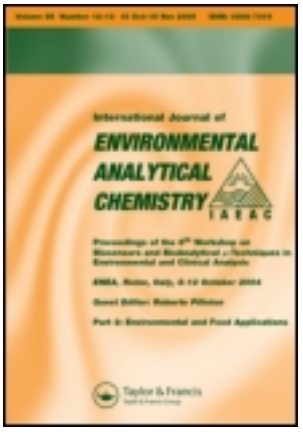

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Acetylcholinesterase based assay of eleven organophosphorus pesticides: finding of assay limitations

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Acetylcholinesterase based assay of eleven organophosphorus pesticides: finding of assay limitations

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The study includes findings about limitations of acetylcholinesterase (AChE) based assay. Eleven organophosphorus pesticides: chlorpyrifos ethyl, chlorpyrifos methyl, DFP, dichlorvos, dimethoate, fenthion, paraoxon ethyl, paraoxon methyl, phosalone, pirimiphos methyl and pirimiphos ethyl were photometrically assayed using AChE as a recognition element. The study was carried out in order to find approachability of AChE based assay. In the first round, common organic solvents were tested for interfering in assay, since samples collection and extraction is a necessary part in samples processing. Isopropanol was found as the most convenient due to minimal inhibition not exceeding 5%. Though all analysed pesticides inhibit AChE *in vivo*, some of them are toxic after metabolisation. We found AChE based assay approachable for assay of DFP, paraoxons, and dichlorvos. These are oxoforms of organophosphorus pesticides. From thioforms of assayed pesticides, only fenthion was able significantly inhibit AChE *in vitro*. Electrochemical biosensor with AChE attached on platinum electrode was used for confirmation of interaction pesticide – AChE and complex stability estimation. DFP, paraoxons and dichlorvos were allowed to interact with AChE in biosensor. These pesticides were settled firmly in AChE active site as no spontaneous recovery of AChE activity was observed.

Keywords: organophosphorus; pesticide; acetylcholinesterase; solven; detection; biosensor; paraoxon; DFP; dichlorvos

1. Introduction

Organophosphorus pesticides (OP) are a wide group of toxic compounds. Although these toxic compounds were recognised in the early nineteenth century, a complex study was carried out by the team of German scientist Dr. Gerhard Schrader in the 1930s and 1940s [1]. The main intention was prepare effective pesticides with commercial impact. At this time, some toxic compounds of 'G series' such as tabun, sarin and soman were also recognised. The most toxic organophosphates of so-called 'V series' were recognised shortly after World War II. Due to high toxicity, these compounds were found effective for military purposes but not for agricultural use [2].

The main toxicological pathway of OP is based on irreversible inhibition of cholinesterases especially acetylcholinesterase (AChE; EC 3.1.1.7) playing an important

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role in cholinergic synapses in way of terminating neurotransmission [3]. OP attacks serine in active site of AChE and irreversible blocks it from the physiological function [4]. Cholinergic crisis could appear when approximately 70% of AChE molecules is inhibited [5].

Many of traditional OP such as paraoxon or malaoxon are highly toxic for both warm-blooded and cold-blooded creatures. In order to reduce OP toxicity towards warm-blooded creatures and remain high toxicity towards cold-blooded animals, thioforms of OP such as parathion and malathion were introduced. Thioforms of OP are quickly converted in insect body into more toxic oxon form employing microsomal mixed function oxidases (MFO); the conversion is very slow in a human body as well as the other warm-blooded creatures [6]. In spite of the above-mentioned facts, nonchlinergic as well as cholinergic toxicology pathway of OP with thio group was described in cell lines lacking enzymatic apparatus necessary to produce oxoform of OP [7]. Moreover, the pathways would differ for each of OP [8].

Assays based on cholinesterases have been extensively developed for the detection of OP or diagnosis of intoxication purposes. Diagnosis of intoxication is commonly used in clinical praxis. Since collection of brain tissue is banned for living individuals, activity of blood cholinesterases play an important role in routine diagnosis. Photometric methods prevail for diagnosis [9]; however, electrochemical determination of intoxication was described as well [10]. Mechanism of organophosphates and carbamates toxicity was employed in several studies relating to its detection *in vitro*. Detection of OP in environment is still desirable. Pure cholinesterases seem to be convenient for construction of detector devices such as biosensors [11], sensors [12] or dipsticks [13]. A novel way of cholinesterase based detectors improvement could consist in advanced immobilisation technologies, material nanotechnologies [14,15], selection of different origin cholinesterases, its modification, and production [16].

