SH groups of plasma proteins, oximolysis – breakdown of DTNBA caused by oxime groups of reactivators) but modifications of the method aimed to reduce or overcome the drawbacks were reported as well (Ždárová Karasová, 2010). The concept of sequential injection analysis (SIA) in the lab-on-valve (LOV) format developed by Ruzicka (Ruzicka, 2000) was employed to automate the manipulation of reactant solutions at μL levels.

2. EXPERIMENTAL

2.1 Chemicals

Lyophilized BuChE from equine serum was purchased from SEVAC (Prague); BTCh, DTNBA, insecticide tetraethyl pyrophosphate (TEPP), insecticide 1-naphthyl-N-methylcarbamate (Sevin) and anhydrous ethanol were obtained from Aldrich; KH_2PO_4 and $Na_2HPO_4.12H_2O$ were obtained from Lachema (Brno), sarin was obtained from the NAGs collection of the Faculty of Military Health Sciences, Hradec Kralove. Stock solution of enzyme was prepared and dispensed into Eppendorf vials. These were frozen immediately, stored at -20°C and thawed at the time of use. Each vial contents was thawed only once. No changes in the enzyme activity were noticed during one day of operation. Reagents were prepared fresh daily, stored at room temperature during experiments, refrigerated overnight. Solutions containing DTNBA were protected from sunlight. Under these conditions no changes in activity were noticed during one week period.

2.2 Apparatus

A PC-controlled micro-SIA apparatus equipped with a lab-on-valve platform (FIAlab Instruments Inc., Bellevue, USA) comprising a 6-port selection valve, a 1.0 ml syringe pump and USB2000 Ocean Optics spectrophotometer was used as the basis of the NAG detection setup. A glass scrubber packed with glass beads (Fig. 1) included three PC-controlled valves; it was manufactured in the lab workshop. A miniature air pump was used to bubble the sample air through the scrubber. Air samples with defined sarin concentrations were prepared by using a dynamic evaporation chamber (Ševelová et al). For scheme of measurement protocol see table 1.

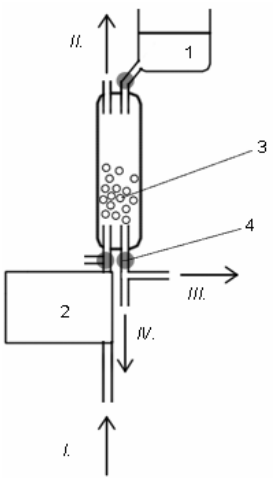

Fig.1: Scrubber Module

1: Fresh enzyme solution container

2: Air pump

3: Glass Beads and absorbing liquid

4: PC-controlled pinch valves

I: air inlet

II: air outlet

III : absorbing liquid to SIA

IV: waste

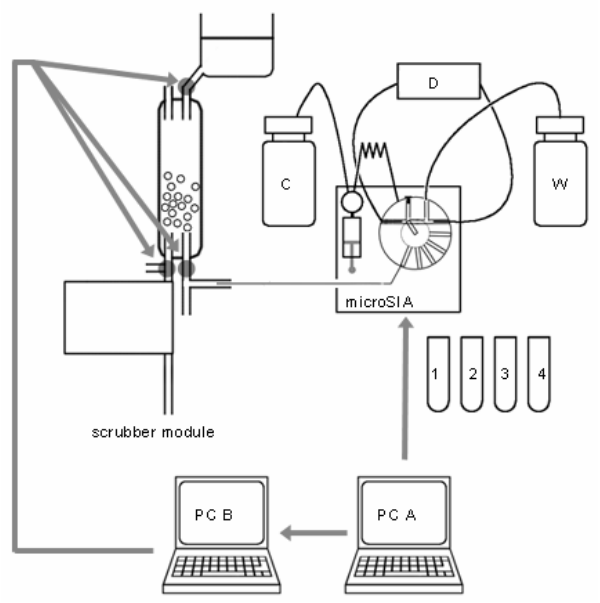

Fig. 2 Scheme of the system, connection of the absorber and SIA units.

Legend:

PC A – controlling microSIA-LOV, PC B – controlling the scrubber valves, C – carrier solution, D – spectrophotometric detector, W - waste. In the given part of the program, PC A acts as an actuator giving signal to PC B to execute a new absorption cycle. 1, 2, 3, 4 – vials containing reagents and substrate solution.

