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The Use of Tetramethylbenzidine as an Indicator in the Enzymatic Quantitative Determination of Ethonium

Abstract

The study considers the possibility of using 3,3',5,5'-tetramethylbenzidine (TMB) as an indicator in the enzymatic analysis for the quantitative determination of quaternary ammonium compounds on the example of ethonium. The feasibility of using TMB as an indicator in the kinetic photometric method has been confirmed. Kinetic curves showing the relationship between the optical density and the ethonium concentration have been constructed. The reaction rates of acetylcholinesterase inhibition by ethonium have been estimated using the tangents of the angles of these curves with TMB as an indicator. The degree of enzyme inhibition has been calculated, and a linear relationship between the ethonium concentration and the degree of inhibition has been determined. This method was applied to determine the ethonium content in 0.1 % ethonium gel. The relative standard deviation of the method does not exceed 2.5 %. The approach suggested offers a reliable and accurate method for the quantitative analysis of ethonium in dosage forms.

Keywords: ethonium; quaternary ammonium compounds; cholinesterase; acetylcholine

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Використання тетраметилбензидину як індикатора в ензиматичному методі кількісного визначення етонію

Анотація

У дослідженні розглянуто можливість використання 3,3',5,5'-тетраметилбензидину (ТМБ) як індикатора в ензиматичному аналізі для кількісного визначення четвертинних амонієвих сполук на прикладі етонію. Підтверджено можливість застосування ТМБ як індикатора в кінетичному фотометричному методі. Побудовано кінетичні криві, що демонструють залежність між оптичною густиною та концентрацією етонію. Швидкість реакції інгібування ацетилхолінестерази етонієм було оцінено за тангенсами кутів цих кривих, із цим ТМБ використано як індикатор. Розраховано ступінь інгібування ферменту, а також визначено лінійну залежність між концентрацією етонію та ступенем інгібування. Цей метод застосовували для визначення вмісту етонію в 0,1 % гелі етонію. Відносне стандартне відхилення методу не перевищує 2,5 %. Запропонований підхід забезпечує надійний та точний метод кількісного аналізу етонію в лікарських формах.

Ключові слова: етоній; холінестераза; ацетилхолін; четвертинні амонієві сполуки

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■ Introduction

3,3′,5,5′-Tetramethylbenzidine (TMB) is a widely used chromogen that is valued for its non-carcinogenic nature and, upon oxidation, gives products with high absorption coefficients. This makes TMB a key component in enzyme-based assays, offering superior sensitivity and low detection limits compared to many commercially available chromogenic reagents. Notably, TMB is the most widely used chromogenic substrate in ELISA procedures that use horseradish peroxidase conjugates. The TMB substrate develops a soluble blue reaction product that can be read at 370 or 655 nm [1].

