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Biosensor analysis of blood esterases for organophosphorus compounds exposure assessment: Approaches to simultaneous determination of several esterases

Larisa Sigolaeva[a], Galina Makhaeva[b], Elena Rudakova[b], Natalia Boltneva[b], Marya Porus[a], Galina Dubacheva[a], Arkadi Eremenko[a], Ilya Kurochkin[a], Rudy J. Richardson[c,*]

[a] Department of Chemistry, M.V. Lomonosov Moscow State University, Moscow 119991, Russia
[b] Institute of Physiologically Active Compounds Russian Academy of Sciences, Chernogolovka, Moscow Region 142432, Russia
[c] Department of Environmental Health Sciences, University of Michigan, 1420 Washington Heights, Ann Arbor, MI 48109-2029, USA

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ABSTRACT

This paper reviews our previously published data and presents new results on biosensor assay of blood esterases. Tyrosinase and choline oxidase biosensors based on nanostructured polyelectrolyte films were developed for these purposes. Experiments were performed on the quantitative determination of acetylcholinesterase (AChE), butyrylcholinesterase (BChE), carboxylesterase (CaE), and neuropathy target esterase (NTE) in samples of whole blood of rats, mice, and humans. Good agreement was found between biosensor and spectrophotometric assays for AChE, BChE, and CaE. No direct comparison could be made for NTE because its activity cannot be measured spectrophotometrically in whole blood. A new method of simultaneous quantitative determination of AChE and BChE in test mixtures is also described. This method represents a bifunctional biosensor for the simultaneous analysis of choline and phenol based on integration of individual sensors. Algorithms for calculation of separate concentrations of AChE and BChE in the mixture were developed. The mean error of calculated component concentrations was ~6% for binary test mixtures. The present work provides a foundation for building multiplexed systems for the simultaneous determination of multiple esterases with applications to biomonitoring for exposures to organophosphorus compounds.

1. Introduction

Defending against organophosphorus compounds (OPs) of known structure as well as those inadvertently produced by destruction of chemical weapons, natural disasters, or terrorist attacks requires rapid, sensitive, and specific detection of them and their biological effects by developing biomarkers and biosensors of exposure. A notorious demonstration of the availability, rapidity, and lethality of these agents was the release of sarin into the Japanese commuter train system in Tokyo, in which blood cholinesterase (ChE) activity was used as a tool for confirming exposure to the agent [1].

OPs phosphylate primary targets, e.g., acetylcholinesterase (AChE; acute toxicity) and neuropathy target esterase (NTE; delayed neuropathy, OPIDN); as well as secondary targets, e.g., butyrylcholinesterase (BChE) and carboxylesterase (CaE), which act as stoichiometric scavengers of OPs. We denote the set of activities of these esterases as well as that of paraoxonase-1 (PON-1), one of the A-esterases that can act as a catalytic scavenger of some OPs by hydrolyzing and thereby detoxifying them, as the "esterase status" of an organism—a determinant of individual sensitivity to OPs and a complex biomarker of exposure [2,3].

Currently, individual activities of blood esterases are determined mainly by using spectrophotometric techniques with corresponding substrates and specific inhibitors: this is a time-consuming process, suffering from high interference from blood hemoglobin and limited sensitivity. The application of modern electrochemical biosensors is a good alternative to routinely used methods, mainly due to high sensitivity achieved in recent years for analytes, such as phenol and choline [4,5].

The layer-by-layer (LBL) technique has demonstrated its promise as a versatile way to form organized multilayer thin films with predictable physical and chemical properties [6,7]. Based on the consecutive adsorption of positively and negatively charged polyelectrolytes on a solid substrate, the technique provides control of surface properties and enables the insertion of enzymes or other functional elements into the assembly [8,9]. Recently, we used LBL technology to create new amperometric biosensors for phenol and choline by integrating tyrosinase and choline oxidase into self-assembled polyelectrolyte layers [10,11].

