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Multichannel spectrophotometry for analysis of organophosphate paraoxon in beverages

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Multichannel spectrophotometry was performed to assay for paraoxon in spiked beverages. A 96-well microplate was used for this purpose. The measuring protocol was based on inhibition of the enzyme acetylcholinesterase by paraoxon that resulted in decreased or no reaction of the enzyme product thiocholine with Ellman's reagent (5,5′-dithiobis [2-nitrobenzoic acid]). The above assay was practically tested using spiked drinking water, mineral water, and coffee. Analytical parameters such as the limit of detection, time, and sample size consumption were adequate. The limit of detection for beverages ranged from 32 to 48 ppb corresponding to 0.32-0.48 ng of paraoxon in absolute values. The described assay seems to be convenient in terms of practical use.

Key Words: Acetylcholinesterase, cholinesterase, biosensing, pesticides, assay, Ellman.

Introduction

The toxic effects of organophosphorus insecticides have long been intensively studied. Affected acetylcholinesterase (AChE) can result in cholinergic excess; furthermore, inactivation of butyrylcholinesterase (BuChE) is another critical effect.[1] Active centres of enzymes AChE and BuChE include histidine, glutamate, and serine.

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The inhibition mechanism is based on phosphorylation of the serine hydroxy group resulting in cholinesterase dysfunction.[2,3] Reactivation of the cholinesterase activity is possible by the action of some compounds. The quaternary pyridine aldoximes, in particular, seem to be quite useful.[4,5]

Paraoxon (diethyl p-nitrophenyl phosphate) is a toxic compound with LD_{50} lower than 10 mg/kg. Its main toxicological pathway is the inhibition of cholinesterases in a way presented in Figure 1. It also influences the body through muscarinic and nicotinic receptors.[6] Paraoxon may be metabolised in the body from another pesticidal compound, i.e. parathion (diethyl p-nitrophenyl thiophosphate), in which the sulphur atom of the phosphate group is displaced by oxygen.[7] Exposure to paraoxon results in convulsions, poor vision, vomiting, and dyspnoea. Advanced poisoning leads to lung oedema and subsequent respiratory arrest.

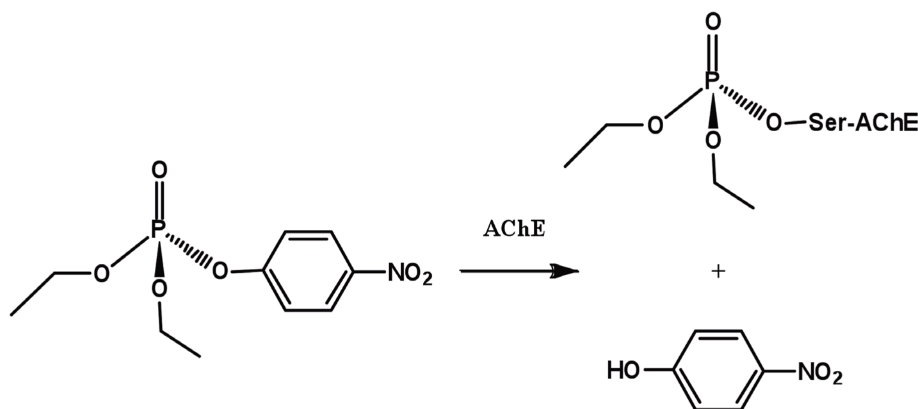

Figure 1. The toxic pathway of paraoxon. The active centre of acetycholinesterase (AChE) is phosphorylated by paraoxon leaving the p-nitrophenol as a result of the reaction.

