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The Quantitative Determination of Etonium by the Enzymatic Kinetic-Spectrophotometric Method

Abstract

The use of the cholinesterase enzyme as a component of the analytical system has made it possible to develop a new kinetic-spectrophotometric method, which is alternative to the pharmacopoeial method, for determining the quaternary ammonium salt – ethonium in the substance and a dosage form. This method is characterized by high sensitivity and specificity, and is relatively cheap. The relative standard deviation of the procedure does not exceed 2.7 %, and the limit of quantification is 17 ng mL^{-1}.

Keywords: surfactant; ethonium; cholinesterase; acetylcholine

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Кількісне визначення етонію ферментативним кінетико-спектрофотометричним методом

Анотація

Використання ферменту холінестерази як компонента аналітичної системи дозволило розробити нову, альтернативну фармакопейній, кінетико-спектрофотометричну методику визначення четвертинної амонійної солі етонію в субстанції та лікарському засобі. Методика характеризується високою чутливістю і специфічністю та є відносно дешевою. Відносне стандартне відхилення методики не перевищувало 2,7 %, а межа визначення становила 17 нг мл$^{-1}$.

Ключові слова: поверхнево-активні речовини; етоній; холінестераза; ацетилхолін

■ Introduction

Ethonium (1,2-ethylene-*bis*-(N-dimethylcarbdecyloxymethyl)ammonium dichloride, **Figure 1**) is a *bis*-quaternary ammonium salt. It exhibits several valuable biomedical properties, e.g. displays the bacteriostatic and bactericidal activity against streptococci, staphylococci, and other microorganisms, acts as an antitoxic agent against the staphylococcal toxin, has a local anesthetic effect, stimulates wound healing, etc. [1].

According to the features described, ethonium has found application as an antimicrobial, analgesic, and regenerative agent in treating various diseases of the skin and mucous membranes, such as bedsores, cracked nipples, vaginitis, burns, trophic ulcers, radiation lesions, etc. [2].

It is manufactured as a substance and is included as an active ingredient in various medicinal formulations, for instance, 1% ointment and 1% or 0.5% gel for external application; 0.02–1% solutions for the treatment of wounds, ulcers, keratitis, and other eye lesions; the ethonium paste for the use in dental practice [3].

The ethonium content control in any substance or preparations can be achieved by several
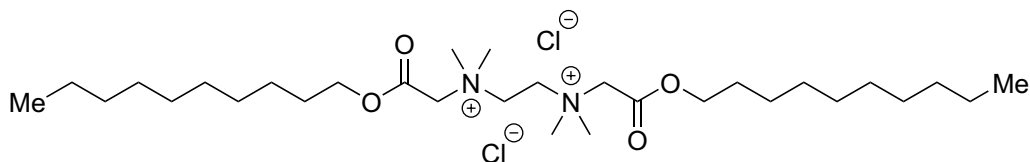
Figure 1. The structural formula of ethonium

analytical methods, including titration with perchloric acid in a non-aqueous medium [4], bichromatometry and mercurimetry [5, 6], the photometric and extraction-photometric determination in the form of associates with inorganic or organic dyes [7–11], gas-liquid chromatography [12, 13], and ionometry [14]. Although the methods mentioned are relatively simple, their general drawback is the low selectivity (except for the ionometry method). Thus, in the analysis of ethonium by bichromatometric or mercurimetric methods, the determination is carried out with respect to the chloride anion, and not to the organic cation. The method of ionometry involves the preliminary manufacturing of an indicator electrode, which is very inconvenient. Therefore, despite a number of existing methods for the quantitative determination of ethonium, the development of simple, accessible, and selective methods is still an open question.