* Corresponding author. Tel.: +1 734 936 0769; fax: +1 734 763 8095.
E-mail address: rjrich@umich.edu (R.J. Richardson).

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This paper reviews our previously published data [12,13] and presents new results on biosensor assay of blood esterases. Here we demonstrate the applicability of the previously developed choline oxidase and tyrosinase biosensors for the analysis of individual activities of AChE, BChE, CaE, and NTE in whole blood using esters of choline and phenol as esterase substrates. To validate the new method, biosensor measurements were compared with standard spectrophotometric methods. In addition, we produced a bifunctional biosensor system for the simultaneous analysis of choline and phenol, optimized its detection, and developed an algorithm for quantification of binary test mixtures (AChE + BChE).

2. Materials and methods

2.1. Materials

Tyrosinase from mushroom (EC 1.14.18.1), activity 2870 U/mg for L-tyrosine; AChE from bovine erythrocytes (EC 3.1.1.7), activity 0.31 U/mg; BChE from horse serum (EC 3.1.1.8), activity 264 U/mg; 20% (w/w) aqueous solution of poly(dimethyldiallylammonium chloride) (PDMDAACl, MW 400–500 kDa); eserine hemisulfate; paraoxon; phenol; choline; acetylcholine chloride; butyrylcholine chloride; phenyl acetate; butyrylthiocholine iodide; and graphite rods (d = 3 mm) were from Sigma. Ethopropazine hydrochloride; 1-naphthyl acetate; acetylthiocholine iodide; tetraisopropylpyrophosphoramide (iso-OMPA); and bis-*p*-nitrophenyl phosphate were from Sigma–Aldrich. Choline oxidase from Alcaligenes species (EC 1.1.3.17), activity 13.5 U/mg for choline; and glutaraldehyde (50% (w/v) aqueous solution) were from Fluka. Phenyl valerate and mipafox were from Oryza. All other chemicals were of analytical grade and used without further purification. All aqueous solutions were prepared using deionized Milli-Q water.

2.2. Blood sampling

White outbred male mice and Wistar male rats were used in the experiments. The animals were sacrificed by decapitation under CO_2-induced anesthesia. Trunk blood from each animal was collected immediately in glass vials containing citrate (3.8% (w/v) sodium citrate, 0.2 ml citrate/ml blood). Blood from 4 anonymous human (female) donors stabilized with citrate (0.2 ml citrate/ml blood) was used in the experiments. Blood samples were aliquoted, frozen in liquid nitrogen, and stored at −70 °C before use.

2.3. Preparation of blood for analysis

Frozen blood samples were thawed in an ice-water bath. Whole blood hemolysates (1:100, v:v) were prepared by adding 1 volume of blood to 99 volumes of ice-cold diluting buffer (see below for buffer for each esterase). Aliquoted hemolysates were frozen immediately in liquid nitrogen to facilitate complete hemolysis and kept at −20 °C until analysis.

2.4. Preparation of tyrosinase/choline oxidase-based biosensors

Polished graphite rods used as is (for tyrosinase entrapping) or electrochemically modified by MnO_2 (for choline oxidase entrapping) were first immersed in PDMDAACl solution for 10 min, rinsed with deionized water, and dipped into enzyme solution for 10 min followed by rinsing with deionized water. Cross-linking by glutaraldehyde was carried out in aqueous solution of 1% (w/v) glutaraldehyde for 60 min followed by rinsing thoroughly with deionized water. Sensors were stored under air at +4 °C and used for analysis during the next several days. PDMDAACl was used as a 0.5% (w/v) solution in 0.05 M sodium phosphate (pH 7.0). Tyrosinase and choline oxidase were prepared at concentrations of 0.1 mM in 0.05 M sodium phosphate (pH 7.0) and at 0.1 mM in 50 mM Hepes with 30 mM KCl (pH 7.5), respectively.