Detection techniques for organophosphates and carbamates may be based on instrumental methods. For example, mass spectrometry was successfully employed to assay multiple pesticides with the limit of detection of approximately 1 μg/mL;[8] the tandem of liquid chromatography together with mass spectrometry proved useful for pesticides in spiked lemons as well as raisins with the limit of detection (LOD) of 0.01 mg/kg.[9] Chromatographic techniques are also considered useful for pesticides. The importance of thin layer chromatography was recently reviewed.[10] Liquid chromatography may detect concentrations as low as 0.0083 μg/L of pesticides in groundwater.[11] Gas chromatography was used for pesticides in milk.[12] The standard spectrophotometric Ellman's method may also be used to assay for pesticides.[13–15] Such a test was used for monitoring pesticides in surface waters [16] and, furthermore, it was employed to examine the cholinesterase activity in canine sera.[17] When the enzymatic activity was inhibited by metal ions, Ellman's assay for the cholinesterase activity was commonly used with another chromogen, i.e. o-nitrophenyl acetate.[18] The cholinesterase activity may be measured by m-nitrophenol reaction with the formed acetate.[15] Another way of detection of toxic compounds is that of biosensors presented recently.[19] Biosensors for pesticides based on cholinesterase were thoroughly reviewed[20] and one of this type of biosensor was used for a pharmacological study of a cholinesterase reactivator and its impact.[21] Nevertheless, cheap and reliable multichannel methods for paraoxon and pesticides, in general, remain a challenge for practical use. In the present work, we introduce the multichannel spectrophotometry technique to assay for the pesticide paraoxon. Paraoxon is a pesticide in common use and the risk of contamination of

water supplies remains in some countries. Spiked beverages and drinking water were chosen as commodities that will probably contain paraoxon when water supplies are polluted.

Materials and methods

Reagents

Paraoxon-ethyl (paraoxon hereinafter in the text) was obtained from Labor Dr. Ehrenstorfer-Schafers (Augsburg, Germany) as pure liquid. Human recombinant acetylcholinesterase (AChE; EC 3.1.1.7) - lyophilized, acetylthiocholine chloride (ATChCl), and Ellman's reagents (DTBN) - 5,5′-dithiobis (2-nitrobenzoic acid) were purchased from Sigma-Aldrich (St. Louis, MO, USA). AChE was diluted with deionised water up to the final activity 0.1 U/μl; the prepared solution was kept in a fridge until use. All other chemicals were of the highest purity. Millipore was used to produce the deionised water throughout the experiments.

Drinking water and beverages

Packed mineral water and packed drinking water were purchased in a local shop and employed without any adjustments. The coffee drink tested was prepared from ground coffee by mixing 5.0 g of the powder with 200 mL of boiling water and letting it cool down. Beverages were spiked with paraoxon in serial dilutions of 1 – 10 – 100 – 1000 – 10,000 – 100,000 - 1,000,000 parts per billion (ppb). Deionised water spiked in the same manner was used for optimisation purposes.

Measuring procedure

Microplates with 96-wells (Gama, Ceske Budejovice, Czech Republic) were chosen for all experiments, and 1 mM ATChCl and 0.4 mg/mL DTBN were prepared as solutions in phosphate buffered saline (PBS). The 2 solutions were mixed 1:1 into the stock solution shortly before measuring. A total of 10 μL of the measured sample was injected per well, along with 1 μL of enzyme solution (lyophilised powder suspended in deionised water; activity adjusted accordingly to optimisation), followed by incubation for 10 min. A total of 40 μL per well of stock solution was injected after the incubation and absorbance at 412 nm was measured immediately by the absorbance reader MRX (Dynatech Laboratories, Chantilly, VA, USA) and then at 3, 7, 5, and 10 min intervals. Three wells filled with DTBN in PBS were used as blanks.

Experimental data evaluation

The data were processed using the Origin 6.1 software (Northampton, MA, USA). Experimental points were fitted according to the following function:

$\Delta A = \frac{(\Delta A_1 - \Delta A_2)}{(1+e^{(c-IC_{50})/dc})} + \Delta A_2$ (equation 1)

The absorbance A is expressed as a function of the concentration c. Logarithm of paraoxon concentration in ppb is taken for the value c. Values ΔA_2 and ΔA_1 represent the absorbance of completely inhibited ($c \rightarrow \infty$) and without inhibition ($c \rightarrow 0$) AChE, respectively. IC_{50} represents concentration of paraoxon

providing 50% inhibition of the AChE activity (point of inflexion). Although the concentrations in equation 1 are logarithmic, they are converted into the ppb scale in the proper text. Standard deviation was calculated for every experimental point (n = 3).

