

Article

## Electrochemical Sensors for the Estimation of the Inhibitory Effect of Phenylcarbamates to Cholinesterase

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Academic Editor: Igor Medintz

Received: 9 October 2015 / Accepted: 2 December 2015 / Published: 10 December 2015

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**Abstract:** The inhibitory effect of nine phenylcarbamates with various substituents was studied. For this purpose, electrochemical sensors were applied under two different conditions: if an enzyme was present in the solution or if the enzyme was immobilized onto the electrode surface. In both cases, 3-[(ethoxycarbonyl)amino]phenyl (4-chlorophenyl)carbamate was found as the most effective inhibitor for butyrylcholinesterase. The best inhibitors for acetylcholinesterase were determined as well, depending on the used method. Thus, 3-[(butoxycarbonyl)-amino]phenyl phenylcarbamate with the enzyme present in the solution and 3-[(ethoxycarbonyl)amino]phenyl (3-methylphenyl)carbamate when the enzyme was immobilized onto the electrode surface were evaluated as the most effective inhibitors. Michaelis constants as well as maximum reaction rates were calculated and assessed.

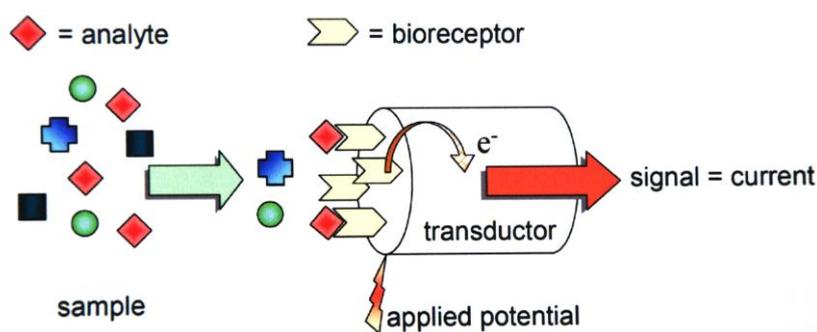
**Keywords:** biosensors; immobilization of cholinesterases; phenylcarbamates; inhibition; IC<sub>50</sub>; kinetic parameters

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## 1. Introduction

Recently, electrochemical sensors (biosensors) were introduced for the determination of various analytes in certain environments, agriculture, biological samples, industrial processes, *etc.* Within their preparation, the selection of proper immobilization techniques of an enzyme onto the surface of electrodes plays a significant role as it supports the development of more economic organization of operations in the food and bio-product industry [1,2]. The purpose of a biosensor is to provide rapid, real-time, accurate and reliable information about the analyte under the test [3].

The most typical part of the electrochemical biosensors is the presence of a suitable enzyme in the biorecognition layer—acetylcholinesterase (AChE) or butyrylcholinesterase (BChE)—providing an electroactive substance (thiocholine in our case) for detection by a physico-chemical transducer providing a measurable signal as a result of a bio-recognition event between the bio-receptor and its target analyte [4]. The reaction between the analyte and bioreceptor produces a new chemical, the release of heat, the flow of electrons, and changes in pH or mass. An example of the enzyme biosensor is given in Figure 1.



**Figure 1.** Scheme depicting the functional principle of the enzyme biosensor.

Nowadays, several techniques for the immobilization of an enzyme onto the surface of an electrochemical sensor are utilized. In 1962, the first biosensor was described by Clark and Lyons [5] in which glucose oxidase was entrapped between two membranes. Since that time, a lot of effort has been put into the development of reliable devices. In fact, the immobilization of enzymes is the most important step in the development of an enzyme biosensor [6]. The easiest and the least denaturing method is physical adsorption, when the enzyme is deposited onto the surface of the electrode and attached through weak bonds such as van der Waals and electrostatic interaction [7]. The most widely used procedure is that in which the enzyme is covalently linked to the surface of a transducer. In such cases, a stable covalent bond is formed between functional groups of the enzyme and the transducer [8].