2.5. Electrodeposition of MnO_2 onto graphite rods

Graphite rods were modified by an H_2O_2-sensitive layer of MnO_2 at a constant potential of +600 mV *vs* Ag/AgCl reference electrode in 0.1 M NH_4Cl (pH 9.5) containing 1 mg/ml $MnCl_2$. After 15 min of deposition, the electrodes were washed with deionized water, incubated at 60 °C for 1 h, and stored at room temperature for at least 3 months until use.

2.6. AChE/BChE assay

AChE/BChE activity was determined in blood hemolysates (prepared in 0.1 M sodium phosphate pH 7.5) with 1 mM of acetylthiocholine/butyrylthiocholine and 0.4 mM DTNB for the spectrophotometric method of Ellman [14] or with 1 mM of acetylcholine/3 mM of butyrylcholine for the biosensor method. AChE assay was carried out in the presence of ethopropasine (0.02 mM, 10 min preincubation), for elimination of BChE activity in whole blood [15,16].

2.7. CaE assay

Spectrophotometric and biosensor determinations of CaE activity in blood hemolysates (prepared in 0.1 M sodium phosphate, pH 8.0) were carried out with 1 mM of phenyl acetate. 1-Naphthyl acetate (1 mM) was also used as a substrate in spectrophotometric CaE assays. To discriminate CaE activity 10 min preincubation with inhibitors of PON-1/arylesterase (2 mM EDTA) and ChEs (40 μM eserine) was used [17,18].

2.8. NTE assay

NTE activity was assayed according to the differential inhibition method of Johnson [19] with an electrochemical endpoint as described previously [20,21]. Briefly, blood hemolysate samples were incubated at 37 °C with 50 μM paraoxon (sample B) or 50 μM paraoxon plus 250 μM mipafox for 20 min (sample C), followed by incubation with phenyl valerate (final apparent concentration 0.54 mM) for the next 40 min at 37 °C. The reaction was stopped by addition of 50 μl of 1% (w/v) aqueous SDS. Phenol product was assayed amperometrically after 20-fold sample dilution in 50 mM sodium phosphate with 100 mM NaCl (pH 7.0). NTE activity was calculated as the difference in phenol production between samples B and C.

2.9. Spectrophotometric esterase activity determinations

All spectrophotometric esterase determinations were carried out using a Gilford-250 spectrophotometer in 3 ml 1-cm pathlength cuvettes at 25 °C by recording the increase in absorbance due to accumulation of the specified chromophore. The amount of 1:100 (v:v) blood hemolysate was 0.8–1.0 ml in the 3 ml incubation mixture, representing a final blood dilution of 1:300–1:375 (v:v). All data were automatically corrected for spontaneous hydrolysis of each substrate measured at the same time in the reference cuvette. For AChE and BChE assays the wavelength was changed to 436 nm (ε_{436} = 10,600 M^{-1} cm^{-1}) [15] to reduce interference from the high absorbance of hemoglobin at 412 nm. In the CaE assay, when phenyl acetate was used as the substrate, appearance of phenol product was measured at 270 nm (ε_{270} = 1310 M^{-1} min^{-1}); when 1-naphthyl acetate was used as substrate, appearance of 1-

naphthol product was monitored at 332 nm (ε_{322} = 2200 M^{-1} cm^{-1}) [22,23].

2.10. Electrochemical measurements

Electrochemical measurements were performed with an IPC-TWIN bipotentiostatic system (Institute of Physical Chemistry of the Russian Academy of Science) in a 1-ml electrochemical cell, supplied with Ag/AgCl as a reference electrode and tyrosinase and choline oxidase sensors as two working electrodes operating at −150 mV and +350 mV *vs* Ag/AgCl for phenol and choline detection, respectively. Sensors were switched on separately (single-channel mode for individual esterase analysis in blood hemolysates) or simultaneously (dual-channel mode for simultaneous assay of AChE/BChE mixtures). Individual esterase analysis in blood hemolysates was carried out after fixed-time incubation of blood hemolysate with specified substrate and reaction conditions for each esterase; the reaction was stopped by addition of 100 μl of 1% (w/v) aqueous SDS. Products released were assayed amperometrically after 20–50-fold dilution of samples in 50 mM sodium phosphate with 100 mM NaCl, pH 7.0 (for phenol) or in 50 mM Hepes with 30 mM KCl, pH 7.5 (for choline), respectively. The analytical signal was determined as a value of steady-state baseline current change (the difference between an average value of steady-state baseline current before and after analyte addition). Activity values were calculated using phenol or choline calibration curves, obtained in the same conditions, and each were corrected for spontaneous hydrolysis of substrates, determined separately.