Results and discussion

Measuring optimisation

Two ways of optimisation were considered important, i.e. the time of pre-incubation and the enzymatic activity per well. Paraoxon solution in deionised water was used during the optimisation procedure. LOD was taken as the optimised parameter. It was estimated as a point on the calibration curve corresponding to the triplicate of deviation of the blank value (S/N = 3). The time of pre-incubation with DTBN and ATChCl can influence not only the limit of detection but also economic aspects. Four intervals, i.e. 3, 5, 7, and 10 min, were consequently measured to obtain the calibration scale for the inserted activity 0.01 U. Absorbance for the calibration scale and the given time intervals are presented in Figure 2.

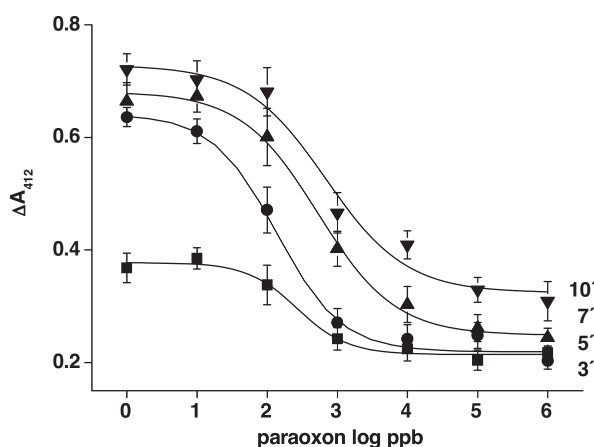

Figure 2. Optimisation of time pre-incubation for the paraoxon assay by an assay based on cholinesterase. Experimental points express 3 min (■), 5 min (●), 7 min (▲), and finally 10 min (▼) of pre-incubation prior to reading the absorbance. Error bars indicate standard deviation of 3 independent measurements.

Equation 1 parameters were computed for every interval. The lowest LOD was achieved for 5 min of pre-incubation, i.e. 26 ppb. Moreover, 7 and 10 min of pre-incubation provided the LOD of 135 and 138 ppb, respectively. The worst LOD of 141 ppb was estimated for 3 min of pre-incubation. The time interval of 5 min was chosen for the subsequent experiments. However, longer times could be useful for measuring higher levels of paraoxon concentration. For this reason, the time interval of 7 min was also used. Better resolution in the higher paraoxon concentrations is obvious from the IC_{50} at 7 min of pre-incubation. It is 148 and 537 ppb after 5 and 7 min of pre-incubation, respectively. Approximately above the calibration point of 300 ppb, the quantification is better at this time interval. The time of pre-incubation is important for accumulation of sufficient amounts of thiocholine and yellow products of Ellman's reagent. On the other hand, excessively long

incubation can suppress differences between partially inhibited cholinesterases in solution and the non-inhibited ones.

The activity inserted into one well was the second thing to optimise. Three different activities of cholinesterase of 0.001, 0.01, and 0.1 U were inserted into wells and followed by 5 min of pre-incubation and 7 min of incubation for reaction visualisation. Three calibration scales for the tested activities are included in Figure 3.