Cholinesterases (ChEs), *i.e.*, both acetylcholinesterase and butyrylcholinesterase, differ in substrate specificity and susceptibility to various kinds of inhibitors [9]. These enzymes play an important role in neurotransmission upon the hydrolysis of acetylcholine (ACh) to choline (Ch) and acetic acid [10]. Both ChEs belong to a large protein family containing the  $\alpha/\beta$  hydrolase fold. Individual amino acid

residues involved in determining the molecular basis of the differences in substrate and inhibitor specificity of AChE and BChE have been identified in the acyl pocket, located at the bottom of a deep catalytic gorge; the peripheral anionic site, located at the lip of the gorge; the oxyanion hole; and the choline-binding site of the hydrophobic patch, also located within the gorge [11]. As described [9], the peripheral anionic site is a very important structural element, responsible for the binding of many inhibitors.

Alzheimer's disease (AD) is one of the most common forms of dementia in adults. It is an age-dependent neurodegenerative disorder that destroys human brain cells, causing problems with memory, thinking, and behavior. The physiopathology of AD is very complex; a promising therapeutic strategy is based on the cholinergic hypothesis. At present, four drugs are used for the symptomatic treatment of AD, and three of them are classified as inhibitors of ChEs. Inhibitors of acetylcholinesterase should increase the efficiency of cholinergic transmission by preventing the hydrolysis of released acetylcholine [12]. Nowadays, suitable AChE inhibitors are the drugs most frequently used for AD treatment. Likewise, substituted carbamates seem to be suitable for AChE inhibition. For example, Rivastigmine (marketed under the trade name Exelon), a parasymphathetic or cholinergic agent for treatment of moderate dementias of the Alzheimer's type or Parkinson's disease, belongs to carbamate compounds as well [13].

In this paper, nine phenylcarbamates with two different substituted carbamate groups are examined. Inhibition efficiency and kinetics parameters of tested carbamates were determined by using electrochemical sensors in two ways, either with an enzyme present in the solution or with an enzyme immobilized onto the electrode surface.

## 2. Experimental Section

### 2.1. Chemicals, Reagents, Stock and Standard Solutions

Acetylcholinesterase (E.C. 3.1.1.7) lyophilized from electric eel (eeAChE), acetylthiocholine iodide, butyrylcholinesterase (E.C. 3.1.1.8) lyophilized powder from equine serum (eqBChE), and DTNB (5,5'-dithiobis-2-nitrobenzoic acid, Ellman's reagent) were all from Sigma-Aldrich Prague (Czech Republic). Dioxan p.a. was purchased from Lach-Ner (Czech Republic). The 0.1 mol·L<sup>-1</sup> phosphate buffer saline (PBS) pH 7.4 was prepared from NaCl, KH<sub>2</sub>PO<sub>4</sub>, Na<sub>2</sub>HPO<sub>4</sub>·12 H<sub>2</sub>O and KCl (all purchased from Penta, Czech Republic).

To prepare each of the ChE solutions, about 10 mg of the corresponding enzyme was dissolved in 20 mL of PBS and kept at -6 °C in plastic tube in the freezer. The actual activity of the ChEs was verified every day using Ellman's method.

The substituted phenylcarbamates were synthesized and both their structure and purity were verified; corresponding compounds are listed in Table 1. All inhibitors were dissolved in dioxan p.a. to obtain 10 mmol·L<sup>-1</sup> solutions and then diluted with deionized water as needed.

