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The determination of the cholinesterase activity using 3,3′,5,5′-tetramethylbenzidine as an indicator

Aim. To develop a new method, which has a good reproducibility of the experimental results, is fast, cheap and provides safe working conditions during the analysis, in order to determine the activity of cholinesterase.

Experimental part. The light absorption of the test and control samples was measured using a CPhC-3-01 photoelectric photometer (420 nm, l = 3 cm). The reaction rate was characterized by the value of the optical density of the solution in 10 min (the *fixed time method*). Measurements were performed at +37 °C, the temperature of the reaction mixture was maintained by thermostating in water, the pH of the solutions was monitored potentiometrically using a glass electrode. The determination was repeated five times with each solution of a certain concentration of the enzyme. According to the average values obtained, the calibration graph of the specific activity of the enzyme (in international units – activity unit (AU/mg) – kmol/min to 1 mg of the substance) on the optical density of the solution was constructed. Using the mean value of five measurements of the optical density of the test solution the specific activity of the enzyme (U) was found by the calibration graph.

Results and discussion. The essence of the method is the photometrical measurement of the rate of the enzymatic hydrolysis of acetylcholine in a buffer medium using 3,3′,5,5′-tetramethylbenzidine (TMB). The enzymatic hydrolysis reaction of the substrate was performed at pH 8.3, and in 10 min after the start the rate of enzymatic hydrolysis of acetylcholine was measured. The linear dependence of the optical density on the specific activity of the enzyme (U) was observed in the range of 3.5–28 AU/mg (activity unit/mg). The activity of the enzyme, according to the average results of 5 measurements, was 27.9 AU/mg. The declared activity the enzyme in accordance with the quality certificate was 28 AU/mg. The limit of quantification was 0.2 AU/mg. Metrological characteristics of the method were as follows: RSD = 1.8 % (n = 5; P = 0.95), accuracy – 0.45 %. These values indicate that the method proposed for determining the activity of cholinesterase is characterized by high sensitivity, reliability and reproducibility of the results. At the same time, it was proven that there was no systematic error in determining the activity of cholinesterase by the method developed.

Conclusions. As a result of the research conducted a new method for determining the activity of the cholinesterase enzyme has been developed; it is characterized by high sensitivity, reliability and reproducibility of the results, and also provides safe working conditions during the analysis.

Key words: acetylcholinesterase; acetylcholine; photometric detection; 3,3′,5,5′-tetramethylbenzidine

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Визначення активності холінестерази з використанням 3,3′,5,5′-тетраметилбензидину як індикаторної сполуки

Мета. Розробити новий метод визначення активності холінестерази, який має хорошу відтворюваність результатів експерименту, є швидкий, дешевий і забезпечує безпечні умови праці під час аналізу.

Експериментальна частина. Світлопоглинання досліджуваного та контрольного зразків вимірювали на фотоелектричному фотометрі КФК-3-01 (420 нм, l = 3 см). Швидкість реакцій характеризували значенням величини оптичної густини розчину через 10 хв (*методом фіксованого часу*). Вимірювання здійснювали за +37 °С, підтримання температури реакційної суміші забезпечували за допомогою водяного термостата, рН розчинів контролювали потенціометрично за допомогою скляного електрода. Визначення оптичної густини розчину повторювали п'ять разів із кожним розчином певної концентрації ензиму. За отриманими пересічними даними будували градуювальну залежність питомої активності ензиму (у міжнародних одиницях (АО/мг) – кмоль/хв до 1 мг субстанції) від оптичної густини розчину. За пересічним з п'яти визначень значенням оптичної густини випробуваного розчину за допомогою градуювального графіка знаходили питому активність ензиму (U).

Результати та їх обговорення. Суть методу полягає у фотометричному вимірюванні швидкості ферментативного гідролізу субстрату ацетилхоліну в буферному середовищі з використанням 3,3′,5,5′-тетраметилбензидину (ТМБ).

