

Myeloperoxidase-mediated oxidation of organophosphorus pesticides as a pre-step in their determination by AChE based bioanalytical methods

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Abstract In order to improve the sensitivity of assays for inhibitors of the enzyme acetylcholine esterase (AChE), an effective method was developed for the conversion of the organophosphate pesticides (OPs) diazinon, malathion, chlorpyrifos, azinphos-methyl and phorate into more toxic inhibitors. This was accomplished by converting them from the thio form into their oxo form using the enzyme myeloperoxidase. The oxo forms, which are the only products of conversion, were determined by AChE bioassays, using either the free enzyme, or a flow injection analysis manifold with immobilized AChE and spectrophotometric detection. All modified OPs exhibited inhibitory power at ppb levels and within 10 min. The method is considered to represent an excellent means for improving the sensitivity of assays for determination of OPs.

Keywords Organophosphate pesticides · Myeloperoxidase · Oxidation · Bioanalytics · Acetylcholinesterase

Introduction

Among various organic and inorganic pollutants, organophosphorus pesticides (OPs) widely used in agriculture are

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very dangerous and harmful because of their toxic nature [1]. Their toxic effects are attributed to the irreversible inhibition of the enzyme acetylcholinesterase (AChE) [1–5]. They also inhibit the other enzymes playing an important role in biochemical processes [6]. Moreover, phosphorothionate molecules undergo to chemical transformations after metabolic oxidation which leads to oxon derivatives (phosphates) formation, where the sulfur atom from the thionate group was replaced by an oxygen atom [2, 7, 8]. The products of thio OPs oxidation are more toxic than the original pesticides. However, OPs hydrolysis leads to detoxification, although the products of this process are not completely harmless [9]. Therefore, the close monitoring and removal of OPs from environment is necessary. Several methods, either independent or in conjunction with others, have been used for removal of OPs by adsorption [10–13] including also oxidation with ozone [14], chlorine [8] and biological degradation [15].

There is growing interest in the rapid and accurate monitoring and determination of OPs in environment. Besides complicated and time-consuming conventional techniques, such as gas chromatography [16] and high-performance liquid chromatography (HPLC) [17–19], and more convenient multisyringe flow injection analysis (MSFIA) [20], electrochemical method [21], various cholinesterase (ChE) based bioanalytical methods and biosensors for in situ detection of OPs were developed [22–26]. The principle of the biosensors is based on the fact that the activity of cholinesterases is inhibited by OPs and the degree of inhibition is dependent on the concentration of anti-ChE in contact with the enzyme. However, oxidized forms of OPs show stronger inhibitory action towards AChE than parental, unoxidized compounds [4] and the limit of their detection depended often on the oxidation pre-step of the OPs containing sample [27–29].

The oxidations of OPs were performed either directly in water samples or in solvents which were diluted in working buffer prior the analysis. For this purpose N-bromosuccinimide (NBS) [30–33] or bromine [28, 29, 34, 35] were introduced as selective and rapid oxidants to enhance the inhibition in determination of thio OPs by biosensors. Moreover, other methods of oxidations were also developed, but were mainly intended for synthesis of oxons from thiophosphates using nitric acid, peroxide, peroxi acids, chlorine, ozone [36–40]. In these studies, the efficiency of oxidation reaction was unknown, and the possibility of enzyme inhibition by certain undesirable products was not addressed.

The metabolic activation of phosphorotioate molecules, which is performed by cytochrome P450 enzymes, yields oxon derivatives. Besides cytochrome P450 systems, peroxidative oxidation of OPs has also been documented with other enzymes, such as horseradish peroxidase [41], chlorperoxidase [42, 43] and soybean lipoxygenase [44]. Enzyme myeloperoxidase (MPO) acts as oxidant enzyme in the process of inflammation and atherogenesis [45]. MPO is relatively nonspecific with respect to its reducing substrates. This enzyme is able to oxidize different substrates among which anilines and phenols [46–48].

In this work the ability of MPO to biocatalyze the transformation of OPs (diazinon, malathion, chlorpyrifos,

azinphos-methyl and phorate, Scheme 1) from thio to oxo forms was examined. The aim was to use MPO mediated oxidation of thio OPs to oxo OPs without any preconcentration or extraction step under normal laboratory conditions in order to improve the sensitivity of AChE based bioanalytical assays for their detection.