Tab.1 Measurement protocol.

step	SIA measurement protocol
1	SIA washes the detection cell, aspirates and flushes 500 µl of water.
2	Spectrometer performs a reference scan.
3	SIA aspirates 300 µl water + 8 µl of blank enzyme solution + 2 µl of substrate - reagent solution
4	Enzyme reacts with thesubstrate – reagent solution for 60s.
5	SIA delivers 75 µL to spectrophotometer.
6	Spectrometer performs reaction product measurement at 412 nm.
7	SIA flushes the holding coil and empties the detection cell.
8	SIA aspirates 600 µl of enzyme solution from absorber and flushes it to prevent sample contamination.
9	SIA aspirates 300 µl water + 8 µl of enzyme solution from absorber.
10	PC A, controlling the SIA part, sends signal to PC B, controlling the scrubber, to remove the inhibited enzyme solution from the scrubber and to start a new cycle of absorption of sarin from the air.
11	SIA aspirates 2 µl of substrate - reagent solution.
12	Enzyme reacts with substrate - reagent solution for 60s.

13	SIA delivers 75 µL to spectrophotometer.
14	Spectrometer performs reaction product measurement at 412 nm.
15	SIA flushes the holding coil and empty the detection cell.

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step	absorber protocol
1	The scrubber is filled with approx. 5ml of enzyme solution.
2	Air sample bubbles through the absorbing liquid for 20 s.
3	Enzyme solution is flushed to collection point from which it is consequently aspirated by SIA.
4	PC B waits for the signal from PC A.
5	The residual enzyme solution is flushed into waste, the scrubber is washed by filling and emptying with the enzyme solution into absorption space.
6	New absorption process starts.

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120 *2.3 Optimization and procedures*

121 Initially the SIA conditions for the Ellman's reaction were optimized apart from the scrubber by using less
122 hazardous BuChE inhibitor solutions, namely Sevin and TEPP, with respect to concentrations and volumes of
123 reactants aspirated, time of BuChE – inhibitor interaction (20 – 600 s) and inhibited BuChE – (Ellman's
124 reagent + BTCh) incubation time (15 – 120 s). Inhibition curves relating the percentage of BuChE retained
125 activity (E_A%) to the concentration of the inhibitors (10^{-9}- 10^{-4} mol l^{-1}) were measured; the E_A % was calculated
126 as (h_x/h_0).100 where the h_x and h_0 are the peak heights of the thionitrobenzoate at 412 nm obtained with the
127 inhibited and native uninhibited enzyme injections respectively. After this, the inhibition curve of BuChE with
128 sarin solutions was measured. Consequently the dependence of the level of inhibition of enzyme on the time of
129 BuChE – sarin interaction (300-700 s) was also examined.

130 To study the effect of the air contaminated with sarin on the E_A %, the air sample containing 1 µg/L of sarin
131 was pumped through the scrubber (packed with glass beads) containing 5 mL of absorbing liquid (enzyme
132 activity 87U; 0.12 mg mL^{-1} of BuChE in phosphate buffer, pH 7.40) at the flow rate of 80 $l.h^{-1}$ for 20 s.
133 Thereafter 8 µl of the absorbing liquid and 2 µl of the reagent (2.5 mmol BTCh + 0.25 mmol DTNBA) were
134 aspirated, allowed to react for 60 s and sent to the detector at a flow rate of 4 µL s^{-1} (peak height h_x); the h_0 was
135 obtained by aspirating 8 µl of uninhibited enzyme solution (0.12 mg ml^{-1} of BuChE in phosphate buffer of pH
136 7.4) instead of the absorbing liquid. Also in this measurement the dependence of the level of inhibition of

enzyme on the duration of BuChE – sarin interaction (300-700 s) was evaluated.

3. RESULTS AND DISCUSSION

3.1 Inhibition curves

Optimum SIA conditions for the measurement of the inhibition curves of BuChE with Sevin as relatively weak inhibitor (carbamate) and TEPP as much stronger inhibitor (organophosphate) were following - carrier water, order of the aspirated zones: 4 µl of the inhibitor solution, 8µl of 0.12 mg ml^{-1} BuChE in phosphate buffer pH 7.4, another 4 µl of the same inhibitor solution (sandwich mode) and inhibition time 60 s; thereafter 8 µl of reagent solution (2.5mmol l^{-1} BTCh + 0.25mmol l^{-1} DTNBA) aspirated and after the incubation time of 60 s the zones with the yellow reaction product thionitrobenzoate were pushed at 4µl s^{-1} into the detector channel of the LOV module. TEPP and sevin are pesticides employed as model structures of organophosphate- or carbamate-induced cholinesterase inhibition. They differ both in structure and kinetics of their action. The inhibition curves of Sevin (curve 1 and 2) and TEPP (curves 3 and 4) for inhibition times 60 s and 600 s are shown in Fig. 3. It can be clearly seen that even at 60 s inhibition time TEPP can be reliably detected (causing 10% BuChE inhibition) at 0.1 µmol l^{-1} concentration levels with repeatability characterized by RSD 3.6 % (n=5). . Sevin is apparently weaker inhibitor. By 3 orders higher concentration of sevin is necessary to achieve the same level of inhibition as TEPP. For both inhibitors their effect is incubation time-dependent. By increasing the incubation time lower detection limits can be achieved. Regarding total inhibition of enzyme, incubation time of 600s is sufficient for both TEPP (concentrations $>1.10^{-5}$M) and sevin (concentrations $>1.10^{-3}$M).