Реакцію ферментативного гідролізу субстрату проводили за рН 8,3, а через 10 хвилин після початку вимірювали швидкість ферментативного гідролізу ацетилхоліну. Лінійна залежність оптичної густини від питомої активності ензиму (U) спостерігалась в інтервалі 3,5–28 АО/мг. Активність ензиму за пересічними результатами 5 визначень становила 27,9 АО/мг (заявлена у сертифікаті якості питома активність становить 28 АО/мг). Межа кількісного визначення – 0,2 АО/мг. Метрологічні характеристики опрацьованого способу були такими: RSD = 1,8 % (n = 5; Р = 0,95), правильність – 0,45 %. Це свідчить, що запропонований спосіб визначення активності холінестерази крові характеризується високою чутливістю, достовірністю і відтворюваністю результатів. Водночас було доведено відсутність систематичної похибки під час здійснення визначення активності холінестерази опрацьованим методом.

Висновки. У результаті проведених досліджень було опрацьовано новий метод визначення активності ферменту холінестерази, який характеризується високою чутливістю, достовірністю і відтворюваністю результатів, а також дозволяє забезпечити безпечні умови праці під час виконання аналізу.

Ключові слова: ацетилхолінестераза; ацетилхолін; фотометричні методи аналізу; 3,3′,5,5′-тетраметилбензидин

Introduction

Improving the healthcare system around the world and increasing the average age of the population in the developed countries cause a growing demand of new treatment methods of complex biological disorders, such as neurodegeneration, dementia and cancer [1]. Physicians and pharmaceutical chemists have been searching for new treatment approaches based on compounds that can interact with two or more targets simultaneously to have synergistic clinical effects from a single compound. Such a way reduces the risk of side effects and eliminates the problems associated with taking several drugs simultaneously.

Supporting this approach, enzymes remain the main target for drug development as changes in the activity of enzymes lead to desired effects. Even with the increase in the use of drugs for receptors to modulate signals from outside the cell 47% of all current drugs inhibit enzyme targets [2].

A new approach that has recently been introduced to medical chemistry is called the creation of multi-target-directed ligands (MTDL). It is based on treatment using the combination of enzyme modulators. For instance, the combination of cholinesterase and monoamine oxidase inhibitors is employed to reduce the rate at which the neurotransmitters are broken down, thereby increasing their concentration in the brain to treat problems of Alzheimer's disease. Another example is application of monoamine oxidase together with a metal ion to cure Parkinson's disease. For cancer treatment, mixtures containing substances with kinase inhibiting and cyclooxygenase inhibiting properties have been proposed [3–5].

A wide application of the treatment strategies involving enzyme inhibitors leads to an urgent need of simple, sensitive and selective methods of the enzyme activity analysis [4].

Acetylcholinesterase is an important enzyme of the nervous system. It stops the transmission of nerve impulses by catalysing the hydrolysis of the neurotransmitter acetylcholine (ACh).

There are two types of cholinesterase: acetylcholinesterase, also called specific cholinesterase or real cholinesterase (the systematic name is acetylcholine-acetylhydrolase, EC 3.1.1.7) and non-specific cholinesterase (pseudocholinesterase, the systematic name is acylcholine-acylhydrolase, EC 3.1.1.8). Real cholinesterase is found mainly in erythrocytes, nerve and muscle tissues; pseudocholinesterase – in the blood serum, liver, pancreas. Acetylcholinesterase and pseudocholinesterase differ in some properties and primarily in the substrate specificity. The most specific substrate for real cholinesterase is ACh. Pseudocholinesterase does not have the strict substrate specificity and together with ACh also hydrolyses other substrates, such as benzoylcholine, succinylcholine and others. The reaction of the ACh hydrolysis catalyzed by cholinesterase is given in Scheme 1.

The simplest methods of measuring the cholinesterase activity described in the literature are photometric ones; they are based on the enzymatic hydrolysis of ACh with the formation of acetic acid (Scheme 1) and the subsequent measurement of light absorption of the reaction mixture in the presence of an acid-base indicator [6]. The photometric method involving acetylcholine chloride as a substrate and phenolic red as an indicator has been approved to be the common one [7].

