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ELECTROPHORETICALLY MEDIATED MICROANALYSIS TECHNIQUE AS A TOOL FOR THE RAPID SCREENING OF NOVEL ACETYLCHOLINESTERASE INHIBITORS

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Abstract: An electrophoretically mediated microanalysis technique was developed for the rapid screening of acetylcholinesterase inhibitors. Activity of four original compounds, *N*-benzylpiperidine, carbamoyloxyphenyl derivatives and reference inhibitor – tacrine was determined and compared with the data obtained from Ellman's assay. Percentage of enzyme inhibition detected at inhibitor concentration of $3.33 \cdot 10^{-4}$ M was between 0 and 78% and was the highest for tacrine. The comparison of data obtained from the EMMA assay and Ellman's test proved inhibitory activity of novel compounds.

Keywords: capillary electrophoresis, electrophoretically mediated microanalysis (EMMA), acetylcholinesterase inhibitors, *N*-benzylpiperidine derivatives, carbamoyloxyphenyl derivatives

Drug discovery requires fast and simple screening methods for testing biological activity of new agents towards specific targets i.e. receptors, ion channels or enzymes. The latter are among others crucial for the research of the anti-Alzheimer's drugs. Thanks to the cholinergic hypothesis explaining the cognitive deficits occurring during the Alzheimer's disease (AD), inhibitors of acetylcholinesterase (AChE) and lately also butyrylcholinesterase (BuChE) were introduced as an effective method of the symptomatic treatment of mild and moderate AD (1). Donepezil, rivastigmine and galantamine improve learning and memory by increasing cholinergic neurotransmission in brain but there is also a growing evidence showing that due to their cholinesterases inhibiting activity they can affect different mechanisms leading to the development of the disease, such as β-amyloid aggregation, regional cerebral blood flow, tau phosphorylation and oxidative stress (2, 3). Therefore, the search for new cholinesterase inhibitors is well-grounded and undertaken by many laboratories (4).

AChE and BuChE inhibitory activity can be measured according to the slightly modified spectrophotometric Ellman's test (5). This method is based on the reaction between the product of enzymatic hydrolysis, the thiocholine, and specific chromogenic reagent, 5,5'-dithiobis(2-nitrobenzoic acid) (DNTB). Depending on how active the enzyme is, a yellow product of varied color intensity is obtained and its absorbance is measured.

An urgent need for replacing the classical colorimetric Ellman's test with more effective method for biological screening was noticed during designing new inhibitors of AChE and BuChE, synthesis and the evaluation of their biological activity.

A chemiluminescence method and its modifications were used for acetylcholinesterase activity determination by Israël and Lesbats (6, 7) and Birman (8). The acetylcholinesterase activity is determined on the basis of an increase in light emission, produced by the accumulation of the product of hydrolysis – choline. Bartolini et al. used an immobilized enzyme reactor (IMER) for description of reversible and pseudo-irreversible inhibitors (9). Kinetic parameters and mechanism of action was evaluated by the application of enzyme reactor containing acetylcholinesterase immobilized on a Convective Interaction Media monolithic disk (AChE-CIM-IMER). IMER was constructed by covalent binding of enzyme with an ethylenediamine (EDA) monolithic disk. Then, it was inserted

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into a HPLC system. Enzyme activity was measured by determining the product peak area. The method is useful for screening and for a description of inhibitor.

Marston applied TLC plates to make a simple and rapid AChE and BuChE assay for extract from natural products (10). When enzyme was active, the naphthyl acetate used in this method as indicator was hydrolyzed to naphthol and the purple-colored diazonium dye was formed with Fast Blue B salt. All cholinesterases (ChE) inhibitors gave white spots on the dye-colored background of the TLC plates.

Some researchers used capillary electrophoresis for enzymes activity determinations. CE has a number of advantages over other methods, including high-efficiency separation, short analysis time, small amount of the required sample and therefore, cost-effectiveness. A CE-based technique named electrophoretically mediated microanalysis (EMMA) is a tool used for enzyme assays such as analysis of lactate dehydrogenase (LDH) in human erythrocytes (11) or determination of the protein tyrosine phosphatase (PTP) inhibitors in natural extracts (12). Tang et al. applied CE for acetylcholinesterase activity determination, using EMMA and immobilized capillary enzyme reactor techniques (13, 14).

Recently, we have designed and synthesized several series of new compounds as potential cholinesterases inhibitors (15, 16). Therefore, a method suitable for their fast biological screening was searched and the ability to adopt the capillary electrophoresis (CE) for this purpose was tested. In this paper, a method for the rapid screening of novel acetylcholinesterase inhibitors using EMMA assay is presented.