Additionally, many recent reports support and justify the wide incorporation of TMB into analytical practice [2]. Thus, *Sil et al.* proposed a one-step simple method for nanolevel detection of ascorbic acid based on the inhibitory activity of ascorbic acid on horseradish peroxidase and hydrogen peroxide supported by redox properties of TMB [3]. TMB was also used for the uric acid detection with MnO_2 nanosheets. In mildly acidic conditions, MnO_2 oxidizes TMB to its blue oxidation product applied to human urine samples, achieving recovery rates of 93.1–102.4% with a relative standard deviation below 3% [4]. In 2022, *Zhang et al.* developed a colorimetric detection system for Cu^{2+} ions based on TMB and $Ag(S_2O_3)_2^{3-}$ in an aqueous solution. Unlike Ag nanoparticles, which could not oxidize TMB, $Ag(S_2O_3)_2^{3-}$ catalyzed the reaction effectively, with optimal conditions achieved at 800 μM for TMB, 400 μM for $Na_2S_2O_3$, for 40 min, and at 25 °C. This system, with a detection range of 1–100 μM and a limit of 100 nM, shows promise for monitoring the water quality [5]. Important biogenic thiols, such as glutathione (GSH), cysteine (CySH), and homocysteine (HcySH), were analyzed using TMB as a chromogen in a simple spectrophotometric method. Detection limits for GSH, CySH, and HcySH were 1.04, 0.82, and 2.09 μM, respectively, with successful application of the method to human serum samples, achieving recovery rates of 97–112% [6]. *Chandra and colleagues* reported the synthesis of fluorescent carbon quantum dots (M-CQDs) through a simple hydrothermal treatment of mustard seeds [7]. M-CQDs demonstrated peroxidase-like activity, catalyzing the oxidation of TMB in the presence of H_2O_2, thus mimicking the natural horseradish peroxidase activity. The process enabled the colorimetric detection of H_2O_2 in the range of 0.02–0.20 mM with a detection limit of 0.015 mM. Additionally, reduction of oxidized TMB with ascorbic acid allowed for a selective and sensitive detection of ascorbic acid in the range of 10–70 μM, with a detection limit of 3.26 μM. The method was successfully applied to the ascorbic acid detection in fresh fruits. Further advancements in TMB-based systems include the application of oxidized TMB nanobelts enhancing the colorimetric and paper-based sensing of H_2O_2 [8], peroxidase-like nanoenzymes, such as Fe_3O_4 nanoparticles [9], $CoFe_2O_4$ nanoparticles [10], single-atom iron nanozyme [11], MOF-818 nanozyme containing trinuclear copper centers [12], $Ce_2(WO_4)_3$ nanosheets [13], Fe_3O_4@AuNPs [14], etc., all enhancing the H_2O_2 detection sensitivity through the formation of oxidized TMB. The versatility of TMB is reflected in its increasing popularity, with 2,889 publications between 2015 and 2024 in the Scopus® database (www.scopus.com). Notably, the number of publications surged by 1.5 times between 2019 and 2024, demonstrates the growing impact of TMB on analytical chemistry (**Figure 1**).

Previously, our laboratory developed a novel biochemical kinetic-spectrophotometric method for detecting cholinesterase inhibitors from the group of quaternary ammonium compounds (QACs) using the oxidation of *p*-phenetidine by hydrogen peroxide as the indicator reaction [15], including a recent paper reporting the quantification of Ethonium [16]. Considering all the advantages that TMB can provide for the quantification of this group of pharmaceuticals and its central role in the development of sensitive and effective analytical methods, in this study we present the results of using TMB as a chromogen for the quantitative analysis of the QAC Ethonium in a 0.5% gel formulation. The enzyme-kinetic method based on the inhibition of cholinesterase activity and the use of oxidation of TMB with hydrogen peroxide as an indicator reaction was applied.

■ Materials and methods

Reagents and equipment

The following reagents were used in the study:

- ETONIY® (Aethonium) powder (substance) produced by OJSC Farmak, Kyiv, Ukraine. $C_{30}H_{62}Cl_2N_2O_4$; CAS: 21954-74-5; MW 585.736 g mol^{-1};
- 0.5% Ethonium gel, 50 mL, batch No. 74 (Apr 2023) manufactured by APTEKA PAVLOVA