**Table 1.** The overview of tested substituted phenylcarbamates.

Compound Number	R <sup>1</sup>	R <sup>2</sup>	Nomenclature
1.	-H	-CH <sub>3</sub>	3-[(methoxycarbonyl)amino]phenyl phenylcarbamate
2.	-H	-C <sub>2</sub> H <sub>5</sub>	3-[(ethoxycarbonyl)amino]phenyl phenylcarbamate
3.	-H	-C <sub>4</sub> H <sub>9</sub>	3-[(butoxycarbonyl)amino]phenyl phenylcarbamate
4.	-CH <sub>3</sub>	-C <sub>2</sub> H <sub>5</sub>	3-[(ethoxycarbonyl)amino]phenyl (3-methylphenyl)carbamate
5.	-CH <sub>3</sub>	-C <sub>4</sub> H <sub>9</sub>	3-[(butoxycarbonyl)amino]phenyl (3-methylphenyl)carbamate
6.	-OCH <sub>3</sub>	-C <sub>2</sub> H <sub>5</sub>	3-[(ethoxycarbonyl)amino]phenyl (4-methoxyphenyl)carbamate
7.	-OCH <sub>3</sub>	-C <sub>4</sub> H <sub>9</sub>	3-[(butoxycarbonyl)amino]phenyl (4-methoxyphenyl)carbamate
8.	-Cl	-C <sub>2</sub> H <sub>5</sub>	3-[(ethoxycarbonyl)amino]phenyl (4-chlorophenyl)carbamate
9.	-Cl	-C <sub>4</sub> H <sub>9</sub>	3-[(butoxycarbonyl)amino]phenyl (4-chlorophenyl)carbamate

### 2.2. Determination of Cholinesterase Activity

For determination of actual activity of the enzyme, a modified spectrophotometric Ellman's method (ELM), known as a simple, rapid and direct approach to determine the SH and -S-S- groups in proteins, was applied [14]. Cholinesterase activity was measured indirectly by quantifying 5-thio-2-nitrobenzoic acid (TNB) formed within a reaction between the thiol reagent 5,5'-dithiobis-2-nitrobenzoic acid and thiocholine (TCh), which is a product of the substrate (*i.e.*, acetylthiocholine, ATCh) hydrolysis by cholinesterase [15].

Cholinesterase activity was determined using ELM at 25 °C in presence of 0.1 mol·L<sup>-1</sup> phosphate buffered saline (PBS, pH 7.4) in glass cuvette with 1 cm optical path. Into the glass cuvette, the following components were added: 1.184 mL PBS (0.1 mol·L<sup>-1</sup>, pH 7.4), 0.8 mL DTNB (0.5 mmol·L<sup>-1</sup>), 0.008 mL ATCh (0.1 mol·L<sup>-1</sup>). Reaction was initiated by adding 0.008 mL of enzyme (*i.e.*, eeAChE or eqBChE). Product of given enzymatic reaction, TNB, was detected by spectrophotometer with diode array Hewlett-Packard 8453 at the wavelength of 412 nm against comparative solution containing 1.192 mL PBS (0.1 mol·L<sup>-1</sup>, pH 7.4), 0.8 mL DTNB (0.5 mmol·L<sup>-1</sup>), 0.008 mL ATCh (0.1 mol·L<sup>-1</sup>). The dependence of absorbance (at 412 nm) vs. time was observed for 70 s and actual activity of enzyme was calculated. Measurement of activity was carried out in triplicate at least. The average value of daily determined catalytic activity of given enzyme was used to calculate its suitable volume, which had to be added into the initial reaction mixture for determination of inhibitory effect in order to achieve the chosen initial activity in the reaction mixture (*i.e.*, 0.2 U·mL<sup>-1</sup> or 1 U·mL<sup>-1</sup>).

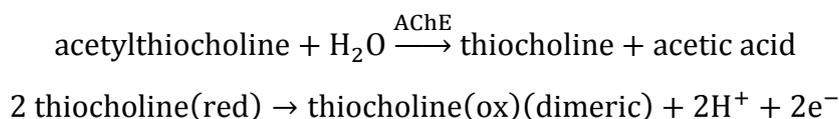
### 2.3. Preparation of Biosensors

The procedure of immobilization was realized using a three-electrode compartment (Pt-AgPd-Pt) from BVT Technologies. As an immobilization technique, the physical adsorption was applied, based

on overspread of proper volume of enzyme at the working electrode surface from the reaction mixture containing activity of enzyme equal to  $1 \text{ U}\cdot\text{mL}^{-1}$ . Each of the sensors was dried, placed into the Petri dish and kept in the fridge.