The Hestrin's hydroxylamine method also belongs to photometric methods [8]. The method is based on the ability of hydroxylamine to react with ACh after precipitation of protein with trichloroacetic acid. As a result of the reaction, hydroxamic acid is formed. The later gives a water soluble yellow-brown complex when reacts with iron(III) chloride in the acidic medium. Its intensity is proportional to the concentration of ACh. The enzyme activity is determined by the difference between the amount of ACh taken for incubation (control ACh) and ACh that is not hydrolyzed during the incubation. The optical density of the solution is measured using a photoelectrocolorimeter with a green light filter against water (540 nm; the cuvette thickness – 5 mm).

The advantages of the Hestrin's method for determining the activity of cholinesterase are the ability to study the kinetics of the enzymatic reaction under different conditions, the method is quite sensitive and allows to work in a wide range of substrate concentrations; it is convenient for determining the activity of cholinesterases of different origin under standard conditions (certain concentrations of the enzyme and the substrate, the standard reaction time under zero order).

However, at present the Hestrin's method is rarely used in clinical practice due to its low accuracy as consistent results can be achieved if the concentration of ACh decreases by 25%. This makes impossible to study the initial section of the kinetic curve [9].

We previously described the method for measurement of the cholinesterase activity under enzymatic conditions. The activity of the enzyme was calculated by the amount of ACh, which was not hydrolyzed
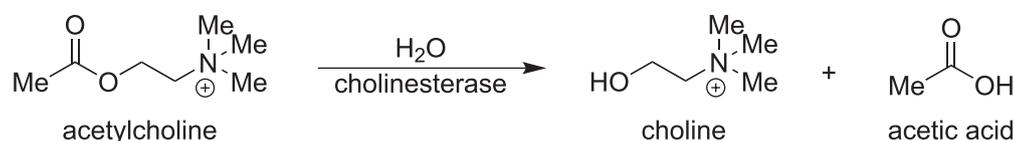
Scheme 1. The hydrolysis of ACh in the presence of cholinesterase

during its incubation with the enzyme. Unreacted ACh was involved in a system of two conjugate reactions: perhydrolysis of ACh (the reaction with an excess of hydrogen peroxide) with the formation of peroxyacetic acid further oxidizing an indicator substance in phosphate buffer at pH = 7.2 giving a colored oxidation product [10]. The optical density of the solution was measured on a photoelectrocolorimeter 4 min after adding 1 mL of 0.05 mol/L solution of sodium phosphate to the mixture of 1 mL of the standard water-acetone solution (1:1) of ACh, 3 mL of 0.1% solution of *o*-dianisidine in acetone and 1 mL of 3% solution of hydrogen peroxide. The last step was to determine the amount of unreacted ACh by the calibration dependence of light absorption of the oxidation product on the concentration of ACh taken after its incubation in the absence of the enzyme.

However, the disadvantage of this method is the use of *o*-dianisidine as an indicator compound, which is a highly toxic substance that has carcinogenic properties. Therefore, *o*-dianisidine causes harmful working conditions violating the principles of "green chemistry". Taking the abovementioned into account the aim of the current research was to develop a new method, which meets the requirements of accuracy and reproducibility of the results of the analysis, as well as provides safe working conditions during the analysis, in order to determine the activity of cholinesterase.

Experimental part

Preparation of the solutions

3,3′,5,5′-Tetramethylbenzidine dihydrochloride monohydrate (TMB) (98.5%, SIGMA) was used. The working solution (0.02 mol/L) was prepared by dissolving the accurate weight of TMB in 40% (*v/v*) mixture of 96% ethanol with double distilled water at +45°C, and stored in a tightly capped vial of dark glass in a cooler (at +8°C). Ethanol (96%) of pharmacopoeial purity was used.

10% Solution of hydrogen peroxide was prepared from 50% solution of hydrogen peroxide by its dilution with double distilled water. The concentration of the working solution of hydrogen peroxide was determined permanganatometrically [11].

To prepare a buffer solution with pH 8.3, disodium phosphate (28.2 g) was dissolved in 900 mL of double distilled water in a 1 L volumetric flask, and 0.2 mol/L hydrochloric acid solution (20 mL) was added; the solution was diluted to the volume.

The solution of acetylcholine chloride with the concentration of 5.4×10^{-3} mol/L was prepared by dissolving 40.0 mg of acetylcholine chloride in 40.0 mL of double distilled water.