Experimental

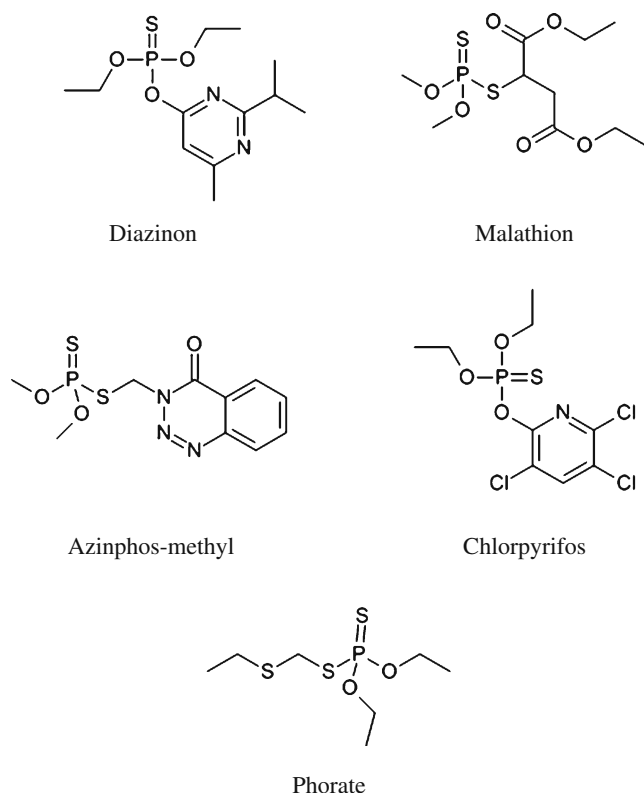
Chemicals and materials

Myeloperoxidase (MPO) from human neutrophils, purified to a purity index (A_{430}/A_{280}) 0.84 was obtained from Planta Natural Products, Vienna, Austria (www.planta.at). Its concentration was calculated using $\epsilon_{430}=91\,000\text{ M}^{-1}\text{cm}^{-1}$ per heme [49]. Catalase, from bovine liver, acetylcholinesterase (AChE, specific activity 0.28 IU/mg) from electric eel, acetylthiocholine iodide (ASChI), 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) and controlled-pore glass (CPG 240, 80–120 mesh) were purchased from Sigma-Aldrich St. Louis, MO, USA (www.sigmaaldrich.com). Potassiumhydrogenphosphate ($\text{K}_2\text{HPO}_4 \times 3\text{H}_2\text{O}$), glutaraldehyde, 3-aminopropyltriethoxysilan, 2-hydroxyiminomethyl-1-methylpyridinium iodide (2-PAM) were purchased from Merck KGaA, Germany (www.merck.com). Hydrogen peroxide solutions were prepared daily by diluting a stock solution and the concentration was determined using $\epsilon_{240}=43.6\text{ M}^{-1}\text{cm}^{-1}$ [50]. The used pesticides were of at least 93% purity and no further purification of chemicals was performed. Malathion, malaoxon, chlorpyrifos, azinphos-methyl and phorate (OPs), were purchased from Pestinal®, Sigma-Aldrich, Denmark, whereas diazinon and diazoxon were purchased from Institute of organic industrial chemistry Poland (www.ipo.waw.pl) and Chemical Co (www.thechemco.com). The pesticide working solutions were prepared by dilution of the $1 \times 10^{-3}\text{ M}$ stock solutions in ethanol. The final working solutions contained 1 % ethanol to improve the solubility of the pesticides. The pesticide stock solutions were held in refrigerator until used. All chemicals were used without further purification. Deionized water was used throughout.

0.05 M phosphate buffer, pH 8, was used as a carrier buffer in the FIA experiments. The reactivation of immobilized enzyme was done using 4 mM 2-hydroxyiminomethyl-1-methylpyridinium iodide (pralidoxime, 2-PAM) [25, 26, 51].