Previously, a new enzymatic kinetic-spectrophotometric method for determining the quaternary ammonium salts was developed in our laboratory. This allows to use the ability of salts to inhibit the catalytic activity of cholinesterase [15]. The degree of inhibition was determined using two conjugated reactions of the acetylcholine perhydrolysis followed by the oxidation of *p*-phenetidine with the peroxyacetic acid formed. Adhering to the research direction in this work, we present the results of the quantitative determination of ethonium in a medicinal product by the enzymatic kinetic-spectrophotometric method.

■ Materials and methods

Reagents and equipment

ETONIY® (Aethonium) powder (substance, 99.9%) was produced by OJSC Farmak, Kyiv, Ukraine, CAS 21954-74-5, MW 585.736 g mol⁻¹.

Ethonium gel, 0.5%, 50 mL, batch No. 74 (Apr 2023) was manufactured by APTEKA PAVLOVA Ltd (Odesa, Ukraine). Its composition is ethonium (active pharmaceutical ingredient) – 0.5 g; glycerin – 20.0 g; propylene glycol – 20.0 g; PEG 400 – 50.0 g; PEG 1500 – 10.0 g; purified water – 10.0 mL. The quantitative content of the active substance according to the certificate was 4.4525% (*w/w*).

p-Phenetidine, 98% (Sigma-Aldrich, batch A0281408); *p*-Phenetidine hydrochloride was prepared by dissolution of *p*-phenetidine in chloroform followed by precipitation of the salt by gaseous hydrogen chloride.

Disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$), puriss. p.a. (“ReaChem”, Kharkiv, Ukraine) was used.

Stabilized hydrogen peroxide, 30–40% solution, puriss. p.a., (LLC Inter-Synthes, Boryslav, Ukraine) was used; the content of hydrogen peroxide was determined permanganatometrically according to the State Pharmacopoeia of Ukraine [16].

Acetylcholine chloride (Pharm Grade), 0.2 g per amp/5 mL, was produced by “Vector” – State Science Center of Virology and Biotechnology (Russia).

A dry cholinesterase (EC 3.1.1.8) powder from horse serum (SMU “Biomed”, Russia), 80 mg in an ampoule (VI class, activity 28 AU mg⁻¹) was used in the study. 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.

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 ($\lambda = 358$ nm, $l = 10$ mm).

Preparation of solutions

Solution of 0.2 M phosphate buffer (pH 8.35)

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

0.5% p-Phenetidine hydrochloride (p-Ph) solution

p-Phenetidine hydrochloride (0.50 g) was dissolved in double-distilled water (80 mL) in a 100 mL volumetric flask and diluted to the volume with the same solvent.

Solution of 10% Hydrogen peroxide

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 permanganatometrically.

Solution of cholinesterase (ChE)

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

Solution of acetylcholine chloride (ACh)

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, 4.0 mL of water was pipetted and added to the ampoule, and then shaken until acetylcholine was completely dissolved. Then the solution was transferred into a 200 mL volumetric flask and diluted to the volume with double-distilled water.

Stock Solution of Ethonium (ET) (1×10^{-4} mol L^{-1})

An accurately weighed powder of the ethonium substance containing 0.058574 g of the main ingredient 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.

Work Standard (WS) Solution of Ethonium 1×10^{-6} mol L^{-1}

A 10 mL aliquot of *Stock Solution* of the drug (1×10^{-4} mol L^{-1}) was transferred into a 1000 mL 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 (10.00 mL, pH 8.35) is added to each of the four 20 mL graduated test tubes with a ground joint stopper. Then 0.50, 1.00, 2.00, and 3.00 mL of the ethonium WS solution was added to the test tubes followed by 2.00, 1.50, 0.50, and 0.00 mL of double-distilled water, respectively. After that 0.50 mL of the ChE solution was added to the test tubes, the content was thoroughly shaken, and the test tubes were kept in a thermostat at +38 °C for 10 min. Shortly thereafter, the ACh solution (1.00 mL) was added to each test tube, the mixture was shaken thoroughly and thermostated for 10 min at +38 °C again. Then a 10% hydrogen peroxide solution (1.60 mL)

was added to each test tube and incubated for 10 min at +38 °C. Further, the *p*-phenetidine hydrochloride solution (1.00 mL) was added, and the solution was diluted to the volume with double-distilled water, shaken thoroughly, and scanned photometrically on a spectrophotometer at a wavelength of 358 nm in a 1 cm cuvette every minute over 15 min. The phosphate buffer was used as a reference solution. The relative rate of the reaction [[(ChE + ET) + ACh] $+ H_2O_2 + p$-Ph] (*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 experiment #1 "ACh"