Although cholinesterase based devices seem to be promising for routine applications useful for detection of OP presence in the environment, some important discrepancies are perceptible. Since toxicity mechanism is based on metabolic activation in some cases, cholinesterases used in analytical devices such as biosensors, dipsticks and photometrical kits are not approachable for its detection. Thioform of OP should be spontaneously oxidised into oxoform during assay or using a convenient reagent such as N-bromosuccinimide [17]. This step is complicating the assay because it also denaturates AChE and could cause false-positive reading of OP [18]. The present study is aimed at estimation of approachability of AChE based assay. Eleven representatives OP were selected for assay purposes. Moreover, the effect of organic solvents as an important part of samples processing is included as well. Both oxo- and thioforms of OPs were used in the study in order to find the limitations of AChE based assay.

2. Experimental

2.1 *Chemicals*

Human recombinant AChE (lyophilised powder 3,200 IU mg^{-1}), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and acetylthiocholine iodide (ATCh) were purchased from Sigma-Aldrich (Czech Republic branch). Pesticides chlorpyrifos ethyl (O,O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate), chlorpyrifos methyl (O,O-dimethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate), diisopropyl fluorophosphate (DFP;

2-(fluoro-(1-methylethoxy)phosphoryl)oxypropane), dichlorvos (2,2-dichloroethenyl dimethyl phosphate), dimethoate (O,O-dimethyl S-methylcarbamoylmethyl phophorodithioate), fenthion (O,O-dimethyl O-4-methylthio-m-tolyl phosphorothioate), paraoxon ethyl (diethyl 4-nitrophenyl phosphate), paraoxon methyl (dimethyl 4-nitrophenyl phosphate), phosalone (6-chloro-3-(diethoxyphosphinothioylsulfanylmethyl)-1,3-benzoxazol-2-one), pirimiphos methyl (O-2-diethylamino-6-methylpyrimidin-4-yl O,O-dimethyl phosphorothioate) and pirimiphos ethyl (O-2-diethylamino-6-methylpyrimidin-4-yl O,O-diethyl phosphorothioate) were chosen for the experiments. Pesticides in analytical purity were obtained from Labor Dr. Ehrenstorfer-Schafers (Augsburg; Germany). All other chemicals (solvents, buffers) were obtained at the standard analytical purity. Deionised water was prepared in the Millipore system.

2.2 *Photometric assay*

The primary goal of experiments was not to develop assay but compare approachability of AChE for assay purposes. The experiments were realised *in vitro* on a multichannel absorbance plate reader Sunrise (Tecan, Salzburg, Austria) and 96-wells polystyrene microplates (Nunc, Roskilde, Denmark). Photometric assay was realised as a modified Ellman's protocol [19]. This assay was chosen as a quick method with good reproducibility. Activity of AChE was adjusted up $0.002\,\mathrm{IU}\,\mu\mathrm{L}^{-1}$ in 50 mM phosphate buffer pH 7.4. One well was fulfilled with 5 μL of AChE solution, 40 μL of freshly prepared solution containing $0.4\,\mathrm{mg\,mL}^{-1}$ of DTBN and 1 mM ATCh in phosphate buffer, and 5 μL of given OP. Activity of AChE was followed as catalysed production of 2-nitro-5-thiobenzoate anion strongly absorbing at 412 nm. The absorbance was measured after 5 minutes incubation period and started at the exact time by addition of ATCh. Multichannel pipette was used to start reaction at the moment. All measurements were repeated four times.