Oxidation procedure

The desired concentrations of organophosphates in the range from 10^{-4} – 10^{-7} M were incubated in 50 mM phosphate buffer, pH 6.0, with various concentrations of



Scheme 1 The chemical structures of relevant OP compounds

MPO in final reaction volume 0.5–1.0 mL. Reaction was started by addition of 50 μM H_2O_2 , and stopped after incubation by adding catalase (100 $\mu\text{g}/\text{mL}$). The reaction mixtures were centrifuged for 2 min at 10,000 rpm, to separate the supernatant for further analysis. At least three replicates were performed for each compound. The controls without MPO and/or hydrogen peroxide were carried out under the same conditions.

AChE immobilization

55 mg AChE was immobilized on 0.15 g activated controlled pore glass (CPG) according to the procedure described earlier [4, 5, 51]. Briefly, the glass particles activated with 3-aminopropyl-triethoxysilan three-times; afterwards, alkylamino glass was cross-linked with 2.5% glutaraldehyde. The enzyme was dissolved in 3 ml cold phosphate buffer and added to the pre-treated glass in a stream of nitrogen. After filtration, the immobilized enzyme was washed with phosphate buffer and then with cold water. The immobilized beads were stored at 4 °C in phosphate buffer (pH 6.0) until use.

Determination of AChE activity in the presence of OPs and their oxo forms

The inhibition of AChE by the pesticides both, oxidized and unoxidized, was measured using modified Ellman procedure [4, 52] in the absence (control) and presence of inhibitors. The experiments were performed by *in vitro* exposure of free enzyme (2.5 IU) to inhibitors in final volume 0.65 mL. The incubation time in the presence of inhibitors was 20–60 min, before the reaction was initiated and followed during 8 min. Acetylthiocholine iodide (ASChI) was applied as an enzyme substrate in combination with 5,5-dithio-bis-2-nitrobenzoic acid (DTNB) as a chromogenic reagent. The product 5-thio-2-nitrobenzoate, formed by the reaction of thiocholine (product of the enzymatic reaction) with DTNB, was measured spectrophotometrically at 412 nm (in buffer solution).

Simultaneously, the effect of the selected compounds before and after oxidation on immobilized AChE activity was investigated by using a flow-injection analysis (FIA) manifold [4, 25, 26, 51]. The inhibition of the enzyme was determined by comparing the enzyme activity before and after passage of 200 μL pesticide solution of desired concentration through the bioanalytical column for a given period of time. The procedure was as follows: the carrier buffer (0.05 M phosphate, pH 8.0) was passed through the sensor system until the base line was stabilized and then the mixture consisting of ASChI and DTNB was injected through 200 μL injection loop into the carrier stream and the response was recorded [4, 51]. The procedure was

repeated at least three times, and the average value of the signal (initial activity) was used for calculations. Then the sample containing the pesticide was injected. The determination of the final enzyme activity was carried out by another injection of the substrate and the remaining enzyme activity was calculated as the ratio of the signal intensities before and after the injection of the sample.

All experiments were performed in triplicate, and the results present mean value \pm SE. Preliminary studies showed that OPs and their products of oxidation, or their combinations, did not interfere with quantification of the yellow product, 5-thio-2-nitrobenzoate.

FIA-AChE bioassay

The flow-injection system consisted of a HPLC pump (Dionex AMP-1), an injection valve (Waters U6K) equipped with a 200 μL injection loop, detection unit (UV VIS detector, accuracy $\pm 2 \times 10^{-5}$ absorbance units at 412 nm) connected to PC and a bioanalytical reactor (21 \times 3 mm peak column) filled with AChE immobilized on CPG. The carrier buffer (phosphate buffer, pH 8.0) was pumped through a flow-through cell. The flow rates which were used in these experiments were 0.1 mL/min and 0.5 mL/min.

Each bioanalytical column was used for several determinations of pesticide. 13 mg of glass beads with immobilized enzyme was weight before being filled in the bioanalytical column, making the analysis more reproducible, since the initial activity was the same all the time. The biosensor was daily calibrated with 3.4×10^{-6} M malaoxon, which induced about 50% inhibition and was afterwards reactivated with 2-PAM.

When the activity of the enzyme dropped to 90% of the initial value, enzyme was reactivated to its initial activity by five consecutive injections of a reactivator (4 mM 2-PAM). When the reactivation by 2-PAM could not reach the initial enzyme activity, the enzyme was replaced with the fresh one.

