

Improved electrochemical analysis of neuropathy target esterase activity by a tyrosinase carbon paste electrode modified by 1-methoxyphenazine methosulfate

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Received 29 October 2004; Revisions requested 12 November 2004; Revisions received 8 June 2005; Accepted 10 June 2005

Key words: biosensor, mediator, 1-methoxyphenazine methosulfate, neuropathy target esterase, tyrosinase

Abstract

A graphite-paste tyrosinase biosensor was improved by adding 1-methoxyphenazine methosulfate as a mediator. Mediator modification enhanced sensitivity to phenol 4-fold and long-term stability 3-fold. Phenol could be detected at 25 nM (S/N=2) using an Ag/AgCl reference electrode. The biosensor was used to measure the activity of a toxicologically significant enzyme, neuropathy target esterase (NTE), which yields phenol by hydrolysis of the substrate, phenyl valerate. Using the new biosensor, blood and brain NTE inhibition by organophosphorus (OP) compounds with different neuropathic potencies were well correlated ($r=0.990$, $n=7$), supporting the use of blood NTE as a biochemical marker of exposure to neuropathic OP compounds.

Introduction

Tyrosinase biosensors can detect phenol electrochemically at sub-nanomolar levels (Kotte *et al.* 1995, Shan *et al.* 2002, Wang *et al.* 2002). Accordingly, such electrodes are widely used, mainly for detection of phenols as environmental pollutants. Phenolic biosensors can also monitor enzymatic processes that yield phenols.

Tyrosinase biosensors have been used for electrochemical analysis of a toxicologically significant enzyme, neuropathy target esterase (NTE) (Sigolaeva *et al.* 1999, 2001, Makhaeva *et al.* 2003). NTE hydrolyzes the substrate, phenyl valerate, to produce phenol. Inhibition of NTE by neuropathic organophosphorus (OP) compounds initiates OP compound-induced delayed neurotoxicity (OPIDN) (Johnson 1982,

Glynn 1999, Huggins & Richardson 1999). Inhibition of brain NTE within hours of exposure to a neuropathic OP compound predicts the onset of OPIDN in adult hens 1–3 weeks later (Johnson 1982, 1990, Lotti 1986, Richardson 1995, 1998). Tyrosinase biosensors enable NTE to be assayed in whole blood, which cannot be done with the standard colorimetric method (Sigolaeva *et al.* 2001). The original tyrosinase carbon-paste biosensor was used to establish correlations of NTE inhibition in blood with that in lymphocytes and brain 24 h after dosing hens with a neuropathic OP compound, *O,O*-di-1-propyl *O*-2,2-dichlorovinyl phosphate. These studies demonstrated that the biosensor NTE assay for whole blood could serve as a biomarker of exposure to neuropathic OP compounds (Makhaeva *et al.* 2003).

The present report describes the preparation of a 1-methoxyphenazine methosulfate (MPMS)-modified tyrosinase carbon-paste electrode for improved electrochemical analysis of NTE activity in blood for the enhancement of biomonitoring exposures to neuropathic OP compounds.

Materials and methods

Chemicals

Mushroom tyrosinase (monophenol monooxidase, EC 1.14.18.1), activity 3800 U/mg for L-tyrosine, graphite powder, phenol, MPMS, and Paraoxon (*O,O*-diethyl-4-nitrophenyl phosphate) were purchased from Sigma. Phenyl valerate was from Oryza (Chelmsford, USA). *N,N'*-di-2-propylphosphorodiamido fluoridate (Mipafox), *O,O*-diethyl *O*-2,2-dichlorovinylphosphate, *O,O*-di-1-propyl *O*-2,2-dichlorovinylphosphate and the *O*-alkyl-*O*-methylchloroformimino phenylphosphonate compounds [(RO)C₆H₅P(O)ON = CCICH₃ (R = alkyl)] were synthesized and characterized in the Institute of Physiologically Active Compounds, Russian Academy of Sciences (RAS) (Russia). All other chemicals were of analytical grade and used without further purification. Aqueous solutions were prepared using deionized water.