As a standard, a purified horse serum cholinesterase enzyme (EC 3.1.1.8, class VI) with the known specific activity of 28 AU/mg (according to the certificate) was used. The manufacturer controlled the activity of a lyophilized powder of cholinesterase by the generally accepted method (according to the certificate) [6].

Preparation of the solution of the working standard sample of cholinesterase (WSS)

The accurate weight of the lyophilized cholinesterase powder (80 mg) was dissolved in 20.0 mL of double distilled water with gentle heating. The solution of the test sample of cholinesterase was prepared similarly. The shelf life of the solutions was 1 day.

The light absorption was measured on a CPhC-3-01 photoelectric colorimeter (420 nm, *l* = 3 cm). The reaction rate was characterized by the value of the optical density of the solution in 10 min (the fixed time method). Measurements were performed at +37°C, the temperature of the reaction mixture was maintained by its thermostating in water, pH of the solutions was monitored potentiometrically using a glass electrode.

Determination of the blood cholinesterase activity by the kinetic-photometric method

Construction of a calibration graph

In each of five 15 mL tubes equipped with a ground stopper the phosphate buffer solution (5 mL, 0.2 mol/L) with pH 8.3 was added, and the tubes were thermostated for 5 min at 37–38°C. Then the aqueous standard solution of cholinesterase (0.50 mL) with the concentration of 4 mg/mL (tube 1), 3 mg/mL (tube 2), 2 mg/mL (tube 3), 1 mg/mL (tube 4) and 0.5 mg/mL (tube 5) was added into each tube, the content was carefully shaken, and the acetylcholine solution (1.00 mL) was then added into each tube. The resulting mixture was immediately stirred vigorously overturning the tubes 5–7 times, the stopwatch was turned on, and the tubes were thermostated at +37–38°C for 10 min. Then to the resulting mixture 5.00 mL of a 10% solution of hydrogen peroxide was added and again thermostated at +37°C for another 10 min. After that 3 mL of 96% ethanol and 0.50 mL of TMB solution were added and stirred. Upon lapse of time the content of each tube was transferred to a cuvette of a CPhC-3-01 photoelectric photometer with a thickness of 3 cm, and the optical density of the reaction mixture was measured at λ = 420 nm. The experiment was repeated 4 times with each solution of a certain concentration of the enzyme. According to the average values obtained the calibration curve of the specific activity of the enzyme in international units (AU/mg – kmol/min to 1 mg of the substance) *versus* the optical density of the solution was constructed.