2.3 *Biosensor performance*

Biosensors were performed in order to determine stability of complex AChE-OP due to firmly intercepted AChE and used washing steps. Previously optimised protocol was adopted for these purposes [20]. Electrochemical sensor strip with platinum working (circle shaped with 1 mm diameter), platinum auxiliary and Ag/AgCl reference electrodes was used in the experiments. AChE (the same origin AChE as used in chapter 2.2) was immobilised in the form of gelatin membrane. A 1 μL of AChE 2 IU in 50 mM phosphate buffer pH 7.4 with bovine serum albumine (BSA) $1\,\mathrm{mg\,mL}^{-1}$ and 0.1% gelatin was applied on the working electrode and left to dry in the refrigerator. Prepared biosensor was overlaid with given OP (10 μL) and left to interact for 5 minutes. After that, the surface was gentle splashed with phosphate buffer in a reaction cell and the outputting current as a function of AChE activity was measured in a stirred cell with volume 2 mL fulfilled with 1 mM acetylcholine chloride in phosphate buffer. Electrochemical detector device EmStat (PalmSens, Houten, Netherlands) was used for biosensor performance. Applied voltage was adjusted up 450 mV against Ag/AgCl reference electrode. All measurements were repeated four times.

2.4 *Data processing*

The data achieved in photometric experiments were processed in order to calculate percentage of inhibition for given OP. Calculation of percentage of inhibition was chosen as an better way to estimate inhibition than the measurement of enzyme activity since the activity would change due to storage and sorption on tube walls. The used formula is depicted bellow:

$$I = \left(1 - \frac{\Delta A_i}{\Delta A_0}\right) \times 100$$

The symbol ΔA_i indicates residual activity of AChE after inhibition. It was estimated as absorbence at wavelength 412 nm. Opposite, symbol ΔA_0 indicates blank application (no inhibition). Blank included the same solvent as OP solution so inhibition by solvent does not affect the processed data.

The data achieved throughout experiments with performance of biosensor were processed in a similar way as photometric assay:

$$I = \left(1 - \frac{i_i}{i_0}\right) \times 100$$

Symbols i_i and i_0 indicate the current provided after OP respective blank application.

Software Origin 8 (OriginLab, Northampton, MA, USA) was used throughout for data processing and statistical analysis of achieved results.

3. Results and discussions

3.1 *Effect of organic solvents*

Organic solvents are irreplaceable in samples collection and processing. Although AChE is a membrane protein with lipophilic regions, solvents affect molecule structure resulting in irreversible denaturation instead of AChE stabilisation [21]. Denaturation process is expected to be proportional to time of acting and solvent concentration. Solvents effect would lower limit of detection for given assay based on AChE; moreover, the data are poorly interpretable. Some finding in this way was described by, for example, Dondoi *et al.* [22] for ethanol, methanol and acetonitrile when pesticides paraoxon and dichlorvos were assayed.

Inhibition effect of methanol, ethanol, propanol, butanol, pentanol, propan-2-ol (isopropanol), polyethyleneglycol (PEG, average Mw 200), 2,5-dimethylfurna (DMF), dimethylsulfoxide (DMSO), tetrahydrofuran (THF), acetonitrile is depicted in Table 1 for solvents concentration 5, 10 and 20%. The solvents were processed in photometrical assay in the same way as the OP sample. The solvents are approachable for pesticides processing and could be potentially performed in sampling process. Although all solvents inhibited AChE in given concentrations, the inhibitory effect was quite diversified. Monovalent alcohols were found inhibiting AChE in a large scale for propanol, butanol and pentanol; however, lower weight alcohols methanol and ethanol were more gentle for AChE based assay. Isopropanol was the best solvent with very low inhibition effect on AChE when compared with alcohols with OH group in position one. DMF, THF and acetonitrile were also insufficient for analysis purposes. DMSO and PEG inhibited AChE in a lower scale when they did not exceed 5% (DMSO) or 10% (PEG).

Table 1. Percent of inhibition of AChE caused by some organic solvents. Inhibitory effect was investigated photometrically. The error indicate standard deviation ($n = 4$).