Preparation of tyrosinase electrode and assembly of biosensor

The tyrosinase electrode was prepared as described below. Graphite/paraffin oil (3.5:1, w/w) were mixed thoroughly and packed firmly into a plastic holder (2 mm diameter). Electrical contact was established by a 0.2-mm copper wire inserted from the rear. The surface of the electrode was covered by the composite of tyrosinase, graphite, MPMS, and paraffin oil (2:5.6:1:2, by weight), and then the surface was polished. The electrodes were stored dry at 4 °C until use. In experiments with optimization of tyrosinase concentration in carbon composite, tyrosinase, graphite, MPMS, and paraffin oil were [$x:(7.6-x):1:2$, by weight]. The enzyme amount (x) was varied from 0.2 to 2 mg. The components ratio of [$2:(6.6-y):y:2$, by weight] was used for optimization of MPMS con-

centration. MPMS amount (y) was in the range of 0.2–1.4 mg.

Electrochemical measurements

All measurements were performed with an IPC-2000 potentiostat (Institute of Physical Chemistry, RAS). The amperometric current response was recorded at –150 mV vs. the Ag/AgCl reference electrode in a flow-through electrochemical cell after an injection of the samples into a flow of sodium phosphate buffer with 0.1 M NaCl, via an injector with a 50 μ l sample loop (Valve V-7, Pharmacia, Sweden) at a flow rate of 0.25 ml/min. Each measurement was made in triplicate.

The cyclic voltammograms of the electrodes were performed in a 1 ml electrochemical cell with stirring in the *o*-quinone redox potential range. In all cases, a third cyclic voltammogram was used for calculations.

Hen brain NTE assay

A lyophilized hen brain membrane fraction consisting of combined mitochondrial/synaptosomal and microsomal pellets (P₂+P₃) (Richardson *et al.* 1979) preinhibited with paraoxon (40 μ M at 25 °C for 45 min) was used as a source of NTE. It was prepared as described (Makhaeva & Malygin 1999) and stored in sealed ampoules. Before use, ampoule contents were suspended with a Potter homogenizer in 5 ml of working buffer (50 mM Tris/HCl, 0.2 mM EDTA, pH 8.0 at 25 °C).

NTE activity was determined colorimetrically according to the differential inhibition method of Johnson (1977) as modified by Kayyali *et al.* (1991) at 492 nm using a Benchmark Plus Microplate Reader (Bio-Rad, USA). Each measurement was made in triplicate. Protein was determined by the microbiuret method with bovine serum albumin as a reference standard.

Whole blood NTE assay

Whole hen blood stabilized by citrate/EDTA was diluted 10-fold with working buffer and homogenized in a Potter glass/glass homogenizer before NTE assay. The differential inhibition method of Johnson (1977) with an electrochemical endpoint

was used as described in (Makhaeva *et al.* 2003). Each measurement was made in triplicate.

Protein was determined using a Coomassie protein assay kit from Sigma (USA) with bovine serum albumin as a reference standard.

NTE I_{50} determinations

The I_{50} (the concentration that inhibits 50% of the enzyme activity under defined preincubation conditions) for inhibitors against NTE was measured by preincubating a sample of enzyme with 10–12 different concentrations of the OP compound for 20 min at 37 °C, in working buffer. Residual NTE activity was then determined either using the colorimetric (brain NTE) or the amperometric (blood NTE, the MPMS-modified tyrosinase carbon-paste electrode) method. Each measurement was made in triplicate (colorimetry) or in duplicate (amperometry). I_{50} values were calculated using Origin 6.1 software (Northampton, MA, USA). Every value represents the mean \pm SEM from three independent experiments.

Results and discussion

Mediator-modified tyrosinase carbon paste electrode

The tyrosinase carbon paste electrode involves oxidation of phenol via catechol into *o*-quinone by the tyrosinase. Then *o*-quinone is electrochemically reduced to catechol directly at the electrode when the required potential is applied. However, regeneration via mediator gives an increased sensitivity to phenol compared to the direct electro-reduction of *o*-quinone (Figure 1).

We used MPMS as a light-stable mediator for the tyrosinase sensor modification. Various aspects concerning the electrode construction and operation have been studied and optimized as detailed below.

Cyclic voltammetry

Figure 2 shows the cyclic voltammograms of the mediator-modified tyrosinase electrode in 10 μ M phenol solution at various potential scan rates in the range between 2 and 40 mV/s.

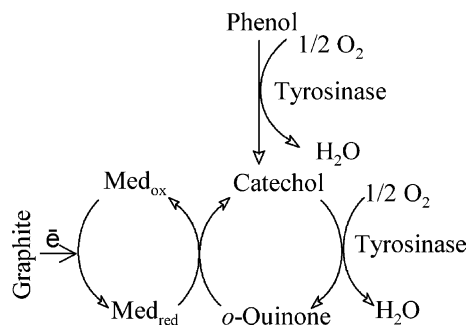


Fig. 1. Principle of amperometric phenol detection by 1-methoxyphenazine methosulfate (MPMS)-modified tyrosinase carbon-paste electrode.