EXPERIMENTAL

Instruments

A Beckman CE system (P/ACE MDQ) equipped with diode-array detector (DAD) was used for all separations. It was controlled by 32 Karat Software version 8.0. An uncoated fused-silica capillary with total length of 30 cm (20.2 cm to detection window) and internal diameter 50 μm (external diameter 375 μm) was purchased from Beckman. The capillary and samples were termostatted at 37°C. Detection was performed at UV 230 nm.

Reagents and chemicals

Acetylcholinesterase (AChE) from *Electrophorus electricus* (425.96 U/mg solid), acetylthiocholine chloride (ATCh) and magnesium sulfate were purchased from Sigma-Aldrich, sodium tetraborate from Fluka and hydrochloric acid from POCh. A solution of AChE (0.4 mg/mL) was prepared with 10 mM borate buffer pH 8.0 with magnesium sulfate addition (20 mM) (17). Ten mM borate buffer was prepared by dissolving 201.2 mg of sodium tetraborate in 100 mL deionized water and adjusted to pH 8.0 with hydrochloric acid, and then 240.7 mg of magnesium sulfate was dissolved in this solution. A solution of ATCh (10 mM) was prepared with 40 mM borate buffer pH 8.0 and solutions of inhibitors (1 mM) with deionized water. Forty mM borate buffer was prepared by dissolving 804.9 mg of sodium tetraborate in 100 mL of deionized water and adjusted to pH 8.0 with hydrochloric acid. All solutions were filtered through 0.45 μm Millex filters. Background electrolyte (BGE) was 40 mM borate buffer pH 8.0.

Procedures

Capillary

A new capillary was pretreated with 0.1 M NaOH solution for 30 min, followed by water for 3 min and running buffer for 3 min. After this procedure, assay was running.

Assay

Procedure of assay was composed of several steps:

1. Reversed rinsing capillary with borate buffer (50 psi, 3 min)
2. Injection of enzyme (0.3 psi, 4 s)
3. Dipping ends of capillary and electrodes in buffer – cleaning the ends
4. Injection of water (in blank) or inhibitor (0.3 psi, 4 s)
5. Dipping ends of capillary and electrodes in buffer – cleaning the ends
6. Injection of substrate (0.3 psi, 4 s)
7. Mixing of all reagents by applying the voltage of 1 kV for 15 s
8. Separation of product – thiocholine (TCh) from unreacted substrate – acetylthiocholine (ATCh) – 12 kV, 4 min.

For every rinsing and separation 40 mM borate buffer was used. After every injection, the ends of capillary were cleaned by dipping in buffer because it was very important to avoid contamination of capillary and solutions. There were three kinds of assay: first, for blank sample where the maximum activity of enzyme was determined, second, for reference inhibitor – tacrine which was used for simple method validation and third, for four selected compounds. Procedure for each sample (blank or with inhibitor) was repeated three times.

Acetylcholinesterase is an enzyme which normally hydrolyzes acetylcholine but for the assay acetylthiocholine was used because it and product of its hydrolysis can be directly determined due to UV absorption with maximum at 230 nm. Solutions of substrate and enzyme were introduced into the capillary and then were mixed by application of voltage. The enzyme reaction took place and the reaction product and the unreacted substrates were electrophoretically separated and detected. The inhibition percentage was determined for final concentration of inhibitor in capillary equaled $3.33 \cdot 10^{-4}$ M.

RESULTS AND DISCUSSION

Activity of compounds **1** – **4** and a reference inhibitor – tacrine was determined by electrophoretically mediated microanalysis method and compared with the data obtained in the classical Ellman's test. For these studies we used four novel compounds, which represent different chemical structures (Fig. 1) (15, 16). Compounds **1** and **2** have been designed as a dual binding site inhibitors with *N*-benzylpiperidine moiety, responsible for the interactions with the catalytic binding site connected by alkyl chain with *N*-phtalimido fragment or *N*-indolyl- moiety, which may be able to bind in the peripheral active site (PAS) of AChE and therefore to prevent the AChE-induced Ab-aggregation. Compounds **3** and **4** represent carbamate derivatives with the carbamoyloxyphenyl fragment responsible for the interactions with the catalytic binding site of the enzyme.