#### 2.4. Determination of Cholinesterase Inhibition in the Presence of Substituted Phenylcarbamates by Using Electrochemical Sensors and Biosensors

The inhibition efficiency of the substituted carbamates under the test was evaluated by measurement of the value of  $\text{IC}_{50}$  (50% inhibitory concentration, *i.e.*, the concentration of inhibitor necessary for reduction of enzyme activity or reaction rate to 50%). For determination of  $\text{IC}_{50}$ , electrochemical screen-printed three-electrode sensors (Pt-AgPd-Pt) from *BVT Technologies* were used, applying square-wave voltammetry as a detection method. Using acetylthiocholine as a substrate for ChEs, thiocholine is produced during enzymatic reaction. Thiol-containing compounds are oxidized at the electrode surface, then the anodic oxidation current of thiocholine is measured [16]. Hydrolysis of ATCh and oxidation of originated TCh may be described as follows:



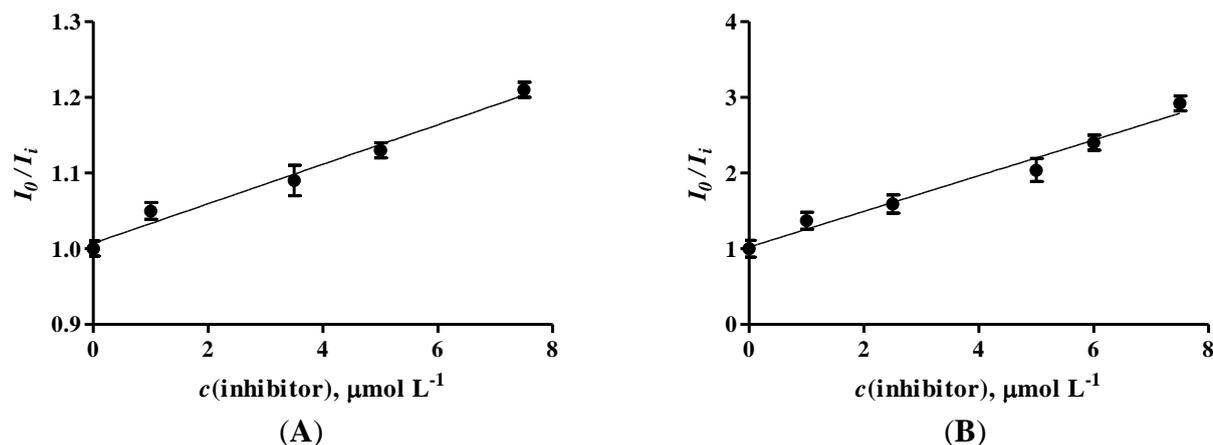
When the enzyme in the solution was present, a reaction mixture in absence of inhibitor was prepared by mixing of PBS ( $0.1 \text{ mol}\cdot\text{L}^{-1}$ , pH 7.4), enzyme eeAChE or eqBChE (activity in final reaction mixture was  $0.2 \text{ U}\cdot\text{mL}^{-1}$ ), and ATCh (concentration in final reaction mixture was  $1 \text{ mmol}\cdot\text{L}^{-1}$ ). The reaction mixture in presence of inhibitor contained PBS ( $0.1 \text{ mol}\cdot\text{L}^{-1}$ , pH 7.4), enzyme (its activity in final reaction mixture was  $0.2 \text{ U}\cdot\text{mL}^{-1}$ ), inhibitor (concentration in final reaction mixture varied in the range of  $1\text{--}7.5 \text{ }\mu\text{mol}\cdot\text{L}^{-1}$ ), and ATCh (concentration in final reaction mixture was  $1 \text{ mmol}\cdot\text{L}^{-1}$ ).