AChE and BChE activities were measured simultaneously using one phenolic substrate (phenyl valerate or phenyl acetate) and one choline substrate (acetylcholine or butyrylcholine). The optimum concentrations of substrates were determined to be as follows: acetylcholine, 1.5 mM; butyrylcholine, 1 mM; phenyl acetate, 0.5 mM; and phenyl valerate, 0.2 mM. The mixture of substrate solutions was added to the electrochemical cell and spontaneous hydrolysis of each substrate was recorded in kinetic mode. Then AChE, BChE or their mixture (AChE + BChE) was added to the electrochemical cell and enzymatic hydrolysis was recorded simultaneously. All measurements were made in 50 mM Hepes with 30 mM KCl, pH 7.5, at 25 °C. The initial rate of each enzymatic hydrolysis was calculated as the difference of the reaction rate after esterase mixture addition and the reaction rate for the spontaneous hydrolysis of the substrate.

2.11. Data and statistics

Data are represented as mean values ± SEM from at least two independent measurements with at least two replicates of each measurement.

3. Results and discussion

3.1. Biosensor development

To create biosensor assays of blood esterases, we recently developed tyrosinase and choline oxidase biosensors built on nanostructured polyelectrolyte films for phenol and choline detection. These sensors enabled activity determination for AChE, BChE, and CaE using choline and phenol esters as substrates [12]. Analytical responses of sensors were directly related to liberated phenol or choline concentrations.

In the phenol detector, tyrosinase is oxidized by oxygen and then reduced by phenol to produce quinone, which is reduced to catechol at an electrode to produce an amperometric signal. Similarly, in the choline detector, choline oxidase catalyzes the oxidation of choline by oxygen to liberate hydrogen peroxide, which is detected amperometrically. The detection limits for phenol and choline were 10 nM [11] and 100 nM, respectively. The coefficients of variation ([SD/mean] × 100) for 10 measurements of phenol and choline were 6% [11] and 1%, respectively.

3.2. Determination of individual esterase activities in blood

Biosensor detection was optimized for analysis of individual activities of AChE, BChE, CaE, and NTE in whole blood using esters of choline and phenol as substrates. First, the characterization of phenol and choline sensors for measurements in the presence of whole blood was carried out. The influence of various dilutions of blood on responses of tyrosinase and choline oxidase biosensors for standard concentrations of phenol (10^{-5} M) and choline (10^{-4} M) was tested. No effect of whole blood on the sensors was observed if the whole blood dilution was at least 1:100. Thus, the biosensors were suitable for measurements in blood and we were able to use 1:100 (v:v) blood hemolysates for further analysis.

Experiments to determine activities of individual esterases showed that the detection limit of biosensor methods was about 1–2 nmol/(min × ml blood) (tyrosinase sensor) and 10–20 nmol/(min × ml blood) (choline oxidase sensor) when the final blood dilution was at least 1:2000 (v:v). Mean time required for single phenol and choline measurements was 2 min and 3 min, respectively. Owing to the high sensitivity and fast response times of biosensor methods [11], rapid measurements could be carried out in blood samples that were highly diluted, which minimized interference from extraneous (non-analyte) substances in blood (e.g., ascorbate or urate). Activities of AChE, BChE, CaE, and NTE in whole blood from humans, rats, and mice were measured using phenol and choline biosensors in single-channel mode (Table 1). To validate the biosensor measurements of blood esterases, it was necessary to compare the results with those obtained using standard spectrophotometric methods; these results are also shown in Table 1 for all esterases except NTE, which cannot be measured spectrophotometrically in whole blood. There was good overall agreement between the biosensor and spectrophotometric results for each specific esterase.