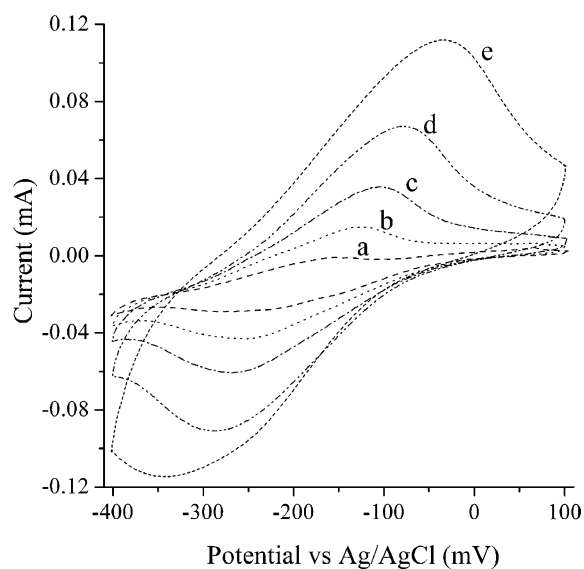


Fig. 2. Cyclic voltammograms of the 1-methoxyphenazine methosulfate (MPMS)-modified electrode at various potential scan rates. Conditions: tyrosinase and MPMS contents in carbon paste were 2 mg and 1 mg, respectively; 0.05 M phosphate buffer solution with 0.1 M NaCl; pH 7.0; 10 μ M phenol. The potential scan rates were (a) 2, (b) 5, (c) 10, (d) 20 and (e) 40 mV/s vs. Ag/AgCl.

Both the oxidation and reduction potential values depended upon the scan rate. In addition, the cathodic and anodic peak currents coincided, and the nonlinearity depended upon the square root of the potential scan rate (data not shown). Thus, the redox system considered represents quasi-reversible character, and the polymeric quinoid products that can irreversibly bind and poison the electrode surface apparently do not readily form.

Optimization of carbon paste composite

The influence of tyrosinase and mediator concentrations in the carbon composite on analytical properties of the modified electrodes was studied by screening electrode response to the addition of standard phenol solution. The electrodes were prepared as described above. The maximum response was achieved with 2 mg tyrosinase. Further increasing enzyme content resulted in deterioration of the physical properties of the carbon composite resulting in peeling off the active paste layer from the electrode surface. The effect of MPMS concentration in the carbon composite on the electrode response was assessed between 0.2 and 1.4 mg MPMS. The electrodes were prepared as described above. The electrode response was found to increase to a plateau at 0.6 mg of the mediator content. Thus, a signal gain of more than four times was achieved in comparison with the unmodified electrode.

Optimization of measuring conditions

The effect of operating potential on the mediator-modified electrode response was examined in the range between -25 and -250 mV vs. Ag/AgCl in comparison with the unmodified electrode. As seen in Figure 3, the highest signals are obtained at potential values between -175 and -200 mV for both electrode types. Nevertheless, the recommended optimal operating potential is -150 mV, because the background current significantly decreases in that case, while the electrode responses remain high. Furthermore, the relatively low operating potential decreases the influence of interfering electrochemical reactions on selectivity and sensitivity of the assay.

The influence of pH was studied from 4.5 to 9.5. The addition of the mediator to the carbon paste composite did not shift the pH optimum of immobilized tyrosinase (Duckworth & Coleman 1970), which remained at pH 7.0.

Figure 4 shows the influence of ionic strength on the character of response of both the mediator-modified and unmodified electrodes to $10 \mu\text{M}$ phenol. The response of the unmodified electrode increased with rising NaCl concentration up to 0.05 M. Further increases in ionic strength of the buffer solution had no effect on electrode response. The mediator-modified electrode