Apparatus

The spectrophotometric measurements were performed on Perkin Elmer Lambda 35 UV-Vis spectrophotometer, using 1 cm path length cuvette. pH measurements were performed using Metrohm pH meter, model 713 equipped with glass electrode.

Waters ACQUITY Ultra Performance Liquid Chromatography (UPLC) system coupled with a TUV detector controlled by the Empower software was used. Chromatographic separations were run on an ACQUITY UPLCTM BEH C₁₈, column 1.7 μm , 100 mm \times 2.1 mm column (Waters). The analyses of diazinon and diazoxon were done

under isocratic conditions with mobile phase consisting of solvent (A) CHCOOH (0.1 vol % in water) and (B) acetonitrile (J.T. Baker) (25:75, v/v). The elutions were monitored at 245 nm. The analysis of malathion and malaoxon were done under isocratic condition with mobile phase consisting of solvent (A) CHCOOH (0.1 vol % in water) and (B) acetonitrile (J.T. Baker) (60:40, v/v). The elutions were monitored at 210 nm. The eluent flow rate was 0.3 mL min^{-1} and the injection volume was $10 \text{ }\mu\text{L}$.

GC/MS analysis was performed on Agilent 7890A GC system equipped with 7000A QqQ MS, using DB-5 MS column ($30 \text{ m} \times 0.25 \text{ mm} \times 0.25 \text{ }\mu\text{m}$). Injection volume was $1 \text{ }\mu\text{L}$ and injector temperature was $250 \text{ }^\circ\text{C}$ with 10:1 split ratio. Carrier gas (He) flow rate was 1.3 ml/min at $80 \text{ }^\circ\text{C}$ (constant pressure mode). Column temperature was linearly programmed in a range of $80\text{--}300 \text{ }^\circ\text{C}$ at a rate of $10 \text{ }^\circ\text{C/min}$. Transfer line was heated at $280 \text{ }^\circ\text{C}$. Mass spectra obtain in EI multiple reactions monitoring (MRM) mode. Collision energy was 15 eV and collision gas was nitrogen. MRM mass spectra were acquired using characteristic transition for standards and samples.

Results and discussion

Reaction of OPs by MPO

In the study of MPO mediated chemical transformation of OPs malathion and diazinon were examined in more details. In the first series of experiments, the OPs reaction with MPO was performed by exposure $1 \times 10^{-5} \text{ M}$ water solution to 50 nM MPO during 5 min . The reaction of diazinon with MPO is schematically presented in Scheme 2.

The reaction products were analyzed by UPLC and GC/MS measurements and chromatograms were compared with those of authentic standards. The identification of the reaction products yielded only one major product—the oxo form of pesticides. In the presence of MPO the sulfur atom was replaced by an oxygen atom in the organophosphorous pesticides, transforming the phosphorothioate group to an oxon derivative (Scheme 2) [42]. Table 1

presents results obtained by GS/MS analysis of $1 \times 10^{-5} \text{ M}$ diazinon and malathion after oxidation. GS-MS/MS mass spectra were acquired in multi reaction mode (MRM) using characteristic transition for standards and samples. Presence of diazoxon and malaoxon in sample was confirmed comparing retention times and MS data of standards obtain under the same conditions. The results were also confirmed by UPLC analysis.

As an example, the UPLC chromatograms obtained for $1 \times 10^{-5} \text{ M}$ diazinon before and after incubation with MPO are presented in Fig. 1. Also, UPLC chromatograms of diazoxon that were recorded during 60 min , after the reaction was stopped by catalase, confirmed that there were no other products of conversion, i.e. a further cleavage of oxo form was not observed (Fig. 1) as found in the case of application of some inorganic oxidants [39]. It is worthily to notice that the formation of diazinon hydrolysis product, 2-isopropyl-6-methyl-4-pyrimidinol (IMP), which is usually found due to UV treatment of diazinon [6, 33], was not found in any of tested samples. These results are also in accordance with those obtained when chlorperoxidase was used for biocatalytic oxidation of some OPs. No hydrolysis and halogenated products were found in the chlorperoxidase oxidation of OPs [42, 43]. These oxidations are similar to those performed by cytochromes P450 in in vivo and in vitro systems. The mayor difference is, however, that a further cleavage of oxons, which is typical of P450 catalyzed reaction, was not observed with MPO, as well with chlorperoxidase. Oxidation by inorganic oxidants, such as aqueous chlorine led to hydrolysis of diazoxon, which was much slower process [8]. For oxidation of diazinon and other OPs, previous studies focused on ozonization and showed that the oxidation process was followed by further hydrolysis of diazoxon to IMP and diethyl phosphate [53].