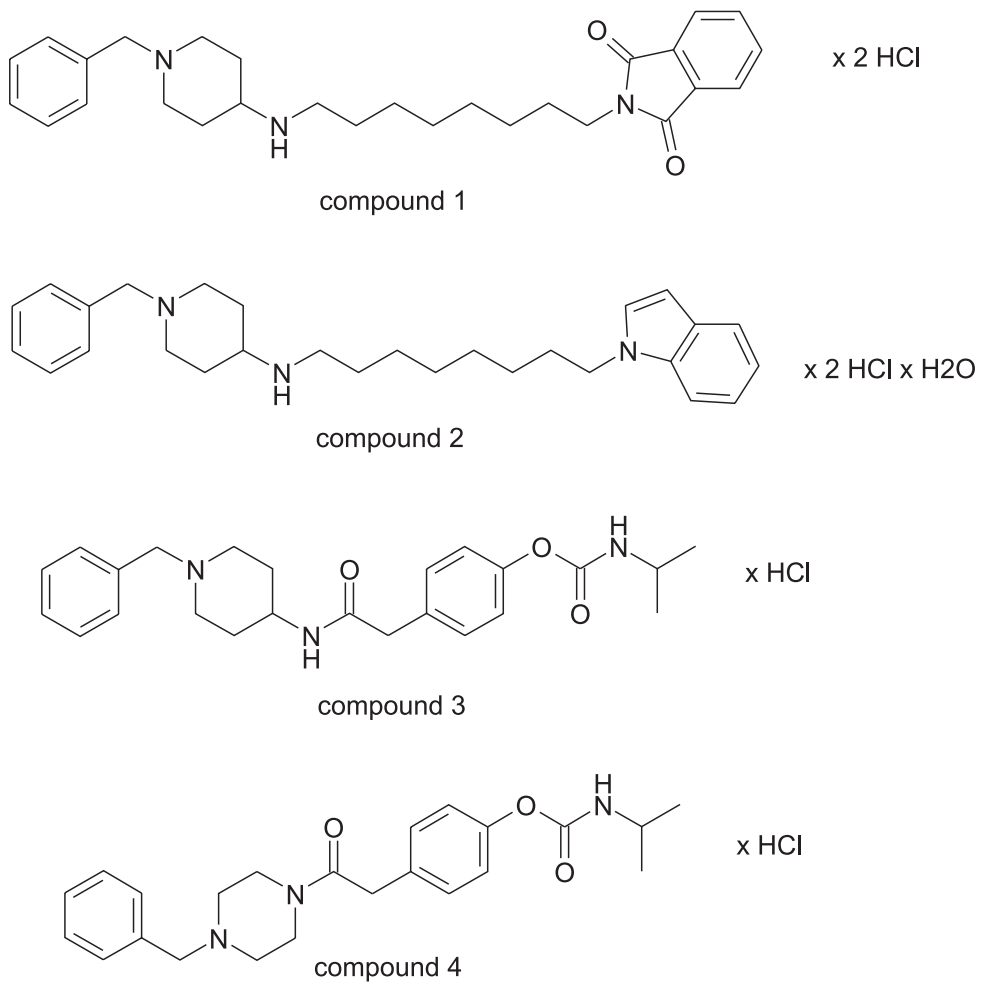

Figure 1. Chemical structure of the investigated potential acetylcholinesterase inhibitors