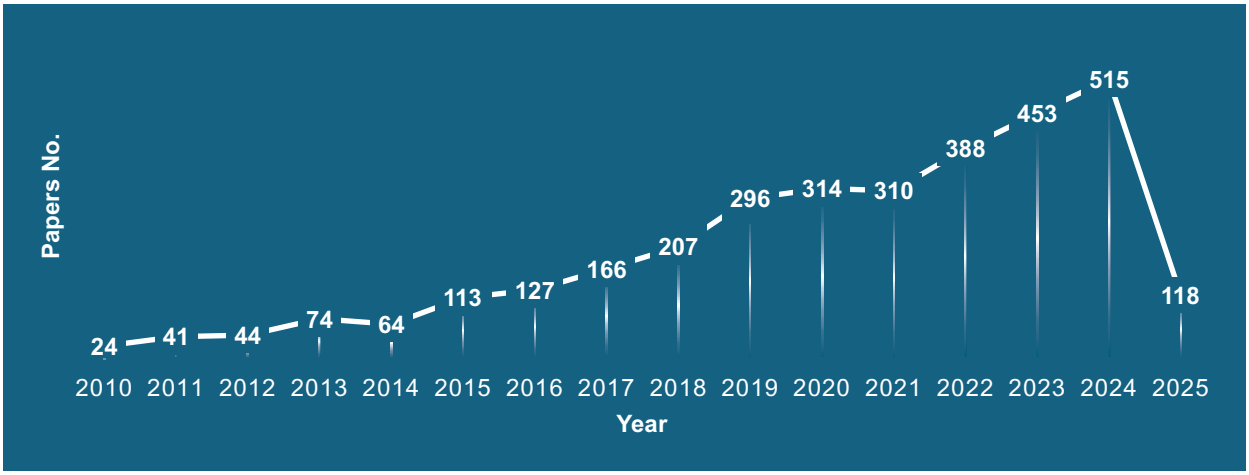

Figure 1. The number of papers per year for the query "3,3',5,5'-tetramethylbenzidine" (title, abstract, keywords) according to the Scopus® database (2010-2025)

Ltd (Odesa, Ukraine) with the composition of ethonium (active pharmaceutical ingredient) 0.5 g; glycerol – 20.0 g; propylene glycol – 20.0 g; PEG 400 – 50.0 g; PEG 1500 – 10.0 g; purified water – 10.0 ml. The content of the active substance, according to the certificate, is 4.4525% (*w*/*w*);

- 3,3',5,5'-Tetramethylbenzidine dihydrochloride (TMB), $C_{16}H_{20}N_2 \cdot 2HCl$; 98.5 % (Sigma-Aldrich); MW 313.27 g mol^{-1};
- Disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$), puriss. p.a. ("ReaChem", Kharkiv, Ukraine);
- Stabilized hydrogen peroxide, 30–40 % solution, puriss. p.a., (LLC Inter-Synthes, Boryslav, Ukraine) with the content of hydrogen peroxide determined using permanganatometry according to the State Pharmacopoeia of Ukraine [17];
- Acetylcholine chloride (Pharm Grade), 0.2 g per amp/5 mL, manufactured by the State Science Center of Virology and Biotechnology "Vector";
- A dry cholinesterase (EC 3.1.1.8) powder from horse serum (SMU "Biomed"), 80 mg in an ampoule (VI class, activity 28 AU mg^{-1}). The catalytic activity of 1 activity unit (AU) is manifested in such an amount of this enzyme preparation that converts 1 μmol of the substrate in 1 min under specified reaction conditions.
- Ethanol 96% *v*/*v* (USP, BP, Ph.Eur.) pure, pharma grade.
- High-purity double distilled water was used throughout the experiment.

The pH measurements were performed with a combined glass electrode (SP20B) together with an EAL-1M3.1 reference standard silver chloride electrode.

The absorbance measurements were performed on an SF-26 spectrophotometer (λ = 420 nm, l = 10 mm).

Preparation of solutions

0.2 M Phosphate buffer solution (pH 8.35)

Disodium hydrogen phosphate dodecahydrate (35.75 g) was dissolved in a 500 mL flask using double-distilled water. 0.1 M Solution of hydrochloric acid (19 mL) was then added. The pH of the final solution was controlled potentiometrically.

0.02 M TMB solution

The substance of TMB (0.6265 g) was dissolved in 40% (*v*/*v*) ethanol solution in a 100 mL volumetric flask and diluted to the volume with the same solvent. The mixture was heated to 45 °C for complete dissolution and was stored in a tightly closed dark glass bottle in a cool place.

10% Hydrogen peroxide solution

The solution was prepared from a 30–40% solution of hydrogen peroxide by dilution with the required amount of double distilled water. The content of hydrogen peroxide in a 10% working solution was determined by permanganatometry.

Cholinesterase (ChE) solution

The accurately weighed content of an ampoule containing the cholinesterase powder (80 mg) was dissolved in double-distilled water (20.0 mL) when heating gently on a water heater. The shelf life of the solution was 1 day.

Acetylcholine chloride (ACh) solution

The solution with the initial concentration of $5.4 \cdot 10^{-3}$ mol L^{-1} was prepared by dissolving the ampoule content (0.2 g of acetylcholine) in 200 mL of double-distilled water. For this purpose, the ampoule was opened, and 4.0 mL of water was pipetted and added to the ampoule, and then shaken until acetylcholine was completely dissolved.