In case the enzyme was immobilized (its final activity was  $1 \text{ U}\cdot\text{mL}^{-1}$ ), the reaction mixture in absence of inhibitor was prepared by mixing of PBS ( $0.1 \text{ mol}\cdot\text{L}^{-1}$ , pH 7.4) and ATCh (concentration in final reaction mixture was  $1 \text{ mmol}\cdot\text{L}^{-1}$ ). The mixture in presence of inhibitor contained PBS ( $0.1 \text{ mol}\cdot\text{L}^{-1}$ , pH 7.4), inhibitor (concentration in final reaction mixture varied in the range of  $1\text{--}7.5 \text{ }\mu\text{mol}\cdot\text{L}^{-1}$ ), and ATCh (concentration in final reaction mixture was  $1 \text{ mmol}\cdot\text{L}^{-1}$ ).

For both methods, the total volume of reaction mixture was 0.55 mL.

After 5 min of incubation, the dependence of current *vs.* potential was registered. For each reaction, uninhibited and inhibited, current corresponded to the reaction rate. The peak of oxidized thiocholine was observed at about 700 mV. Each measurement was repeated three times.

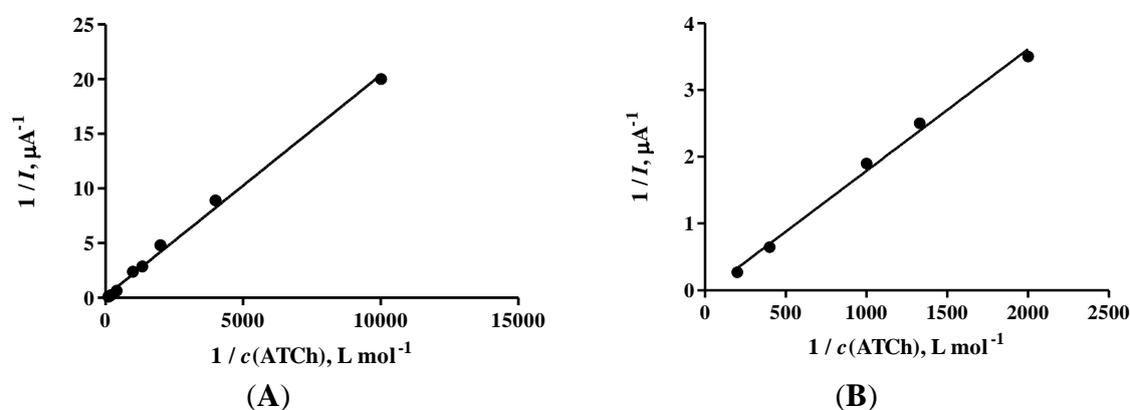
A graphical dependence  $I_0/I_i$  (*y*) *vs.* inhibitor concentration (*x*) was constructed ( $I_0$ : current height of thiocholine peak for uninhibited reaction,  $I_i$ : current height of thiocholine peak for inhibited reaction). The regression curve  $y = kx + q$  was calculated and used for evaluation of corresponding  $\text{IC}_{50}$  values (*x* in regression equation for  $y = 2$  coming out from the  $\text{IC}_{50}$  definition). Each measurement was repeated twice. An example of the  $\text{IC}_{50}$  determination is shown in Figure 2A when the enzyme was present in the solution and in Figure 2B when the enzyme was immobilized onto surface of electrochemical sensor.



**Figure 2.** The dependence  $I_0/I_i$  ratio on concentration of the 3-[(methoxycarbonyl)amino]-phenyl phenylcarbamate when the enzyme was present in the solution (A), or that of 3-[(ethoxycarbonyl)amino]phenyl (3-methylphenyl)carbamate when the enzyme was immobilized onto the surface of the sensor by adsorption (B). ATCh concentration,  $1 \text{ mmol}\cdot\text{L}^{-1}$ ; eeAChE activity,  $0.2 \text{ U}\cdot\text{mL}^{-1}$  (A), or  $1 \text{ U}\cdot\text{mL}^{-1}$  (B); inhibitor concentration,  $1\text{--}7.5 \mu\text{mol}\cdot\text{L}^{-1}$ . Measured in  $0.1 \text{ mol}\cdot\text{L}^{-1}$  phosphate buffer, pH 7.4.