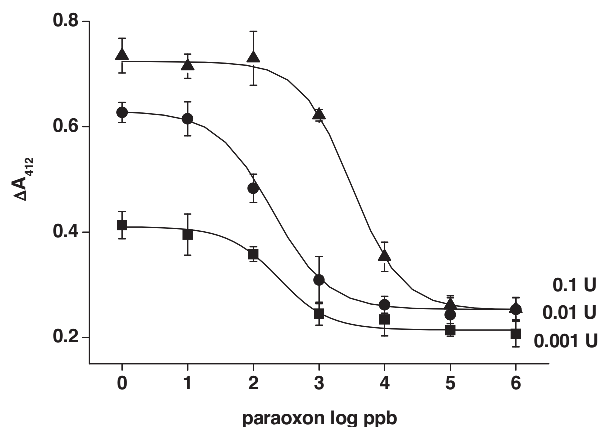

Figure 3. Plots showing optimisation of the cholinesterase activity. Inserted activities per wells of 0.001 U (■), 0.01 U (●), and 0.1 U (▲) are indicated inside the figure. Standard deviation (n = 3) is expressed by error bars.

The lowest activity per well, i.e. 0.001 U, was not found to be sufficient. The parameter ΔA_1 reached only 0.410 ± 0.023 and the LOD was 135 ppb. The highest activity was found to be sufficient for reaching high ΔA_1 although the LOD of 468 ppb was very high. The activity of 0.01 U was chosen as optimum. The obtained LOD in this experiment was 35 ppb; the parameter ΔA_1 was equal to 0.632 ± 0.020. The primary aim of optimisation was to study the ration AChE to inhibitors ration impact on the limit of detection. However, the data obtained confirm the effect of time of incubation as presented in Figure 1 rather than the impact of numbers of AChE active centres.

Measuring of spiked beverages

Three spiked beverages were chosen for the experiment. Packed mineral and drinking waters represented drinking supplies. The coffee drink served as a beverage that could be prepared from contaminated water. In the same manner as in the optimisation part, the LOD was considered the main evaluated parameter. Absorbance was measured after 5 min; however, a 7-min interval was also employed based on the results from the previous part.

The best LOD was achieved for paraoxon in drinking water, i.e. 32 ppb (0.32 ng in absolute values) after 5 min of pre-incubation; a slightly higher LOD of 48 ppb (0.48 ng in total) was obtained for paraoxon in the mineral water. Nevertheless, the calibration curve for mineral water was quite similar to that of the drinking water: the IC_{50} for 5 min of pre-incubation was 120 ppb in comparison with 129 ppb when drinking water was measured. Calibration curves for paraoxon in drinking and mineral waters are included in Figures 4 and 5, respectively.

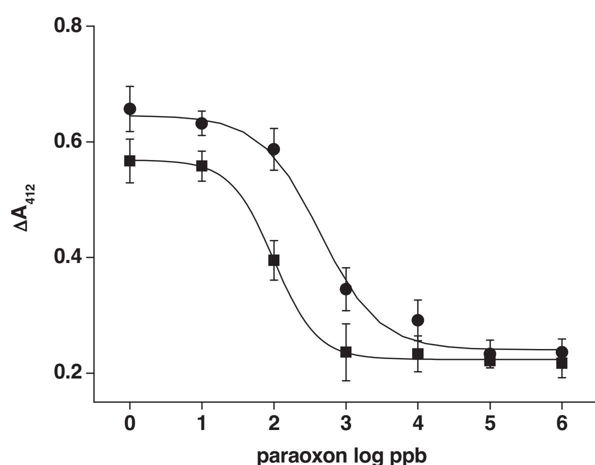

Figure 4. Calibration curves for paraoxon in drinking water. The first curve (experimental points ■) is for 5 min of pre-incubation, the second curve (●) is for 7 min of pre-incubation. Error bars indicate standard deviation (n = 3).