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Then the solution was transferred into a 200 mL volumetric flask and diluted to the volume with double-distilled water.

0.1% Ethonium solution

1.0000 g of the Ethonium substance was dissolved in a 1 L flask using double-distilled water, the solution was heated to 40–45°C, 9.0 g of sodium chloride was added to the solution, and diluted to the volume.

Stock Solution of Ethonium (ET), 1×10^{-4} M

The accurately weighed powder of the Ethonium substance (0.058574 g) was dissolved in 500 mL of double-distilled water in a 1000 mL volumetric flask. The solution was diluted to the volume with the same solvent at +20 °C and mixed thoroughly.

Ethonium Work Standard (WS) solution, $1\cdot10^{-5}$ M

The accurately weighed powder of the ethonium substance (0.58574 g) was dissolved in 500 mL of ethanol solution in double-distilled water ($EtOH/H_2O$ 30:70 *v*/*v*) in a 1 L volumetric flask and diluted to the volume at +20 °C and mixed thoroughly. Using a pipette, 10 mL of the resulting solution was taken and transferred to a 1 L volumetric flask and diluted to the volume with double-distilled water.

Ethonium WS Solution, 1×10^{-6} M

A 10 mL aliquot of *Stock Solution* of the drug (1×10^{-4} mol L^{-1}) was transferred into a 1 L volumetric flask and diluted to the volume with double-distilled water at +20 °C.

The procedure for constructing the kinetic curves

Part 1 – working experiments «ACh + (ChE + ET)»

The buffer solution (2.00 mL, pH 8.35) was added to each of the five 20 mL graduated test tubes with a ground joint stopper. Then 0.50, 1.50, 3.00, 4.50, and 6.00 mL of the ethonium 1×10^{-6} M WS solution was added to the test tubes, followed by 0.50 mL of the cholinesterase solution. The content was thoroughly shaken, and the test tubes were kept in a thermostat at +38 °C for 10 min. After that 1.0 mL of acetylcholine solution and 5.90, 4.90, 1.90, 0.40 mL, and 5.4 mL of double-distilled water were added to the five test tubes, respectively. The content was mixed thoroughly and incubated again for 10 min at 38 °C. Then, a 10% hydrogen peroxide solution (3.20 mL) was added to each of the test tubes, and the latter were incubated for 10 min at +38 °C. After that 3.0 mL of 96% ethanol and 0.50 mL of the TMB solution were added, and the solution was shaken thoroughly and scanned photometrically on a spectrophotometer at a wavelength of 420 nm in a 1 cm cuvette over a 15-minute period. The phosphate buffer was used as a reference solution. The relative rate of the reaction [[(ChE + ET) + ACh] + H_2O_2 + TMB] (*tg*α (Inh), min^{-1}) was determined as the slope of a linear section of the "*optical density (A) vs time (t, min)*" kinetic curve.

Part 2 – control experiments #1 "ACh"

The buffer solution (2.00 mL, pH 8.35) was added to a 20 mL graduated test tube, followed by 6.9 mL of double-distilled water, 1.0 mL of the acetylcholine solution, and 3.2 mL of the hydrogen peroxide solution. The solution was incubated at 38 °C for 10 min. After that 3.0 mL of 96% ethanol and 0.5 mL of the TMB solution were added to the test tube. The solution was shaken thoroughly and scanned photometrically on a spectrophotometer at a wavelength of 420 nm in a 1 cm cuvette over a 15-minute period. The phosphate buffer was used as a reference solution. According to the plotted "*optical density* (*A*) vs *time* (*t*, min)" kinetic curve, the relative rate of the reaction [(ACh + H_2O_2) + TMB] was determined as a slope of a linear section of the curve (*tg*α (ACh), min^{-1}).