AChE test for detection of OPs oxidation products

In order to find out the optimal conditions for conversion of OPs, from thio to oxo form, the influence of MPO in the concentration range from $10\text{--}100 \text{ nM}$ on 1×10^{-4} , $1 \times 10^{-5} \text{ M}$

Scheme 2 Reaction of diazinon with MPO in the presence of H_2O_2

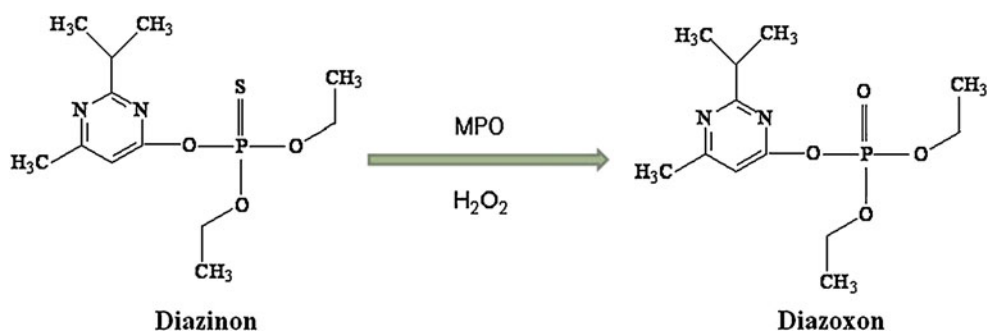


Table 1 Identification of OPs and their products of oxidation by GS/MS

Compound	Mr	m/z	^a RA	^b RT
Diazinon	304	179, 137, 273	100	13.625
Diazoxon	288	137, 273	35	13.299
Malathion	330	125, 127, 173	100	15.462
Malaoxon	314	127	47	14.664

^aRelative abundance (RA)^bRetention Time (RT)

and 1×10^{-6} M OPs during 1–30 min of incubation was investigated using AChE test. In these experiments the incubation time of oxidized sample with AChE was 20 min. It should be noticed that OPs were 10 fold diluted in AChE test, compared to initial untreated solution samples. The results obtained for diazinon and malathion are presented in Figure S1A,C and S1B,D (figures can be found in the Supporting Information), respectively, as the response of AChE (the percent of activity) to the oxidized and unoxidized OPs. The percent of AChE activity after exposure to ten fold diluted oxidized samples of diazinon and malathion, which were incubated with MPO for 5 min, decreased from 5–100% by increasing MPO concentration, depending also on the initial concentration of pesticide. However, the inhibition of AChE activity was ascribed to oxo form of related pesticides, since the inhibition induced by the same initial concentration (below 1×10^{-5} M) of the untreated parent compounds was almost negligible [4]. Based on the obtained results, 100 nM MPO was found as the optimal concentration to be used in further experiments. The AChE activity in the presence of the oxidized OPs compared to non oxidized samples decreased with the increasing the incubation time, indicating the increasing the concentration of oxo forms. The saturation level of oxo

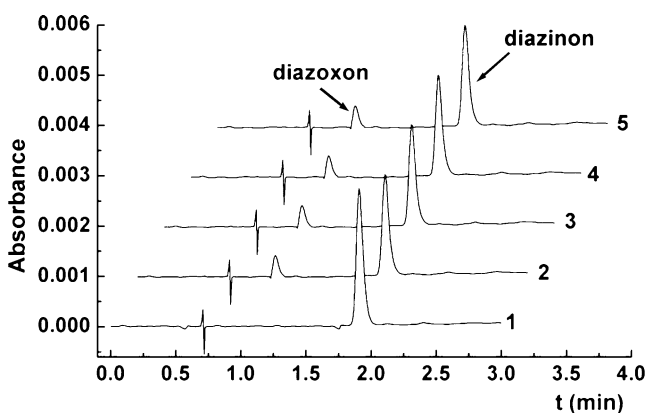


Fig. 1 UPLC chromatograms of 1×10^{-5} M diazinon before (1) and after (2–5) 5 min of incubation with 50 nM MPO. Oxidized samples (2–5) were followed within 60 min. The time interval between two consecutive chromatograms was 20 min

forms was reached after 10 min incubation of parent compounds with MPO.