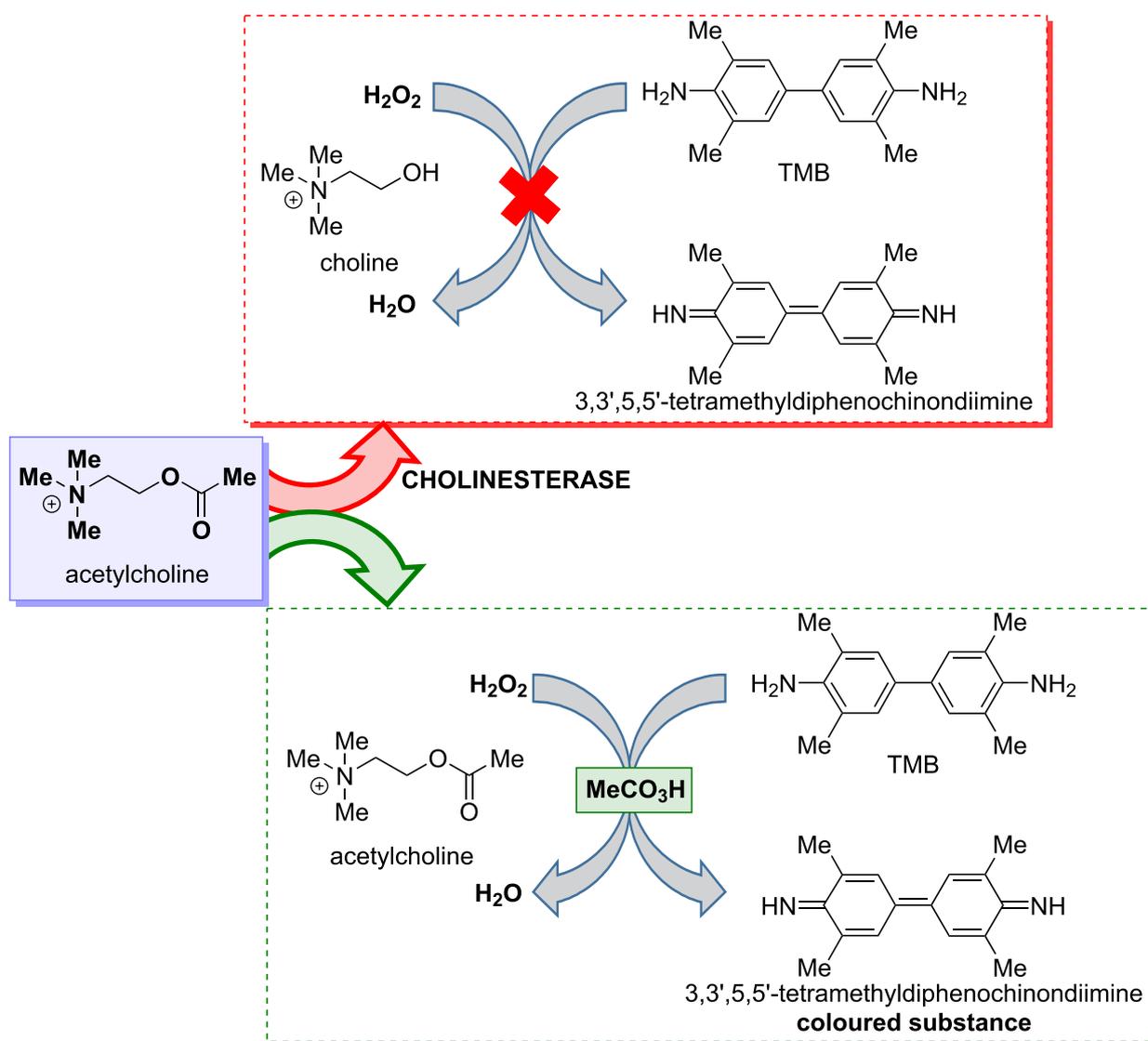### The method for determining the cholinesterase activity by the kinetic-photometric method

The test sample of cholinesterase (80.0 mg) was dissolved in 20.00 mL of double distilled water. 5 mL of 0.2 mol/L phosphate buffer solution with pH 8.3 was added to a 15 mL test tube equipped with a ground stopper, and the tube was thermostated for 5 min at 37–38°C, then 0.50 mL of the test aqueous cholinesterase solution

was added, shaken thoroughly, and 1.00 mL of the acetylcholine solution was introduced in the test tube with a pipette. The resulting mixture was immediately stirred vigorously by overturning the tube 5–7 times, the stopwatch was turned on, and the test tube was thermostated at +37–38°C for 10 min. Then 10% hydrogen peroxide solution (5.00 mL) was added to the mixture, and it was again thermostated at +37°C for another 10 min. Upon lapse of time 96% ethanol (3 mL) and the TMB solution (0.50 mL) were successively added, and the resulting mixture was shaken and thermostated at +37–38°C for another 10 min. After that the content of the tube was transferred to a cuvette of a CPhC-3-01 photoelectric photometer with a thickness of 3 cm, and the optical density of the reaction mixture (A) was measured at $\lambda = 420$ nm. The experiment was repeated five times and a mean value of the optical density from 5 measurements was calculated. The mean value was used to find the specific activity of the test sample of the enzyme (U, AU/mg) by the calibration graph equation.

Results and discussion

In order to determine the cholinesterase activity, TMB was proposed as an indicator substance in the kinetic method with the photometric measurement of the rate of the enzymatic hydrolysis of the ACh substrate in the phosphate buffer at pH 8.3 (the fixed time approach). The pH value of 8.3 was optimal for two reactions proceeding during the analysis: the hydrolysis of ACh in the presence of the cholinesterase enzyme and the indicator reaction of the TMB oxidation with peracetic acid. This fact makes the analytical method developed quite simple to perform. It is worth noting that TMB is a low toxic and rather stable compound [12], thereby we avoid the drawback of the method previously reported [13], and the method proposed meets the requirements of "green chemistry".