It is of interest to compare our results on whole blood esterase activities with published data. Thus, ranges of blood AChE activities (μmol/min/ml) for each species are reported to be as follows: humans (5.0–9.6) [16,24], rats (1.1–2.3) [24], and mice (1.45–2.3) [24,25]. Ranges of blood BChE values (μmol/min/ml) for each species are reported to be as follows: humans (0.9–6.3) [16,24]; rats (0.5–1.3) [24], and mice (1. 5–2.2) [24]. Thus, our values are consistently lower than previously published values, but we find good agreement between spectrophotometric and biosensor methods, with coefficients of variation ranging from approximately 6.7% for human blood AChE to 30% for rat blood AChE.

For CaE, we could find the literature data only for plasma, and it should be noted that true CaE is not found in human plasma [26]. Apparent CaE activity in human plasma is very low; when determined with 1-naphthyl acetate, it is reported to be 0.019 ± 0.001 μmol/min/ml, a value that is 185 times less than that reported for rat plasma, 3.52 ± 0.15 μmol/min/ml [23]. In our experiments, apparent human blood CaE was also less active than rat blood CaE, but only by a factor of 16. A higher level of apparent CaE activity in human whole blood in comparison to plasma may be caused by the presence of CaE in monocytes [27].

Our spectrophotometric experiments showed that using a standard substrate, 4-nitrophenyl acetate, for the CaE assay gives values that are too high for human and rodent serum and blood. This activity was inhibited only about 50% in our experiments after 60 min incubation with iso-OMPA (1 mM) and bis-*p*-nitrophenyl phosphate (0.1 mM) (data not shown), which have been shown

Table 1
Activities of AChE, BChE, CaE, and NTE in whole blood of humans, rats, and mice determined by spectrophotometric or biosensor methods.[a].

	AChE[b]		BChE[c]		CaE[d]		NTE[e]
	spectro	biosensor	spectro	biosensor	spectro	biosensor	biosensor
Human	4.16 ± 0.14 (4)	4.05 ± 0.18 (4)	1.40 ± 0.08 (4)	1.67 ± 0.10 (4)	0.23 ± 0.11 (4)	0.31 ± 0.03 (4)	42.5 ± 4.28 (4)
Rat	0.68 ± 0.07 (4)	0.59 ± 0.09 (4)	0.13 ± 0.02 (4)	0.12 ± 0.02 (4)	3.59 ± 0.43 (4)	3.12 ± 0.09 (4)	15.6 ± 2.34 (3)
Mouse	0.92 ± 0.05 (24)	0.76 ± 0.09 (5)	0.71 ± 0.06 (23)	0.85 ± 0.03 (5)	6.80 ± 0.37 (23)	6.04 ± 1.52 (5)	17.3 ± 2.95 (6)

[a] Data are means ± SEM (*n*).
[b] AChE spectrophotometric (spectro) assay used acetylthiocholine as substrate; biosensor assay used acetylcholine as substrate. Units = μmol/min/ml whole blood.
[c] BChE spectrophotometric (spectro) assay used butyrylthiocholine as substrate; biosensor assay used butyrylcholine as substrate. Units = μmol/min/ml whole blood.
[d] CaE spectrophotometric (spectro) and biosensor assays used phenyl acetate as substrate. Units = μmol/min/ml whole blood.
[e] NTE biosensor assay used phenyl valerate as substrate. Units = nmol/min/ml whole blood.

to be selective CaE inhibitors [3,28]. These results agree with data on the hydrolysis of 4-nitrophenyl acetate by serum albumin [29,30]. When we used another standard spectrophotometric substrate, 1-naphthyl acetate, in the spectrophotometric assay and phenyl acetate in the spectrophotometric and biosensor assays, we obtained CaE activity values in reasonably close agreement with each other; moreover, this activity was fully inhibited by selective CaE inhibitors [3].