### 2.5. Determination of Michaelis Constant ( $K_M$ ) and Maximum Rate ( $V_{max}$ ) for Enzyme in Solution and Enzyme Immobilized on Sensor

The total volume of the reaction mixture was 0.55 mL. Reaction mixture was prepared by mixing of PBS ( $0.1 \text{ mol}\cdot\text{L}^{-1}$ , pH 7.4), enzyme (activity in final reaction mixture was  $0.2 \text{ U}\cdot\text{mL}^{-1}$  for enzyme in the solution and  $1 \text{ U}\cdot\text{mL}^{-1}$  for immobilized enzyme), and ATCh (concentration in final reaction mixture was in range of  $0.1\text{--}10 \text{ mmol}\cdot\text{L}^{-1}$ ). After 5 min of incubation, the dependence of current vs. potential was registered. Kinetic parameters were determined by plotting the dependence of the reciprocal current response on reciprocal ATCh concentration (see Figure 3).



**Figure 3.** The dependence of the  $1/I$  reciprocal current response on reciprocal ATCh concentration for determination of Michaelis constant and maximum rate when the enzyme was present in the solution (A), or when the enzyme was immobilized onto the surface of the sensor by adsorption (B). Measured at ATCh concentration,  $0.1\text{--}100 \text{ mmol}\cdot\text{L}^{-1}$ ; eeAChE activity,  $0.2 \text{ U}\cdot\text{mL}^{-1}$  (A), or  $1 \text{ U}\cdot\text{mL}^{-1}$  (B); and in  $0.1 \text{ mol}\cdot\text{L}^{-1}$  phosphate buffer, pH 7.4.

### 3. Results and Discussion

As described, electrochemical sensors (screen-printed electrodes) of the two types of measuring procedures were prepared and found useful for the determination of inhibition efficiency and kinetic parameters of tested phenylcarbamates using both procedures (either with the enzyme present in the solution or immobilized onto the electrode surface). As tested, the screen-printed sensors were found advantageously applicable for measurements in reaction mixtures of low total volume. Additionally, there is a possibility to use one sensor for a number of inhibitors without adsorption onto the surface of the electrochemical biosensor. A short measurement time was appreciated as well.

The results obtained by both the methods used for the determination of the inhibiting efficiency of the tested phenylcarbamates are summarized in Table 2, expressed as arithmetic means of the 50% inhibitory concentration ( $IC_{50} \pm$  standard deviations).

**Table 2.** The determined values of  $IC_{50}$  ( $\pm$  standard deviation) of tested phenylcarbamates.

Compound Number	Enzyme in Solution		Enzyme Immobilized	
	$IC_{50}$ ( $\mu\text{mol}\cdot\text{L}^{-1}$ )		$IC_{50}$ ( $\mu\text{mol}\cdot\text{L}^{-1}$ )	
	eeAChE	eqBChE	eeAChE	eqBChE
1.	42.1 $\pm$ 6.2	43.0 $\pm$ 8.3	37.3 $\pm$ 2.9	79.8 $\pm$ 8.8
2.	18.0 $\pm$ 3.7	43.7 $\pm$ 1.5	29.4 $\pm$ 3.2	46.8 $\pm$ 6.2
3.	10.7 $\pm$ 0.2	45.8 $\pm$ 8.9	12.4 $\pm$ 4.4	55.8 $\pm$ 1.0
4.	60.9 $\pm$ 4.0	67.7 $\pm$ 8.8	4.1 $\pm$ 0.6	73.9 $\pm$ 4.1
5.	20.0 $\pm$ 1.9	97.8 $\pm$ 1.0	54.8 $\pm$ 0.1	39.8 $\pm$ 0.3
6.	66.6 $\pm$ 7.1	82.3 $\pm$ 6.3	51.0 $\pm$ 8.3	99.1 $\pm$ 8.0
7.	34.8 $\pm$ 1.1	42.1 $\pm$ 1.1	19.5 $\pm$ 2.1	11.4 $\pm$ 0.3
8.	71.8 $\pm$ 3.8	31.9 $\pm$ 2.9	53.3 $\pm$ 1.5	10.6 $\pm$ 0.4
9.	168.0 $\pm$ 4.0	64.5 $\pm$ 7.2	25.2 $\pm$ 7.8	30.7 $\pm$ 2.0