Part 3 – control experiments #2 "Ach+ChE"

2.0 mL of the buffer solution, 6.4 mL of double-distilled water, 0.5 mL of the cholinesterase solution, and 1.0 mL of the acetylcholine solution were successively added to a 20 mL test tube with a ground joint stopper and then thermostated at 38 °C for 10 min. Further 3.2 mL of the hydrogen peroxide solution was added, and the mixture was thoroughly shaken and thermostated again at 38 °C for 10 min. After that 3.0 mL of 96% ethanol and 0.5 mL of the TMB solution were added. Then the solution was scanned photometrically on a spectrophotometer at a wavelength of 420 nm in a 1 cm cuvette over a 15 min period. The phosphate buffer was used as a reference solution. According to the plotted "*optical density (A) vs time (t, min)*" kinetic curve, the relative rate of the reaction [(ChE + ACh) + H_2O_2 + TMB] was determined as a slope of a linear section of the curve (*tg*α (ACh + ChE), min^{-1}).

The relative rates of the reactions (expressed as tangents of the angles of slope) were used to calculate the inhibition degree of the enzymatic hydrolysis of ACh (*U*, %) in the presence of ethonium according to the following equation:

$$U(\%) = \frac{[tg\alpha(\text{Inh}) - tg\alpha(\text{Ach} + \text{ChE})]}{[tg\alpha(\text{Ach}) - tg\alpha(\text{Ach} + \text{ChE})]} \times 100\ \%$$

where *tg*α (*Inh*) (min^{-1}) is the relative reaction rate of the TMB oxidation by peroxyacetic

acid formed during the perhydrolysis of unreacted ACh in the working experiment at various concentrations of the inhibitor (ET);

*tg*α (Ach) (min^{-1}) is the relative reaction rate of the TMB oxidation by peroxyacetic acid formed in the reaction of the ACh perhydrolysis in the absence of the inhibitor and ChE (control experiment #1);

*tg*α (Ach + ChE) (min^{-1}) the relative reaction rate of the TMB oxidation by peroxyacetic acid formed in the reaction of the perhydrolysis of unreacted ACh in the presence of ChE and in the absence of the inhibitor (ET) (control experiment #2).

The calculated values of *U* (%) were used to plot the "*inhibition degree (U, %) vs ethonium concentration (c, ng* mL^{-1}*)*" calibration graph (**Figure 3**).

■ Results and discussion

Previously, a study was conducted to analyze the parameters that may affect the effectiveness of the approach proposed [18]. This allowed us to determine the optimal working conditions and concentrations of the reagents used in this study.

Kinetic curves were plotted using experimental data showing the relationship between the optical density and time, as presented in **Figure 2**.

The calibration graph (**Figure 3**) was constructed in the coordinates of the inhibition degree (*U*, %) *vs* the concentration (*c*, ng mL^{-1}). From **Figure 3**, the linear dependence of the inhibition degree on the concentration of the inhibitor was observed in the interval of 4–60% (R = 0.9) corresponding to the concentration of the inhibitor 17–200 ng mL^{-1}. The LOQ was defined as the concentration corresponding to a 4% degree of inhibition, i.e., 17 ng mL^{-1}.

The method for the quantitative determination of ethonium with TMB as an indicator in a "Ethonium 0.5% gel" formulation

0.1 g (accurate weight) of the ethonium gel was dissolved in 1000 mL of double-distilled water. Then 2.0 mL of the phosphate buffer solution, 5.0 mL of the gel solution, and 0.5 mL of the ChE solution were successively added to a 20 mL test tube. The mixture was thoroughly shaken and incubated for 10 min at 38 °C. After that 1.0 mL of the ACh solution and 1.4 mL of double-distilled water were added, and the content was carefully mixed and incubated again for 10 min at 38 °C. Then, 3.2 mL of the hydrogen peroxide solution was added, and the mixture was incubated again at 38 °C for 10 min. After that 3.0 mL of 96% ethanol and 0.5 mL of the TMB solution were added to the test tube. The optical density of the solution was measured at 420 nm in a 1 cm cuvette for 15 min. According to the plot of the "*optical density* vs *time*" dependence, the tangent of the angle of slope for the linear section *tg*α (Inh) was found in min^{-1}. In parallel, two more experiments were carried out. One of them involved acetylcholine and cholinesterase without the inhibitor, another one was performed without the use of cholinesterase enzyme (*control experiments #1* and *#2* described in the previous section). As a result, the