Spectrophotometric technique does not allow determining NTE activity in whole blood. As we demonstrated earlier, NTE assay in whole blood is possible only using a phenol biosensor [20]. Activity of NTE in human blood determined in this work with the new LBL biosensor was close to that obtained earlier (0.19 ± 0.02 nmoles/min/mg of protein; equivalent to 32.3 ± 3.6 nmol/min/ml blood) [20]. Rat and mouse blood NTE activities were lower than those found for human blood; although the literature values for rodent blood are apparently lacking, it is of interest to note that rat and mouse brain NTE activities are known to be lower than that of human brain [31–34].

3.3. Simultaneous determination of AChE and BChE activities in test mixtures

The possibility of integration of several sensors into a multisensor represents one of the numerous advantages of biosensor approaches. We describe here a multisensor, which allows the two main products of interest from enzymatic hydrolysis (phenol and choline) to be analyzed simultaneously. The measuring conditions were optimized where the presence of phenol and phenolic substrates did not interfere with amperometric choline determination and vice versa [12].

The response of a bielectrode sensor consists of two curves (Fig. 1). The top curve represents the response to the choline oxidase electrode and displays hydrolysis of choline ester. The bottom curve represents the response of the tyrosinase electrode and displays hydrolysis of the phenyl ester. On both curves it is possible to allocate the sites of spontaneous hydrolysis of substrates and hydrolysis of substrates in the presence of ChEs. The initial rate of enzymatic hydrolysis was calculated as the difference between the reaction rates after esterase mixture addition and spontaneous hydrolysis of the substrate.

The initial rate of enzymatic hydrolysis of substrates in the presence of ChEs is calculated according a form of the Michaelis–Menten equation, Eq. (1). Each of the terms in the equation has its commonly accepted meaning:

$$V_0 = \frac{k_2^{\text{eff}} \times S_0}{K_m^{\text{eff}} + S_0} \times E_0 \quad (1)$$

When the reaction proceeds under the action of two enzymes, the equation for the initial rate will be represented as the sum of the initial rates of each enzyme, Eq. (2)

$$V_0^{\Sigma} = v_1 + v_2 = \frac{k_2^{(1)\text{eff}} \times S_0}{K_m^{(1)\text{eff}} + S_0} \times E_0^{(1)} + \frac{k_2^{(2)\text{eff}} \times S_0}{K_m^{(2)\text{eff}} + S_0} \times E_0^{(2)} \quad (2)$$

If we let the multipliers before $E_0{}^{(1)}$ and $E_0{}^{(2)}$ in Eq. (2) be the sensitivity factors, k_s, of enzymes to substrate, S, then the total initial rate of hydrolysis of S will be given by Eq. (3):

$$V_0^{\Sigma} = k_s^{(1)} \times E_0^{(1)} + k_s^{(2)} \times E_0^{(2)} \quad (3)$$

Thus, after determining the sensitivity factors of enzymes to chosen substrates and measuring the initial rates of hydrolysis of a pair of substrates by ChEs, we can calculate the content of each ChE in the sample. Substrate pairs consisting of one phenyl ester (phenyl acetate or phenyl valerate) and one choline ester (acetylcholine or butyrylcholine) were used for AChE and BChE activity determination in the mixture.

Based on the effective kinetic parameters (K_m and V_m) determined for bovine erythrocyte AChE and horse serum BChE for all four substrates (acetylcholine, butyrylcholine, phenyl valerate, and phenyl acetate) [13], the optimum concentrations of substrates were determined to be as follows: acetylcholine, 1.5 mM; butyrylcholine, 1 mM; phenyl acetate, 0.5 mM; and phenyl valerate, 0.2 mM.