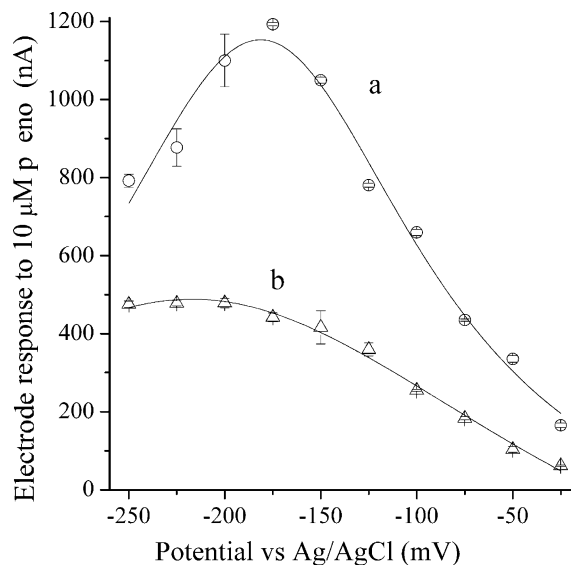


Fig. 3. Effect of the operating potential on the response current of 1-methoxyphenazine methosulfate (MPMS)-modified (a) and unmodified (b) electrodes. Conditions: 0.05 M phosphate buffer solution with 0.1 M NaCl; pH 7.0; $10 \mu\text{M}$ phenol.

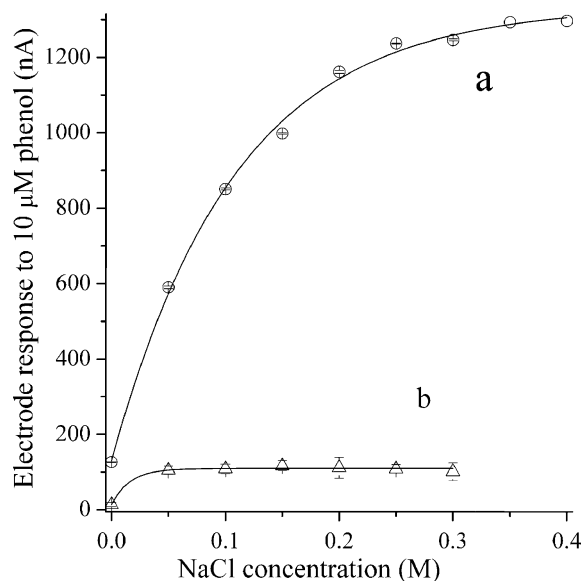


Fig. 4. Influence of NaCl concentration on the response of 1-methoxyphenazine methosulfate (MPMS)-modified (a) and unmodified (b) electrodes. Conditions: 0.05 M phosphate buffer solution containing (0 to 0.35 M) NaCl, pH 7.0; $10 \mu\text{M}$ phenol.

response increased with rising NaCl concentration up to 0.35 M, and exhibited about a two-fold increase in electrode response in a comparison

with standard conditions of measurements at 0.1 M NaCl.

Analytical characteristics of MPMS-modified tyrosinase carbon paste electrodes

The dependence of the MPMS-modified electrode responses on a wide range of phenol concentration is shown in Figure 5. The response was linear up to 50 μM . The detection limit of 25 nM ($S/N=2$) was reached for the mediator-modified electrode, while the detection limit of the unmodified electrode was 100 nM.

The operational stability of MPMS-modified electrodes was examined by serial measurements of 100 μM phenol. After 80 measurements, the electrode retained its initial activity with a coefficient of variation of less than 3%.

The results on functional stability are shown in Figure 6. Data were obtained for electrodes used in the flow-injection system at room temperature during the day and stored overnight at 4 °C in the working buffer solution. The unmodified electrode lost about 80% of its initial activity after 9 days. In contrast, the response of the MPMS-modified electrode decreased 23% after 9 days and 37% after 15 days. In the dry state at 4 °C, the electrodes keep their initial activity for more than 12 months.

The main analytical characteristics of MPMS-modified electrodes are summarized in Table 1.

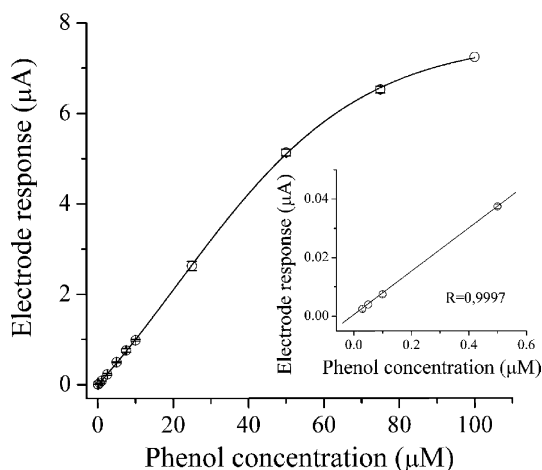


Fig. 5. Calibration curve of phenol at the methoxyphenazine methosulfate (MPMS)-modified electrode. Conditions: 0.05 M phosphate buffer solution with 0.1 M NaCl; pH 7.0; applied working potential -150 mV vs. Ag/AgCl.