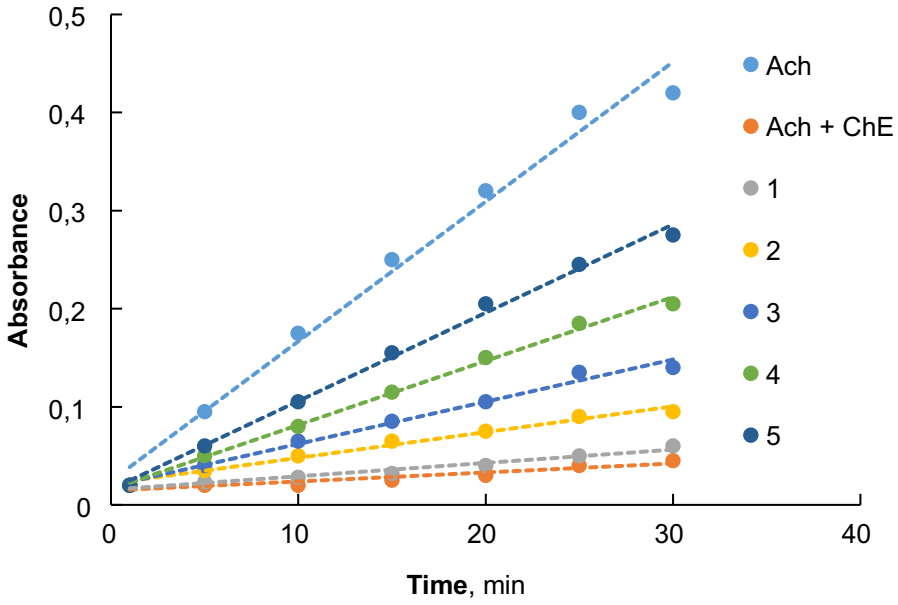

Figure 2. Kinetic curves of the conjugated oxidation of TMB with hydrogen peroxide in the presence of mixtures ACh + ChE + Inh (**1–5**). *c* (Ach) = 3.3×10^{-4} mol L^{-1}; *w*(H_2O_2) =1.92%; c (AChE) = 0.24 mg mL^{-1}. *w* (ET): 17 ng mL^{-1} (**1**); 50 ng mL^{-1} (**2**); 100 ng mL^{-1} (**3**); 150 ng mL^{-1} (**4**); 200 ng mL^{-1} (**5**); *c* (TMB) = 6.25×10^{-4} mol L^{-1}; control experiments *"Ach + ChE"*, control experiments *"ACh"*

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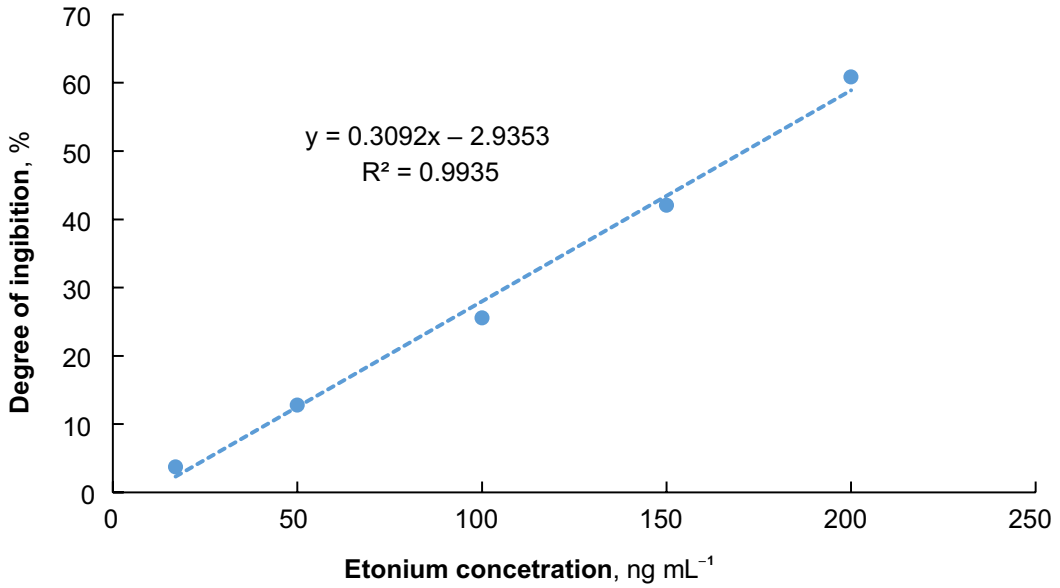