For each pair of substrates, the sensitivity factors of ChEs were determined along with the linear ranges of enzyme concentrations in which they can be measured using the appropriate pair of substrates. For this purpose, the dependencies of enzymatic rate of substrate hydrolysis on the concentration of enzyme were obtained. The sensitivity factor was calculated as the slope of the calibration curves. The sensitivity factors and linear ranges are summarized in Table 2. The lower limit was accepted as the con-

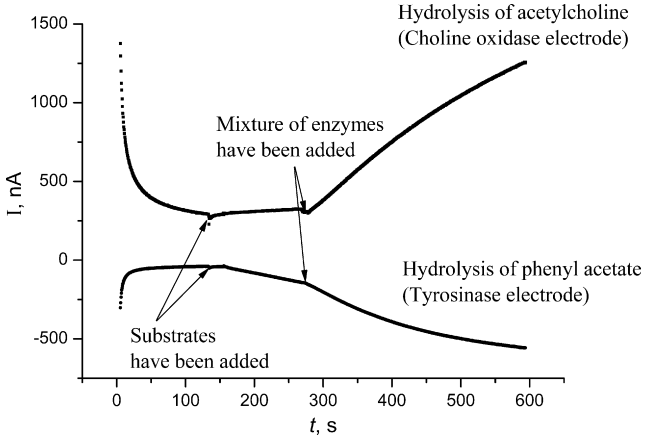

Fig. 1. Typical response to bielectrode sensor. Conditions: bielectrode cell with stirring, concentrations in the cell: AChE, 0.44 nM; BChE, 0.89 nM; acetylcholine, 1.5 mM; phenyl acetate, 0.5 mM; buffer solution, 50 mM HEPES with 30 mM KCl (pH 7.5); room temperature.

Table 2
Results on discriminative analysis of test binary mixtures of AChE/BChE for each pair of substrates: acetylcholine + phenyl acetate, acetylcholine + phenyl valerate, and butyrylcholine + phenyl acetate.[a].

Substrate pairs	AChE (bovine erythocyte)			BChE (horse serum)		
	k_s, A/M s	LR × 10^{10}, M	MD, %	k_s, A/M s	LR × 10^{10}, M	MD, %
Acetylcholine Phenyl acetate	1.1 ± 0.1 0.16 ± 0.02	2.2–32.8	13	1.76 ± 0.03 0.19 ± 0.02	7.7–44.4	4
Acetylcholine Phenyl valerate	0.94 ± 0.01 0	2.2–28.5	5	1.6 ± 0.1 0.63 ± 0.10	2.1–53.2	3
Butyrylcholine Phenyl acetate	0 0.12 ± 0.01	2.2–26.3	7	6.45 ± 0.07 0.17 ± 0.01	7.7–35.5	3

[a] Data for k_s are means ± SEM, n = 2–3 independent measurements in duplicate; A = current in amps, LR = linear range of enzyme concentrations in which they can be determined using this pair of substrates; MD = mean deviation of the activity determination of ChEs calculated as a difference between the specified and calculated concentrations for each ChE taken as the absolute value and expressed as a percentage of the specified concentration, ($[|C_{spec.} - C_{calc.}|/C_{spec.}] \times 100$).

centration of enzyme at which the total rate of substrate hydrolysis was twice the rate of its spontaneous hydrolysis.

Analysis of the data presented in Table 2 has shown that the widest linear range of the concentration of two enzymes is achieved using the substrate pair acetylcholine and phenyl valerate. The lower limit of detection for BChE is obtained for the same pair of substrates. This result is explained by a better sensitivity of BChE to phenyl valerate in comparison with phenyl acetate. This result is in agreement with other published work on a bienzyme biosensor (tyrosinase and AChE, BChE, or NTE catalytic domain) that incorporated esterases into the phenolic sensor to enable detection of esterase inhibitors [35]. In their work, phenyl acetate gave the highest sensitivity for AChE and phenyl valerate gave the highest sensitivity for BChE.