Thus, it can be concluded that all the substituted carbamates in the test can be classified as quite potent inhibitors of both acetylcholinesterase and butyrylcholinesterase. Generally, as observed by both the methods, it can also be concluded that the inhibition effect of mostly all compounds in the test on acetylcholinesterase is significantly higher than that on butyrylcholinesterase.

According to our opinion, some differences of inhibition concentration may be caused by the fact that enzymes originate from different sources. In both of the enzymes, there are some differences in their active sites. Concerning acetylcholinesterase, the acyl pocket consists of two phenylalanine molecules (Phe288, Phe290). In the case of butyrylcholinesterase, these amino acids are replaced by valine (Val288) and leucine (Leu286) molecules. Also, acetylthiocholine as a substrate is more specific for acetylcholinesterase. Differences in the structure of the active site can explain the majority of the activity differences of the two enzymes [9].

Based on the results summarized in Table 2, it is possible to conclude that the lowest values of  $IC_{50}$  for butyrylcholinesterase were estimated for inhibitor #8 (3-[(ethoxykarbonyl)amino]phenyl (4-chlorophenyl)carbamate) by both of the methods used. On the other hand, compound #3 (3-[(butoxykarbonyl)amino]phenyl phenylcarbamate) appeared to be the most effective inhibitor of acetylcholinesterase in the solution, whereas compound #4 (3-[(ethoxykarbonyl)amino]phenyl-

(3-methylphenyl)carbamate) was the best if acetylcholinesterase was immobilized onto the surface of the electrode. However, compound #3 mentioned above exhibited a low  $IC_{50}$  value and, thus, it can also be considered a proper inhibitor for acetylcholinesterase.

Determinations of Michaelis constant and the maximum rate for both the soluble and immobilized enzyme were realized. The results obtained by both the methods are summarized in Table 3.

**Table 3.** The values of Michaelis constant and the maximum rate for the enzyme in the solution and the enzyme immobilized by physical adsorption.

		$K_M$ (mmol·L <sup>-1</sup> )	$V_{max}$ (μA)
Enzyme in solution	eeAChE	38.6 ± 3.7	19.7 ± 1.4
	eqBChE	82.2 ± 15.0	42.9 ± 5.6
Enzyme immobilized	eeAChE	44.0 ± 0.4	25.8 ± 0.2
	eqBChE	61.0 ± 0.9	30.50 ± 0.5

#### 4. Conclusions

It is possible to conclude that physical adsorption affects both the kinetics parameters, *i.e.*, Michaelis constants and maximum rates. The immobilization of eeAChE resulted in an increase of both kinetic parameters, whereas the immobilization of eqBChE caused their decrease.

In case the  $K_M$  after immobilization is increased, a significant substrate diffusion barrier appears between the sample and the reaction layer [7]. Nevertheless, it should be mentioned that kinetic parameters for the immobilized enzyme may differ from those of the soluble enzyme because of, among other reasons, diffusional restrictions and interactions with the carrier or deactivation due to immobilization [17].

Having tested nine new inhibitors of cholinesterases, it is possible to conclude that screen-printed sensors are suitable for studies of the inhibition effect of all phenylcarbamates tested by both methods used within this study.

#### Acknowledgments

Authors wish to acknowledge for financial support of Faculty of Chemical Technology, University of Pardubice. Further, the work was supported by Grand Agency of the Czech Republic under project No. 15-16549S.

#### Author Contributions

K.Vo. prepared and tested biosensors and performed all electrochemical measurements; Š.Š. suggested the experiments and finalized the manuscript; M.S. synthesized and characterized the compounds; K.Vy. supervised the work and wrote the final text as well.

#### Conflicts of Interest

The authors declare no conflict of interest.

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