In further experiments, the AChE inhibition induced by thio OPs before and after oxidation together with their oxo forms, in the concentration range from 1×10^{-4} M– 1×10^{-9} M was evaluated. Results obtained for malathion and diazinon are presented in Fig. 2, as the examples. As can be seen from the inhibition studies, oxo forms exerted for about more than three orders of magnitude stronger inhibitory power, compared to the parent compounds under the same experimental conditions (Fig. 2a and b, curves 1 and 2). Further, OPs in the same concentration range were oxidized by MPO under optimal conditions (10 min incubation with 100 nM MPO). The inhibition of AChE activity obtained using solutions of oxidized OPs is presented in Fig. 2 (curves 3), for comparison. In these experiments the incubation time of OPs with AChE was prolonged to 60 min, in order to decrease the concentration level of OPs which induces the measurable decrease AChE activity (at least 10%).

It is also obvious that after the incubation of OPs with MPO, the inhibition curves were shifted to the lower OPs concentration range, compared to the inhibition curves of

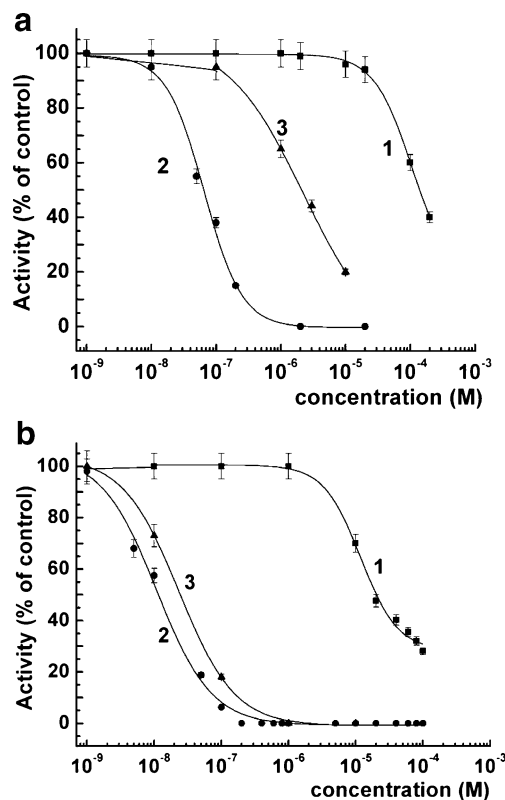


Fig. 2 The concentration dependent inhibition of AChE activity induced by **a** diazinon (1) and **b** malathion (1), before (2) and after (3) exposure to 100 nM MPO during 10 min. The AChE activities are expressed as the mean % activity relative to the corresponding control value in the absence of OPs

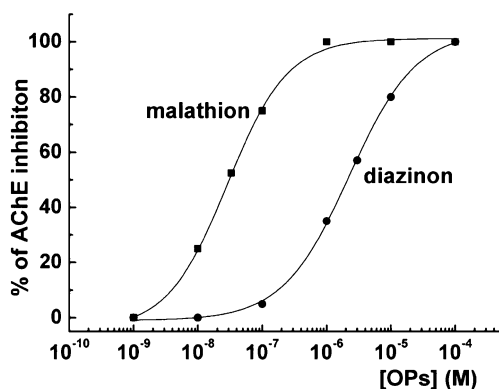


Fig. 3 Plot of percent inhibition of AChE activity vs initial concentration of diazinon and malathion after 10 min oxidation with 100 nM MPO (incubation time between oxidized OPs and AChE was 60 min)

parent compounds due to the formation of oxo forms of OPs (Fig. 2, curves 3).

Determination of OPs using MPO mediated oxidation and AChE test

For the construction of calibration curves for diazinon and malathion, the assumption was made, that the AChE inhibition was achieved only due to the oxons formed after incubation with MPO. Plot of percent inhibition of AChE activity vs. initial OPs concentration was deduced from inhibition curves for oxidized OPs (Fig. 2, curves 3) and inhibition curves of oxones (Fig. 2, curves 2) and is presented in Fig. 3.