The values of the initial rate of enzymatic hydrolysis, and the calculated sensitivity factors for each esterase were introduced into a system of linear equations represented by Eq. (4):

$$\left.\begin{aligned} R_1 &= x_1[\text{AChE}] + y_1[\text{BChE}] \\ R_2 &= x_2[\text{AChE}] + y_2[\text{BChE}] \end{aligned}\right\} \quad (4)$$

where x_1, x_2 are the sensitivity factors of AChE to choline and phenyl esters respectively; y_1, y_2 are the sensitivity factors of BChE to choline and phenyl esters, respectively; and R_1, R_2 are the initial rates of enzymatic hydrolysis of choline and phenyl esters, respectively. Solving this system of linear equations gives the concentrations of AChE and BChE in the mixture.

The deviation of the activity determination of ChEs was calculated as a difference between the specified and calculated concentrations for each ChE taken as the absolute value and expressed as a percentage of the specified concentration, ($[|C_{spec.} - C_{calc.}|/C_{spec.}] \times 100$). The data on deviations determined for 15 test AChE/BChE mixtures [12] using the 3 pairs of substrates are presented in Table 2. The maximum value of average deviation did not exceed 13%, which indicates good accuracy for the suggested method. The minimum deviation was achieved when using acetylcholine and phenyl valerate as substrates. The average deviations in determining ChEs using this pair of substrates were 5% for AChE, and 3% for BChE, respectively [13].

The apparent esterase activity in whole blood samples was investigated using the bielectrode biosensor system and two substrates: phenyl acetate and acetylcholine. The hydrolyzing activities of whole blood samples of human blood toward acetylcholine and phenyl acetate were simultaneously measured by choline and phenol sensors combined with a bielectrode electrochemical cell and bipotentiostat. The electrochemical cell was loaded with a buffer containing the mixture of substrates (the concentrations of the substrates were chosen previously as 1.5 mM for acetylcholine and of 0.5 mM for phenyl acetate) and the rate of spontaneous hydrolysis was measured. Then an aliquot of whole blood was added and the rate of the total hydrolysis of each substrate by blood esterases was measured. The results demonstrate that the apparent activities given by both substrates remain linear if the volume of the added blood does not exceed 10–20 μl per 1000 μl of the measuring media, which corresponds to 1:100 and 1:50 (v:v) blood dilutions. The observed linearity confirms the utility of the analytical measurement of blood esterase activities by this method. Further work will be required to determine species-specific sensitivity factors and to assess the impact on the analysis of varying levels of expression of blood esterases in response to genetic and environmental factors.

4. Conclusions

Biosensors based on LBL technology incorporating choline oxidase or tyrosinase can be used to detect AChE, BChE, CaE, or NTE activity in whole blood using either choline or phenolic substrates. The technology can be extended to bielectrode sensor systems that enable the simultaneous independent measurement of AChE and BChE activities in mixtures with concentrations as low as 0.3 nM with high accuracy. Such results demonstrate the feasibility of using bielectrode sensors for determining ChE activities in whole blood. A multisensor approach significantly expands the types of substrates that can be used for enzyme activity assays resulting in an increase in sensitivity of the analysis and potentially enlarging the number of enzymes assayed in the mixture (e.g., CaE, NTE, and PON1 in addition to AChE and BChE). This is a serious advantage in comparison to a recently described method for simultaneous determination of AChE and BChE in whole blood based on spectrophotometric assay of hydrolysis of acetyl-, butyryl- and propionyl-thiocholine substrates [36]. Whereas the biosensors described here are designed to detect activities of esterases in whole blood, enzymes of interest can also be incorporated into the electrode to create bienzyme biosensors for the detection of anti-esterase inhibitors, demonstrating the versatility of multienzyme biosensors [37]. Thus, the present work has provided a foundation for building multiplexed systems for the simultaneous determination of multiple esterases or the incorporation of multiple esterases into electrodes for the simultaneous analysis of multiple esterase inhibitors.

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