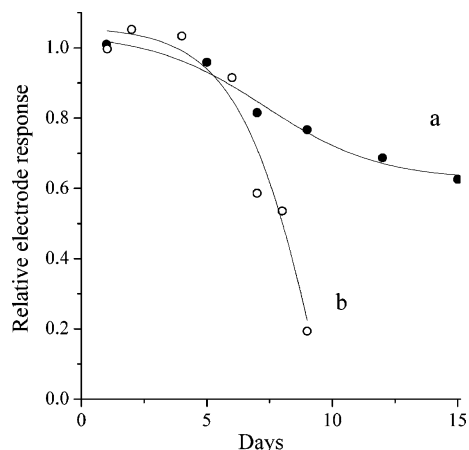


Fig. 6. Storage stability of the 1-methoxyphenazine methosulfate (MPMS)-modified (a) and unmodified (b) electrodes in 0.05 M phosphate buffer solution with 0.1 M NaCl; pH 7.0; 4 °C. Relative electrode response values (ordinate axis) represent the electrode response obtained in a period from 1 to 15 days of storage against the initial electrode response obtained on the first experimental day.

Table 1. Characteristics of the phenol biosensor based on 1-methoxyphenazine methosulfate (MPMS)-modified tyrosinase carbon paste electrode.

Analytical parameter	Value
Linear range	0.025–50 μM
Detection limit of phenol ($S/N=2$)	25 nM
Sample volume	50 μl
Time of analysis	2 min
Work stability	> 60 % during 21 days
Storage stability (dry)	> 12 months
Coefficient of variation of electrode preparation	25%
Coefficient of variation of 80 consecutive phenol assays	3%

Whole blood NTE analysis as a biomarker of neuropathic OP compound exposure

We previously showed that the tyrosinase carbon-paste electrode remarkably improved the sensitivity of the NTE assay in comparison with a colorimetric method (Sigolaeva *et al.* 2001). Due to the high sensitivity of the biosensor method, the influence of interfering blood components (ascorbic acid, tyrosine, and others) was reduced by the high extent of sample dilution, thus allowing NTE to be detected selectively and with high sensitivity in whole blood, for which the usual colorimetric

Table 2. The *in vitro* comparison of sensitivity of hen blood and brain NTE to OP compounds with different neuropathic potential.

Compound	I_{50} , μM	
	Brain NTE ^a (Colorimetry)	Blood NTE ^a (Amperometry)
$\begin{array}{c} \text{O} \\ \parallel \\ (\text{CH}_3)_2\text{CHNH} - \text{P} - \text{F} \\ \parallel \\ (\text{CH}_3)_2\text{CHNH} \end{array}$	3.8 ± 0.3	4.17 ± 0.26 4.22 ± 0.12 (Makhaeva <i>et al.</i> 2002) ^b
$\begin{array}{c} \text{C}_2\text{H}_5\text{O} \\ \diagdown \\ \text{P} \\ \diagup \\ \text{C}_2\text{H}_5\text{O} \end{array} \begin{array}{c} \text{O} \\ \parallel \\ \text{O} - \text{CH} = \text{CCl}_2 \end{array}$	1.1 ± 0.09	1.23 ± 0.12
$\begin{array}{c} n\text{-C}_3\text{H}_7\text{O} \\ \diagdown \\ \text{P} \\ \diagup \\ n\text{-C}_3\text{H}_7\text{O} \end{array} \begin{array}{c} \text{O} \\ \parallel \\ \text{O} - \text{CH} = \text{CCl}_2 \end{array}$	0.038 ± 0.002	0.058 ± 0.005 0.066 ± 0.003 (Makhaeva <i>et al.</i> 2003) ^b
$\begin{array}{c} \text{CH}_3\text{O} \\ \diagdown \\ \text{P} \\ \diagup \\ \text{C}_6\text{H}_5 \end{array} \begin{array}{c} \text{O} \\ \parallel \\ \text{O} - \text{N} = \text{C} \begin{array}{l} \diagup \text{CH}_3 \\ \diagdown \text{Cl} \end{array} \end{array}$	18.2 ± 3.2	31.6 ± 4.1
$\begin{array}{c} \text{C}_2\text{H}_5\text{O} \\ \diagdown \\ \text{P} \\ \diagup \\ \text{C}_6\text{H}_5 \end{array} \begin{array}{c} \text{O} \\ \parallel \\ \text{O} - \text{N} = \text{C} \begin{array}{l} \diagup \text{CH}_3 \\ \diagdown \text{Cl} \end{array} \end{array}$	5.75 ± 0.72	4.9 ± 0.51
$\begin{array}{c} n\text{-C}_3\text{H}_7\text{O} \\ \diagdown \\ \text{P} \\ \diagup \\ \text{C}_6\text{H}_5 \end{array} \begin{array}{c} \text{O} \\ \parallel \\ \text{O} - \text{N} = \text{C} \begin{array}{l} \diagup \text{CH}_3 \\ \diagdown \text{Cl} \end{array} \end{array}$	2.4 ± 0.12	1.95 ± 0.2
$\begin{array}{c} n\text{-C}_4\text{H}_9\text{O} \\ \diagdown \\ \text{P} \\ \diagup \\ \text{C}_6\text{H}_5 \end{array} \begin{array}{c} \text{O} \\ \parallel \\ \text{O} - \text{N} = \text{C} \begin{array}{l} \diagup \text{CH}_3 \\ \diagdown \text{Cl} \end{array} \end{array}$	0.512 ± 0.038	0.501 ± 0.045