Figure 3. The dependence of the degree of inhibition on the concentration of Ethonium in the system (ACh + (ChE + Ethonium), determined by the indicator reaction of the conjugated oxidation of TMB with hydrogen peroxide in the presence of residual acetylcholine. *c* (ACh) = 3.3×10^{-4} mol L^{-1}; *w* (H_2O_2) = 1.92 %; *c* (AChE) = 0.24 mg mL^{-1}, *c* (TMB) = 6.25×10^{-4} mol L^{-1}

Table. The results of the analysis of 0.5 % ethonium gel and 0.1 % solution according to the proposed procedure by the kinetic-spectrophotometric enzyme method

The substance analyzed	ET found ($\bar{x} \pm \Delta\bar{x}$), %[a]	*RSD*, %	The quality certificate data, %	Accuracy, (δ, %)[b]
0.5 % Ethonium gel, 50 mL, manufactured by APTEKA PAVLOVA Ltd (Odesa, Ukraine)	0.443 ± 0.014	2.54	0.445	–0.45
0.100 % Ethonium solution, 1000 mL, prepared *ex tempore*	0.098 ± 0.003	2.46	0.100	–2.00

Notes: [a] Mean of 5 measurements (*P* = 0.95); [b] $\delta = (\bar{x} - \mu) \times 100\ \% \times \mu^{-1}$; μ is the actual content of ET according to the Certificate

other two tangents *tg*α (Ach + ChE) and *tg*α (ACh), respectively, were determined.

The content of ethonium in the gel formulation, (*w*, %) was calculated by the formula:

$$w\ (\%, \text{w/w}) = \frac{0.00059574[tg\alpha(\text{X}) - tg\alpha(\text{Ach} + \text{ChE})]\times 100\ \%}{g\times[tg\alpha(\text{Ach}) - tg\alpha(\text{Ach} + \text{ChE})]}$$

where 0.00059574 – is the mass of Ethonium in a 10.00 mL aliquot of the Stock Solution of the drug, g;

g – is the mass of the ethonium gel sample taken for the analysis, g;

*tg*α (X) – is the relative reaction rate (the tangent of the angle of slope of the kinetic curve), in the working experiment with the sample solution of the drug studied (the Ach – ChE – Inh (X) + (H_2O_2 – TMB) system), min^{-1};

tgα (Ach + ChE) – is the relative reaction rate in the absence of the inhibitor (ET) (the tangent of the angle of slope of the kinetic curve) in the Ach – ChE + (H_2O_2 – TMB) system, min^{-1};

tgα (ACh) – is the relative reaction rate (the tangent of the angle of slope of the kinetic curve) in the working experiment without the use of the inhibitor and cholinesterase, (the Ach – ChE – Inh (WSS) + (H_2O_2 – TMB) system), min^{-1}.

■ Conclusions

This paper proves the possibility of using tetramethylbenzidine as a promising indicator substance for the quantitative determination of surface-active substances of the class of quaternary ammonium compounds.

The quantitative content of Ethonium as an active ingredient in the 0.5% gel formulation and in the 0.1% solution prepared *ex tempore* was determined by the enzyme-kinetic method using the effect of inhibiting the activity of the enzyme cholinesterase. The inhibition was estimated by the residual acetylcholine using the indicator reaction of the 3,3',5,5'-tetramethylbenzidine oxidation with peracetic acid formed in the perhydrolysis of acetylcholine. As a result, RSD did not exceed 2.5% with accuracy δ = –0.45….–2.00 % (δ < RSD). The LOQ value was 17 ng mL^{-1}.

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