The successive reactions of the ACh hydrolysis and TMB oxidation occurring in the mixture analyzed are given in Scheme 2.
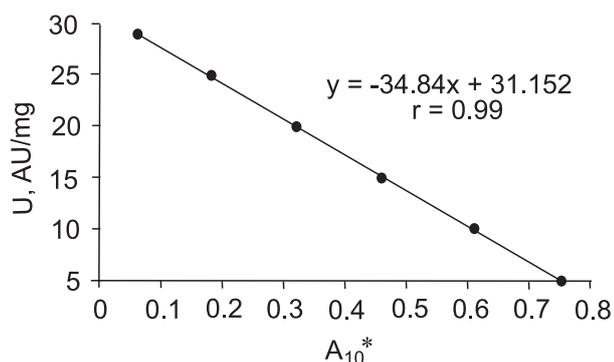

Scheme 2. The main idea of the research

Fig. 1. The calibration curve for determining the specific cholinesterase enzyme activity, 3.5–28 AU/mg

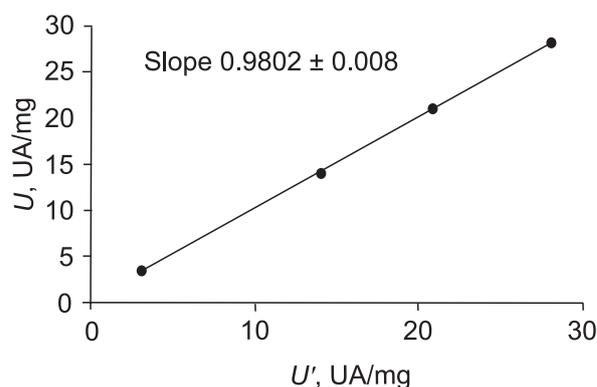Note: * – the fixed time method, 10-minute intervals

Fig. 2. The dependence of the specific activity found on the specific activity according to the unified standard method

Table

The results of the cholinesterase determination
($P = 0.95$, $n = 5$)

The specific activity of the standard, *AU/mg*	The specific activity found, *AU/mg*	Metrological characteristics, ($n = 5$; $P = 0.95$)
28.0*	28.1	$\bar{x} \pm \Delta\bar{x} = 27.9 \pm 0.63$ RSD = 1.84% $\delta^{**} = -0.5$
	28.5	
	27.1	
	27.8	
	27.8	

Notes: * – the activity of cholinesterase in accordance with the quality certificate; ** – as compared to the spectrophotometry detection [6] according to the manufacturer's quality certificate

The calibration graph of the dependence of the optical density of the solution analyzed on the enzyme specific activity and its equation are given in Fig. 1. The linear dependence of the optical density on the specific activity of the enzyme (U) is observed in the range of 28–3.5 AU/mg.

The specific activity of the enzyme (U) was found using the calibration graph presenting the average of five determinations of the optical density of the test solution. It was 27.9 AU/mg, RSD = 1.8%, correctness –0.5% (Table).

According to regression characteristics of the calibration graph (Fig. 1) the limit of quantification was calculated; it was 0.2 AU/mg.

Fig. 2 shows the dependence of the specific activity determined by the kinetic-photocolorimetric method (U) on the specific activity found by the unified standard method (U') [6].

The values of slope of the dependence was equal to 0.98 ± 0.01, and the correlation coefficient r = 0.99. It indicates the equivalence of both methods and the absence of systematic error in determining the activity of cholinesterase by the method proposed [11].

Conclusions

Thus, a new rapid kinetic-photometric method for determining the activity of blood cholinesterase has been developed; it is characterized by high sensitivity, reliability and reproducibility of the results, and also allows creating safe working conditions during the analysis. This method can be used for routine work in scientific laboratories, in the process of performing medical research and diagnosing diseases.

Conflict of Interests: the authors have no conflict of interests to declare.

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