The results presented in Fig. 3 show that the concentrations, which induced measurable AChE activity (at least 10% of control) were below 4×10^{-9} M for malathion, and 2×10^{-7} M for diazinon.

Finally, AChE test was used to determine the concentrations of oxo forms as dependence on MPO concentration

Table 2 Influence of OPs before and after 10 min oxidation with 100 nM MPO on AChE activity

OP	Activity (% of control)			
	Unoxidized	Oxidized		
Initial concentration (M)	1×10^{-5}	1×10^{-7}	1×10^{-6}	1×10^{-5}
Diazinon	100	95±5	65±8	20±4
Malathion	100	20±2	0	0
Phorate	100	88±4	45±5	0
Azinphos-methyl	96	81±6	20±7	0
Chlorpyrifos	100	60±5	30±6	10±2
Syntetic mixture	100	75±4	0	0

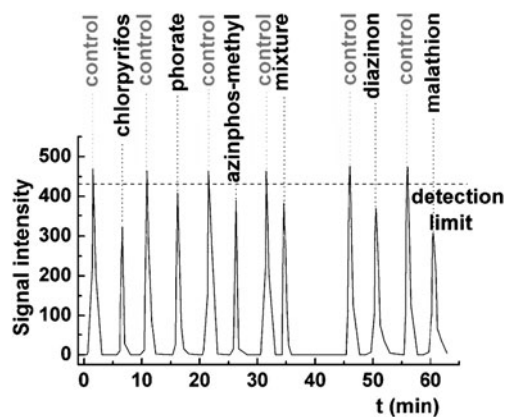


Fig. 4 FIA signals at flow rate 0.5 mL/min before (control) and after enzyme inhibition by oxidized 1×10^{-7} M OPs

and oxidation time. The obtained results were in agreement with those obtained using conventional analytical techniques, as UPLC. The UPLC calibration graphs for diazinon and diazoxon were constructed (data not shown) to evaluate the adequate concentrations of parent compound and oxo form upon the oxidation of diazinon. Table S1 (table can be found in the Supporting Information) shows the results obtained for evaluation of diazoxon concentration as dependence on MPO concentration. It is obvious that the results obtained from AChE test and UPLC determination for initial diazinon concentration above 1×10^{-6} M are in the range of experimental error. However, diazoxon concentration in the oxidized sample which contained below 1×10^{-6} M diazinon before oxidation could not be measured without sample pre-concentration. Moreover, AChE test appeared to be faster and simpler in this case.

The oxidation efficiency as the function of oxidation time was expressed as the oxidation yield based on determined concentrations of oxo forms relative to the initial concentration of the parent compounds. The results for oxidation efficiency as the function of oxidation time

Table 3 Influence of flow rate on the response of sensor enzyme in FIA bioanalytical assay for 1×10^{-7} M unoxidized and oxidized OPs

OPs	Signal intensity (% of control)		
	0.1	0.5	0.1
Flow rate (mL/min)	Unoxidized		Oxidized
Diazinon	100	78±5	70±7
Malathion	100	65±5	59±2
Phorate	100	84±4	77±3
Azinphos-methyl	96	80±3	80±5
Chlorpyrifos	100	67±3	57±4
^a Syntetic mixture	100	80±5	72±2

^a 0.2×10^{-7} M of each mentioned OP

for diazinon and malathion, obtained using AChE test and UPLC measurements, are given in Table S2 (table can be found in the Supporting Information). However, the conclusion can be made that the conversion efficiency increased by lowering diazinon concentration. These results can be also compared to those obtained by treatment of several OPs with 10 molar excess of bromine in acetonitrile [29]. This method yielded about 82–100 % oxons. Further, the ozonization of OPs in aqueous solutions led to the complete degradation within 1 h [54]. N-bromsuccinimide was also introduced as rapid and effective oxidant to enhance AChE inhibition in determination of OPs. However, the complete assays could not be performed in less than 2 h [30, 32, 55].