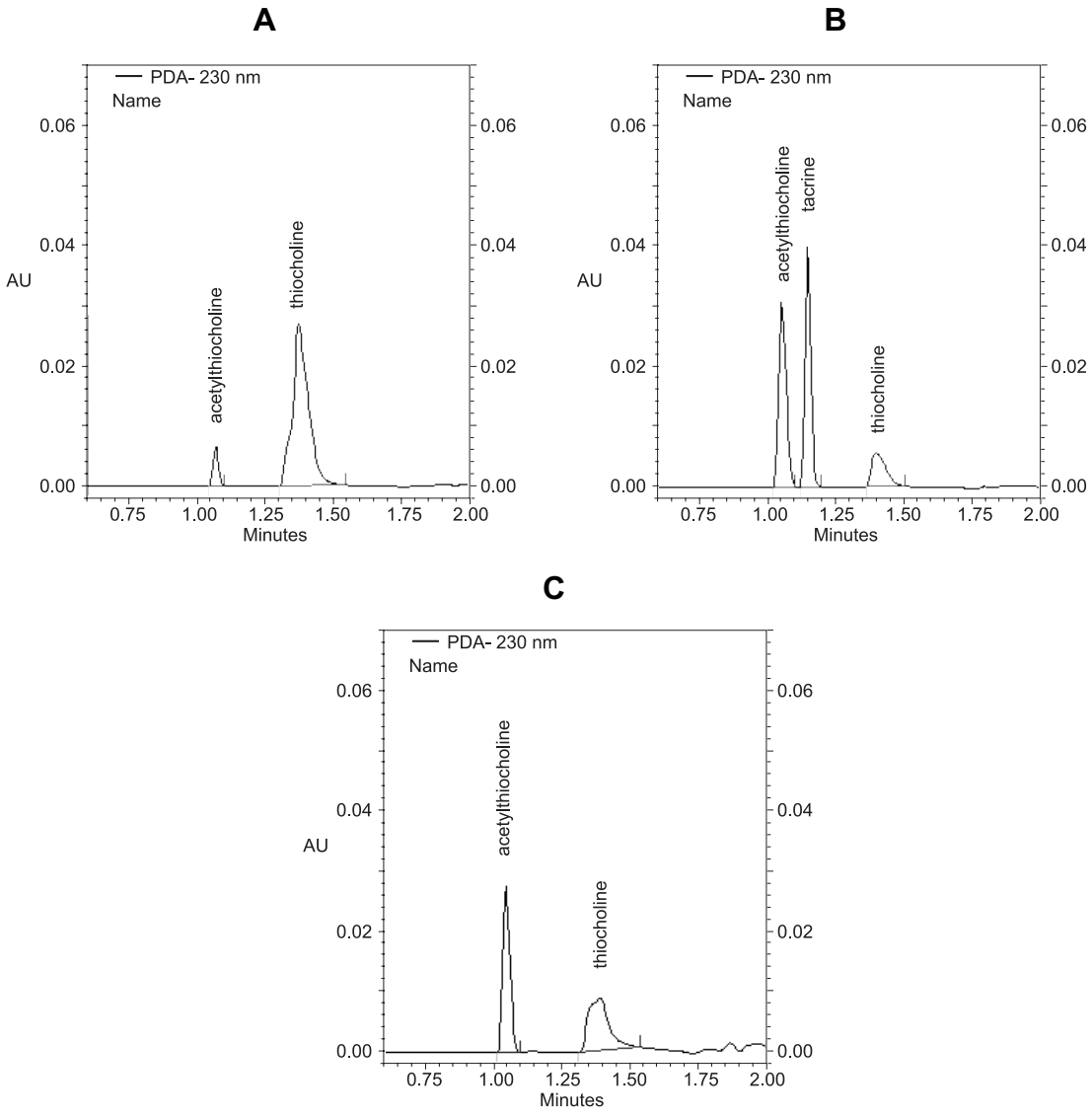

Figure 2. Electropherograms obtained in case of blank sample (**A**), tacrine (**B**) and inhibitor **2** (**C**)

In EMMA, solutions of substrate and enzyme were introduced in the capillary as distinct plugs. Upon the application of voltage, these two zones overlapped each other due to their different electrophoretic mobilities, and the enzyme reaction took place. The resultant reaction product and the unreacted substrates were electrophoretically separated and detected. The area under the peak of product (thiocholine) was measured because it shows enzyme activity. An ability of enzyme inhibition for each compound was calculated from the equation:

$$\text{Inhibition}\% = 100 - \left(\frac{x}{blank} \times 100\right) \qquad (1)$$

where x is the mean of area under thiocholine (TCh) peak from three independent determinations for inhibitor and *blank* is the mean of area under TCh peak from three independent determinations without inhibitor (Table).

Examples of the electropherograms obtained are presented in Figure 2.

Influence of pH and concentration of buffer

It has been reported that pH 8.0 is an optimum for AChE activity and so all determinations were performed in buffer with this pH value. Increased or decreased pH lowered the enzyme activity (5). Tang described usage of borate – phosphate buffer (13, 14). We modified the buffer by replacement of phosphoric acid by hydrochloric acid. Concentration of the buffer was determined experimentally. Forty and

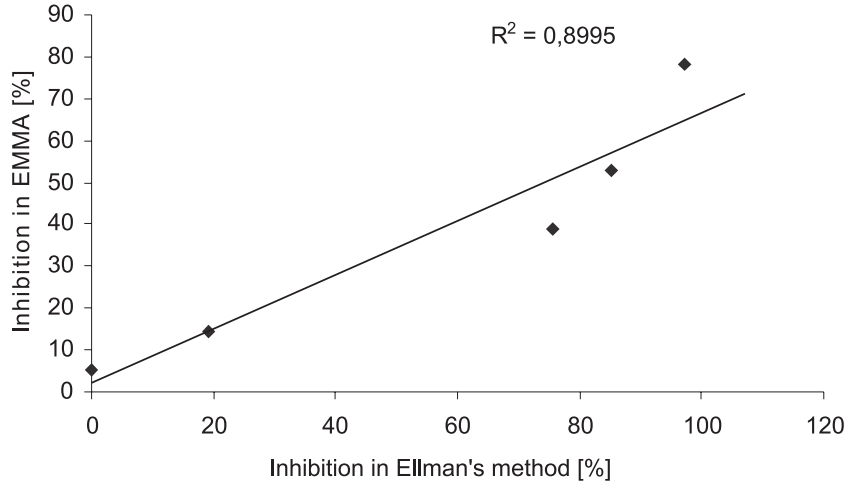

Figure 3. Dependence between inhibition percentage in both methods

Table. Values of inhibition percentage for tested compounds (The mean of migration time for thiocholine was 1.389 min, S.D. = 0.023, n = 24)