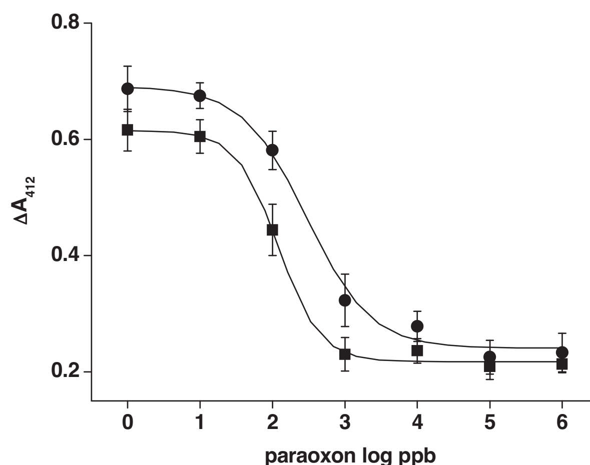

Figure 5. Calibration of paraoxon spiked into mineral water. Curves for 2 pre-incubation times are shown: 5 min (■) and 7 min (●). Standard deviation (n = 3) is expressed by error bars.

Figure 6 presents laboratory assays for paraoxon in the coffee drink. Although we expected some kinds of influences of the coffee ingredients on the paraoxon assay, no striking changes in the calibration curve as compared with drinking and mineral waters were observed. On the other hand, parameters ΔA_1 and ΔA_2 in the coffee calibration curve (0.691 ± 0.029 and 0.242 ± 0.012 for 5 min of pre-incubation) are higher than those in the drinking water (0.570 ± 0.020 and 0.222 ± 0.013) as well as in the mineral water (0.615 ± 0.027 and 0.218 ± 0.011). This fact may be due to partial reactions of coffee components with DTBN; however, the obtained LOD of 34 ppb (i.e. 0.34 ng) seems to be quite good and is similar to that obtained for drinking water contaminated by paraoxon.

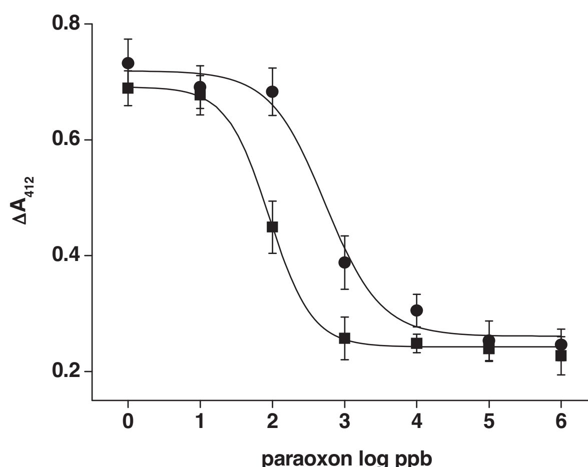

Figure 6. Plots showing calibration for paraoxon spiked into the coffee drink; 5 min (■) and 7 min (●) of pre-incubation are given in the figure. Standard deviation (n = 3) is attributed to the experimental points.

Indeed, we are encouraged by the results obtained. Especially the adequate analytical parameters in different beverages are advantageous. It may be feasible for analyses of a great number of samples assayed at a time. We consider the described multichannel spectrophotometry assay a useful tool of practical importance. As known from previous studies, the AChE based assay is sensitive to inhibition caused by e.g. some metal ions,[22,23] nanoparticles,[24] and aflatoxins.[25,26] Carbamate pesticides are another type of AChE inhibitor. These toxic compounds can also be monitored using an assay with AChE as recognition element. The performance of biosensors can be introduced as an example.[27] The inhibitory impact of the other toxins should not be considered typical interference since the levels in food and beverages of such compounds are also regulated. The substantial fact was the ability to assay inhibitors in a complex matrix without any proven interference or significant shift of limit of detection due to desired parts of these matrices.

Conclusions

Photometric multichannel spectrophotometry based on AChE as a recognition element was tested for the pesticide paraoxon assay in spiked beverages. Although the photometric assay of organophosphates is not a novel idea, multichannel devices such as multichannel spectrophotometers may be advantageous under practical testing conditions of food and beverages considering the size and numbers of samples evaluated at a time, in particular. In this study, a 96-channel plate, which is considered very advantageous when evaluating great numbers of samples, was employed. Regarding the good results achieved, we may suppose common practical usage of the described method in the future.

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