AChE test as described above was also applied to the other OPs (phorate, azinphos-methyl, chlorpyrifos) and their synthetic mixture in tap water, before and after 10 min oxidation with 100 nM MPO. The single initial concentrations were 1×10^{-5} M, 1×10^{-6} M and 1×10^{-7} M, and the synthetic mixtures contained all individual components in the mixture. The results are presented in Table 2. All the oxidized OPs showed the greater percentage inhibition than the corresponding non oxidized samples.

These results show that the chosen method for OPs oxidation can be used to increase the sensitivity of the AChE based enzymatic and sensor analysis of OPs.

The effects of OPs oxidation on the activity of immobilized AChE

The response of the bioanalytical FIA manifold, using immobilized AChE to unoxidized and oxidized OPs was followed in 1×10^{-7} M aqueous solutions of diazinon, malathion, phorate, azinphos-methyl, chlorpyrifos, and also in the synthetic mixture containing 1×10^{-7} M of all components as the sum of their initial concentrations (0.2×10^{-7} M). In the present study, the flow rates were 0.5 mL/min and 0.1 mL/min. The typical FIA signals obtained for oxidized 1×10^{-7} M malathion, diazinon, phorate, chlorpyrifos and azinphos-methyl at flow rate 0.5 mL/min are presented in Fig. 4. Moreover, the unoxidized 1×10^{-7} M OPs did not induce any inhibition of immobilized AChE (Table 3). After inhibition by each single oxidized OP, AChE was reactivated by 2-PAM, at flow rate 0.1 mL/min to restore the initial AChE activity (control value). As the results presented in Fig. 4 show, the same value of control signals was achieved after each reactivation of sensor enzyme.

As a detection limit, the lowering of the control signal for about 10% was considered. It is worthily to notice, that the lower flow rate induced the higher value of the control signals for about 10%, but the intensity of signals after injection of OP solutions decreased (Table 3). The

lower flow rate ensured the prolonged contact time between OPs and sensor enzyme. Based on this finding, the lower flow rate leads to increasing of the FIA manifold detection limit (lowering of the control signal for at least 10 %) for OPs.

The oxidation of OPs for their determination in FIA system was studied by some authors. Chlorperoxidase in citrate buffer was used to oxidize OPs in determination of OPs using bioanalytical assay based on inhibition of AChE which was determined in FIA system by thermal lens spectroscopy [42, 43]. The improved signal was obtained in ionic liquids which were tolerated by the enzyme up to 30% [43]. Bromine was also used as the in situ oxidant for aqueous sample solutions and oxidizes samples were injected directly into FIA system. The efficiency of oxidation was unknown, and the possibility of AChE inhibition by certain undesirable products, including bromine species, was not addressed [28, 29, 34, 35]. The percent of AChE inhibition through use of oxidized OPs increased to between 1.6–18 fold.

The comparison of percent inhibition induced by unoxidized and oxidized 1×10^{-7} M OPs and the standard synthetic mixture, at flow rates 0.5 mL/min and 0.1 mL/min is shown in Table 3. Based on this finding, the lower flow rate leads to decreasing of the detection limit of OPs. It is obvious from Fig. 4 that all investigated OPs at concentration 1×10^{-7} M induced more than 10% inhibition of sensor enzyme activity. Table 3 also presents the detection limits obtained for each single OP at flow rate 0.1 mL/min.

Conclusion

This study demonstrates the applicability of enzyme MPO for the oxidation of OPs as the useful method to improve the sensitivity of AChE-based bioanalytical assays using free or immobilized enzyme. The degree of OPs oxidation depends on the OPs and MPO concentrations, as well as on incubation time between OPs and MPO.

The 10 min incubation time of OPs in phosphate buffer pH 6.0 with 50–100 nM MPO was found to be applicable for OPs oxidation, to produce oxo forms with stronger inhibitory power towards AChE. Since the detection limit of immobilized AChE for OPs after oxidation was below 1×10^{-7} M, the sensitivity of the bioanalytical assay using preoxidation step with MPO is satisfactory. Reactivation of the inhibited enzyme in FIA manifold with 2-PAM restored the enzyme activity almost completely, allowing the repeated use of this sensor for OPs analysis.

The developed procedure might be applied as a convenient screening method for determination of OPs in selected real samples even without complicated clean-up steps, which save time, money and the environment.

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