Solvent concentration	20%	10%	5%
methanol	48 ± 3	33 ± 4	24 ± 2
ethanol	56 ± 4	47 ± 3	21 ± 1
propanol	77 ± 3	70 ± 5	41 ± 4
butanol	75 ± 5	79 ± 5	61 ± 5
pentanol	64 ± 6	63 ± 6	57 ± 3
propan-2-ol	58 ± 3	24 ± 2	5 ± 1
PEG	44 ± 4	20 ± 4	13 ± 2
DMF	80 ± 4	77 ± 3	62 ± 5
DMSO	64 ± 5	43 ± 1	10 ± 1
THF	72 ± 3	68 ± 5	48 ± 4
acetonitrile	79 ± 7	57 ± 6	37 ± 5

Inhibition of AChE could represent a serious obstacle for routine assay. Inhibition caused by OP would not be determined when solvent effect prevails. Isopropanol was chosen as the best solvent for assay of OP. The content of isopropanole in calibrants was adjusted in a way to reach a final concentration of isopropanole in one well just 5%. Here presented data indicate the crucial importance of solvents selection. Moreover, other chemical compounds such as aflatoxins and others would interfere with the assay like the solvents or even in a higher scale [23].

3.2 *Spectrophotometric assay*

OPs in a calibration scale $10^{-10} - 10^{-3}$ M were photometrically assayed. Inhibitions caused by tested pesticides are depicted in Figure 1. Four of OPs were strong inhibitors of AChE. They were DFP, paraoxon methyl, paraoxon ethyl, and dichlorvos. DFP was the strongest inhibitor, as could be seen from the IC50 value (1.70×10^{-6} M). Paraoxon ethyl and paraoxon methyl calibration curves were similarly shaped; nevertheless paraoxon ethyl was found to be the stronger inhibitor according IC50 value. It is probably caused by a higher lipophility of paraoxon ethyl when compared with paraoxon methyl. The last inhibiting pesticide dichlorvos was the weaker inhibitor (2.63×10^{-4} M) of all four named. Limits of detection for the four pesticides are of the same sequence as IC50 values. The lower limit of detection was found for DFP (6.83×10^{-8} M), the higher for dichlorvos (2.63×10^{-4} M). The data are presented in Table 2. The significance of differences was also confirmed using one way ANOVA with Scheffe test (Origin software).

Chlorpyrifos ethyl, chlorpyrifos methyl, dimethoate, fenthion, phosalone, pirimiphos methyl and pirimiphos ethyl were not *in vitro* inhibitors of pure AChE. One exception could be seen in fenthion assay. However, inhibition mechanism in the increased concentration would be based on another phenomenon such as competition. On the other side, spontaneous oxidation is a possible mechanism as well. The purity of pesticides is satisfactory. Moreover, the presence of impurities in stock solutions is regularly confirmed by GC. It was found as slight inhibitor of AChE *in vitro* and two calibration points (10^{-3} and 10^{-4} M) were statistically distinguishable from control (t-test, $P = 0.05$). However, average percentage of inhibition provided by 10^{-3} M fenthion was only

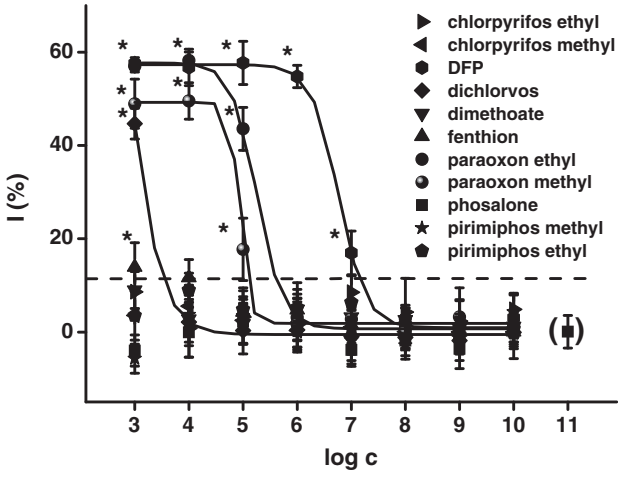

Figure 1. Calibration plot for given organophosphorus pesticides expressed as percentage of inhibition vs. molar concentration of organophosphate. Error bars indicate standard deviations. Dashed line delimitates limit of detection level ($S/N = 3$). Point in brackets was achieved by blank assaying – no OP in well. Error bars indicates standard deviation ($n = 4$). Asterisks indicates significant difference aganst control (ANOVA with Scheffe test; $P = 0.01$).

Table 2. Expression of molar values of limit of detection (LOD) and the half maximal inhibitory concentration (IC50) of calibration curves being depicted in Figure 1.