^aControl NTE activities, nmol/(min×mg protein), mean ± SEM, $n = 3$. Lyophilized preparation of hen brain NTE = 41 ± 2, whole blood = 0.23 ± 0.02.

^b I_{50} was determined using the unmodified tyrosinase carbon-paste electrode.

assay is impossible. Using the unmodified electrode, whole blood samples could be diluted 1:500 (v/v). Dilution of 1:1400 (v/v) was achieved with the MPMS-modified electrode.

Blood NTE activity measurements with the new MPMS-modified tyrosinase carbon-paste electrode were explored as a possible biochemical marker of neuropathic OP compound exposures. The biosensor assay was used to obtain I_{50} values for inhibition of blood NTE activity *in vitro* with a number of standard neuropathic OP compounds. These include mipafox, *O,O*-diethyl- and *O,O*-di-1-propyl *O*-dichlorovinyl phosphates, as well as a series of *O*-alkylphenyl phosphonates of general formula $\text{RO}(\text{C}_6\text{H}_5)\text{P}(\text{O})\text{ON}=\text{CCH}_3\text{Cl}$ where R = Me, Et, *n*-Pr, *n*-Bu. The neuropathic potential in the last series of compounds was shown to increase with R-group length (Makhaeva *et al.* 2001, Malygin *et al.* 2003). The standard colorimetric assay was used to determine the I_{50} for these compounds against a preparation of hen brain NTE. The data presented in Table 2 show that all inhibitors exhibited a similar inhibitory potency *in vitro* toward NTE from hen brain and hen blood. NTE from both sources had the same sensitivity to variations in structure and neuropathic potential of OP inhibitors. A good correlation was found between the values of pI_{50} for blood and brain NTE inhibition by the studied compounds ($r=0.990$, $n=7$). Furthermore, consistent agreement was obtained between two amperometric methods (MPMS-modified and unmodified electrodes) for I_{50} determinations of mipafox and *O,O*-di-1-propyl *O*-dichlorovinyl phosphate against blood NTE. The data support the validity of measurements carried out with the new MPMS-modified tyrosinase carbon paste biosensor and demonstrate that blood NTE inhibition is a promising biomarker of exposure to neuropathic OP compounds.

NTE Analyzer

A pilot device (Analyzer) incorporating the new biosensor for NTE assays was developed. The Analyzer consists of an internal microprocessor, flow-through electrochemical cell supplied with working and reference electrodes, pump, sampler,

vessels for working buffer and waste, liquid crystal display, and controls for manual operation. All functions are controlled and monitored by an external computer. The software provides measurement monitoring, and displays the measured signal in real-time format. Data processing is also possible.

The application of this device is expected to facilitate the rapid assessment of human exposures to neuropathic OP compounds that could arise through occupational, environmental, or accidental means, or from warfare or terrorist acts.

Acknowledgements

The research was supported by Project #1055.2 of the International Science and Technology Center (ISTC), Projects #RB2-2035 and #RB2-2488 of the U.S. Civilian Research and Development Foundation (CRDF), and Grant #DAAD19-02-1-0388 from the US Army Research Office.

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