Sarin inhibition curve was obtained with aqueous sarin solutions. Consequently, 0.2 mL of sarin solution was added into 1.8 ml of enzyme solution (0.012 mg ml^{-1} BuChE in phosphate buffer, pH 7.4) to obtain concentration scale 10^{-5} to 10^{-9} mol l^{-1} of sarin. After 300 s inhibition, enzyme activity was determined by SIA. 8 µl of this enzyme solution and 2 µl of reagent solution were aspirated, allowed to react for 60 s and sent to detector at the flow rate of 4 µl s^{-1}. The enzyme activity was then determined with the same samples in the same way at the times of inhibition 400, 500, 600 and 700 s.

This observation of dependence of inhibition on the time for each concentration of sarin showed that at time of inhibition under 400 s is not possible to reach 0% enzyme activity (Fig. 4).

Inhibition curve resulting from the SIA-LOV detection of sarin in liquid samples under optimum conditions indicated in the experimental section is shown in Fig. 5.

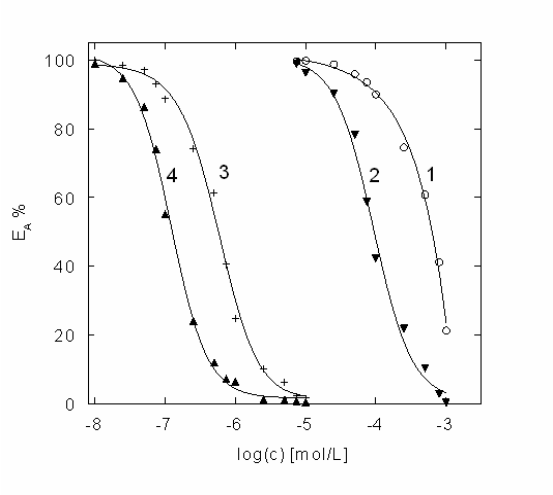

Fig. 3. **Inhibition curves (dependence of E_A% on the concentration of inhibitor) of BuChE as measured by spectrophotometric SIA-LOV technique with inhibitor solutions containing Sevin (1,2) or TEPP (3,4) at inhibition times 60 s (1,3) and 600 s (2,4).** For other conditions see the above text.

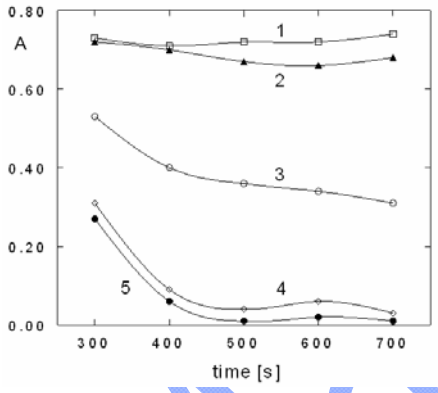

Fig.4 **Dependece of inhibition on the time of interaction between the enzyme and sarin**.

A: absorbance of the reaction product at 412 nm; concentrations of sarin [mol/L]: 10^{-9} (curve 1); 10^{-8} (2); 10^{-7} (3); 10^{-6} (4) and 10^{-5} (5).

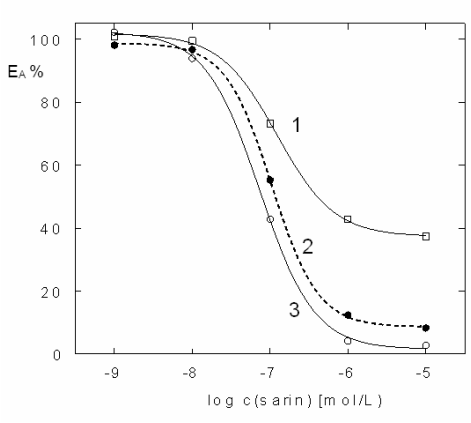

Fig. 5: **The inhibition curves of BuChE with sarin in liquid samples at different times of inhibition.** Inhibition time: 300 s (curve 1); 400 s (2) and 700 s (3). For more detail see the experimental section.