	DFP	Paraoxon ethyl	Paraoxon methyl	Dichlorvos
LOD (mol L^{-1})	6.83×10^{-8}	2.51×10^{-6}	7.76×10^{-6}	2.63×10^{-4}
IC50 (mol L^{-1})	1.70×10^{-6}	5.62×10^{-6}	1.15×10^{-5}	1.41×10^{-3}

13.6% in given conditions. Paraoxons, DFP and dichlorvos provided inhibiton around 50% in the same conditions.

The achieved results indicate good approachability of AChE based assay for testing of oxoforms of OP. On the other hand, OPs being toxic after metabolic activation are not *in vitro* detectable by pure AChE. The analyte should be either chemically modified [17] or metabolised by using contemporary oxidases with AChE. Despite the above-mentioned facts, highly toxic pesticides and nerve agents could be simple detected using devices based on AChE or butyrylcholinesterase [24,25] and the construction of devices such biosensors based on cholinesterases seem to be promising for emerging performance in field conditions as well as laboratory instrumentation. Moreover, AChE based assay could be improved by reactivation step and selective reactivation would be appropriate to partially identify analysed OP, i.e. make this assay more approachable [26]. AChE based assay seems to be possible for routine assay of OP; however, the present methods should be improved. Especially, finding of stabile recombinant AChEs being stable in organic solvents and the method of gentle oxidising OP but not AChE are strongly desirable. AChE based assay would compete to the more elaborative physical assays only when the above-mentioned limitations are resolved.

Table 3. Performance of AChE based biosensor for given pesticides assays. The first line of data indicated by index *a* expresses first performance of biosensor. The second line indicated as *b* was determined after biosensor washing in phosphate buffer for 5 minutes.

	DFP	Paraoxon ethyl	Paraoxon methyl	Dichlorvos
I(%)[a]	56.3 ± 5.2	51.2 ± 4.4	44.7 ± 3.9	38.5 ± 6.2
I(%)[b]	57.4 ± 6.1	55.1 ± 3.9	47.6 ± 2.5	36.3 ± 2.7

3.3 *Estimation of complex AChE – OP stability*

The stability of complex AChE – OP would be an important parameter in AChE based assay. Electrochemical biosensor with AChE captured into gelatin membrane was performed for these purposes. In the first round, immobilised AChE was inhibited by one of the consequent pesticides: paraoxon-ethyl, paraoxon-methyl, DFP, dichlorvos and percentage of inhibition was estimated. After that, biosensor was placed into stirred cell fulfilled with 5 mL of phosphate buffer. Biosensor was allowed to wash with stream of mixed buffer for 5 minutes. The results of assay are attached in Table 3. The stability of complex AChE – OP was proven by the experiment. Changes in inhibition are not significant so we can conclude that interaction AChE – OP is not reversible *in vitro*. This fact could be expected when high toxicity and dealkylation (aging) of captured OP rather than dissociation is considered [27]. Dealkylation is a negative process *in vivo* since oxime drugs are not effective for treatment and only symptomatic manifestation of intoxication could be suppressed [28]. Oxime drugs are also approachable for regeneration of previously inhibited biosenors and in this way could prolong its usability [29]. Here, the stability of complex AChE – OP represents an advantage for AChE based assay. Especially, AChE as part of a biosensor in a flow through apparatus could interact with OP analyte without its gradual depletion [30–32].

4. Conclusions

The presented study was elaborated in order to estimate current limitations of AChE based assay. AChE assay was found approachable for oxoforms of OP. Eleven pesticides were assayed and paraoxon-ethyl, paraoxon-methyl, DFP, and dichlorvos were found as strong inhibitors of AChE. The others were not detectable by AChE based assay. Inability of detectors with AChE as a recognition element to assay thioforms of OP is the main disadvantage for its field application as *in vitro* activation of thioforms of OP is instrumentally difficult. Only one thioform of OP: fenthion was found as a slight inhibitor of AChE. In a conclusion, we could consider AChE as a convenient recognition element for assay of oxoforms of organophoshorous pesticides and nerve agents; especially, if promising devices such as biosensors are taking into account. However, the study appoints at necessary improvement of assay due to limited sensitivity of AChE to thioforms and interference of many compounds including organic solvents. Moreover, development of biosensors is a continual effort to make analytical device smarter for detection of toxins or starting diseases diagnosis [33].

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