Sample	Area under TCh peak (mean, n = 3)	S.D.	Inhibition % (CE)	Inhibition % (Ellman's test)
Blank	111542.3	3637.4	-	-
Tacrine	24592	2561.9	78.0	97.2
1	68223	2716.7	38.8	75.7
2	52413	4301.2	53.0	85.1
3	95329.8	3718.7	14.5	19.2
4	105709.2	4270.2	5.2	0

100 mM borate buffer was tested in our experiments and it was found that 40 mM buffer had been proper for assay as it guaranteed good separations and stability of the enzyme.

Effect of applied voltage

Two different voltage values were applied: 12 and 20 kV. 12 kV was chosen because it guaranteed good separations during 4 min. The current intensity reached 88 μA.

UV detection

A usage of diode-array detector enabled to obtain wide spectrum but due to maximum absorption, 230 nm was chosen as an analytical wavelength.

Incubation time and mixing of reagents

At the beginning of study, the reagents were injected into the capillary hydrodynamically, using pressure. Then the mixture was incubated for 2 min and separated. However, no satisfactory results were obtained because the reagents weren't mixed well. An extension of incubation time didn't give expected improvement of the assay, therefore an electrokinetic mixing was applied. It turned out to be effective even without incubation.

Comparison of EMMA and Ellman's method

Tacrine was used as reference inhibitor. Its inhibitory activity was determined by EMMA and Ellman's method at the same concentration 3.33×10^{-4} M. A percentage of inhibited enzyme was higher in case of classic, spectrophotometric Ellman's method. It might be caused by different proportion of ingredients in the reaction mixture in both methods. An application of various techniques may also lead to observed differences.

To simply validate EMMA method, a screening of 4 new compounds with known inhibitory

activity, and reference inhibitor – tacrine was performed. Activity of each compound was expressed as a percentage of enzyme inhibition. These values were lower than the values obtained from Ellman' test. Correlation between both methods is shown in Figure 3.

All the tested compounds can be put in the activity order: **2** > **1** > **3** > **4** , where **2** is the most active compound and **4** is inactive. Lower values of inhibition percentage in EMMA method was probably caused by different techniques and proportion of reagents in the mixture. Further modifications of this assay are required to obtain the same values in both methods.

Zhang used the quality parameter Z', which described an accuracy of screen system for enzyme inhibitor (18). Z' can be calculated from the equation:

$$Z' = 1 - \frac{3SD_{0\%} + 3SD_{100\%}}{|\bar{A}_{0\%} - \bar{A}_{100\%}|} \qquad (2)$$

where $SD_{0\%}$ is a standard deviation for area under TCh peak without inhibitor (Inhibition % = 0%); $SD_{100\%}$ is a standard deviation for area under TCh peak with reference inhibitor (Inhibition % = 100%) and $\bar{A}_{0\%}$ and $\bar{A}_{100\%}$ are the proper means of area under TCh peak.

Tacrine was a strong inhibitor, its inhibition percentage was 78% so the values obtained for it could be used as $\bar{A}_{100\%}$ and $SD_{100\%}$. The quality parameter Z' had value of 0.79 and was higher than 0.5 what meant that a large difference between blank sample and sample with reference inhibitor had been indicated, implying a good accuracy of EMMA method.

SUMMARY

This work presents the results of preliminary studies on electrophoretically mediated microanalysis method, which will have an application for testing activity of potential acetylcholinesterase inhibitors. The comparison of data obtained from a classical spectophotometric Ellman's test with our EMMA assay gave a satisfactory result. The most active compound in Ellman's assay was also the most active in EMMA, and proper order of inhibitory activity of tested compounds has been retained. However, the percentage of enzyme inhibition was lower than the values determined by Ellman's assay. Thus, this assay requires further development by testing larger library of cholinesterase inhibitors with different chemical structure, as well as new potential inhibitors. In summary, at this stage of studies, presented EMMA method can be used for the rapid screening of compounds for inhibitory activity; it is also easy and not expensive.

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