3.2 Detection of sarin in air

In order to verify ability of conversion of sarin from the air sample (containing 1 µg L^{-1} of sarin) into liquid medium, solution of BuChE in phosphate buffer was used as the liquid phase. The enzyme solution (placed in the scrubber) was purged with the sarin-spiked air sample for 20 s and consequently the enzyme activity was determined in the way described above by the reaction of 8 µl of enzyme solution and 2µl of reagent-substrate solution.

For 300 – 700 s time of inhibition 65 – 45% of original enzyme activity was retained. This is equivalent to 0.1 µmol L^{-1} of sarin in the enzyme solution. This fact shows that only approx. 3% of sarin has been recovered from the air sample. Rapid sample conversion is necessary for all early warning systems. The aim of the SIA setup design was to allow indication of air contamination in almost real time as it is with other devices designed for such purpose. The price paid for this feature is relatively low conversion of the analyte. Decreasing the air flow rate would be desirable for improving the conversion efficiency but this was not possible because of technical limitations of the absorber unit. The results of previous experiments indicate that if enzyme solution is agitated with sarin-contaminated air sample in closed gas burette, the inhibition of enzyme corresponding to 95% conversion of sarin is achieved within approx. 2 min (the 5% loss of sarin is probably caused by its nonenzymatic hydrolysis). Therefore the authors assume that relatively low trapping efficiency of sarin (3%) in the absorption liquid is caused by short and imperfect contact between air and liquid.

4. CONCLUSIONS

It can be concluded that under these conditions the detectable threshold concentration of sarin in liquid medium causing 10% inhibition of BuChE is approximately 1.4 mg m^{-3} of sarin. Considering approx. 3% efficiency of 20 s recovery of sarin from air samples into liquid, the threshold concentration corresponds 0.2 mg m^{-3} of sarin in the air. Effective dose of sarin vapors is EC_{t50} = 2 mg.min/m^3 (causing miosis, the very first recognizable sign of intoxication) while the lethal dose is LC_{t50} = 70 mg.min/m^3 (Riegle D. W., et al., 1994). Hence the sarin vapors can be safely detected by using the proposed SIA device. Our data compare well with data appearing recently for a commercial acoustic wave sensor (30 mg m^{-3}) (Matsushita et al., 2005) or portable ion mobility mass spectrometer (below 0.1 mg/m^{-3}) (Maruko et al., 2006). Comparison of the proposed method with cholinesterase-based field instruments employed by the Czech army is shown in Table 2. While CHP-71 is discontinuously working device based on passing air through the tubes containing enzyme, GSP-11 is continuously working analyzer. In the latter analyzer the enzyme solution is continuously dropped on a moving ribbon which is exposed to the air tested; after that the detection reagent is added and residual enzyme activity is assayed. Both analyzers work in 2-way modes, differing in enzyme consumption, sampling frequency and limit of detection. It can be concluded that our method is comparable in sensitivity with CHP-71 and that it is characterized by reduced need of manual manipulation. Method employing the GSP-11 shows better figures of merit compared to ours because of lower detection limit and lower enzyme consumption. As for portability and mobility, both CHP-71 and GSP-11 are PC-independent, corresponding in size and weight to SIA – LOV apparatus without the absorption module.

Tab. 2 Comparison of SIA-LOV based method with currently used instruments

Parameter	CHP-71 1st range	CHP-71 2nd range	GSP-11 1st range	GSP-11 2nd range	SIA-LOV
Airflow	70 L/h	70 L/h	42-60 L/h	30-42 L/h	80 L/h
Volume of enzyme solution	0.067 ml	0.067 ml	0.04 ml	0.04 ml	5 ml
Enzyme – Inhibitor incubation time	2 min	6 min	0.3 min	2 min	0.3 min
Absorption time	1 min	3 min	0.3 min	2 min	0.3 min
Ratio of volumes of air/enzyme during 1 assay	17412	52238	6375	30000	80
Limit of detection for sarin	50 mg/m^3	0.5mg/m^3	0.05mg/m^3	0.005mg/m^3	1.4mg/m^3

Selectivity of the proposed microSIA-LOV device to NAGs is limited by the fact that any other strong BuChE inhibitors (e.g., insecticides) will give response. On the other hand, this fact can be considered an advantage since any dangerous BuChE inhibitors can be detected in this way. While the tested SIA – LOV method using cholinesterase activity detection according to Ellman's reaction is fast and sensitive and the

scrubber - SIA – LOV enables fully automated collection and testing of the air samples for the presence of sarin, the consumption of enzyme solution in the absorber unit and power consumption are excessive. The low efficiency of scrubber decreases the sensitivity of the system.

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