The control experiment was carried out in the absence of ChE and the inhibitor. The buffer solution (10.00 mL), 3.00 mL of double-distilled water, 1.00 mL of the ACh solution, 1.60 mL of the hydrogen peroxide solution were successively added to a 20 mL graduated test tube with a ground joint stopper. The test tube was thoroughly shaken, and then thermostated for 10 min at +38 °C. After that the *p*-Ph solution (1.00 mL) was added, the solution was diluted to the volume with double-distilled water, and the mixture was thoroughly shaken. Then the solution was scanned photometrically on a spectrophotometer at a wavelength of 358 nm in a 1 cm cuvette every minute over 15 min. The phosphate buffer was used as a reference solution. According to the plotted kinetic curve *"optical density (A) vs time (t, min)"*, the relative rate of the reaction [(ACh + H_2O_2) + *p*-Ph] was determined as a slope of a linear section of the curve (*tg*α (ACh), min^{-1}).

Part 3 – control experiment #2 "ACh + ChE"

The buffer solution (10.00 mL), 2.50 mL of double-distilled water, 0.50 mL of the ChE solution, and 1.00 mL of the ACh solution were successively added to a 20 mL graduated test tube with a ground joint stopper. The content was thoroughly shaken and kept at +38 °C within 10 min. Next, the hydrogen peroxide solution (1.60 mL) was added, and the mixture was thoroughly shaken and thermostated for 10 min at +38 °C. After that, the *p*-Ph solution (1.00 mL) was added, and the solution was diluted to the volume with double-distilled water. Then the solution was scanned photometrically on a spectrophotometer at a wavelength of 358 nm in a 1 cm cuvette every minute over 15 min. The phosphate buffer was used as a reference solution. According to the plotted kinetic curve *"optical density (A) vs time (t, min)"*, the relative rate of the reaction [(ChE + ACh) + $H_2O_2 + p$-Ph] was

determined as a slope of a linear section of the curve (*tg*α (ACh + ChE), min⁻¹).

The relative rates of the reactions determined (expressed as tangents of the slope angles) 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 *p*-Ph 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 *p*-Ph 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) – is the relative reaction rate of the *p*-Ph oxidation by peroxyacetic acid formed in the reaction of the perhydrolysis of unreacted ACh in the presence of ChE and the absence of the inhibitor (ET) (control experiment #2).

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

■ Results and Discussion

Previously, a study was conducted to analyze the parameters that might affect the performance of the approach proposed [17]. This allowed us to determine the optimal working conditions and concentrations of reagents used in the present research.

Kinetic curves constructed were based on the experimental data on the dependence of optical density on time. They are given 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). One can see that within the inhibition interval of 20–80% the dependence on the ethonium concentration is linear. This corresponds to the concentrations of the inhibitor from 17 ng mL⁻¹ to 120 ng mL⁻¹. The limit of quantitation (LOQ) was defined as the concentration corresponding to 20% of the inhibition degree, and it was 17 ng mL⁻¹.

This method was further applied to determine the content of ethonium in a 0.5% gel formulation and a 0.1% solution. The results of these experiments are presented below.

The method for the quantitative determination of ethonium in a 0.5% gel formulation

0.1 g (accurate weight) of the ethonium gel was dissolved in 1000 mL of double-distilled water. Then, 10.0 mL of the phosphate buffer solution,
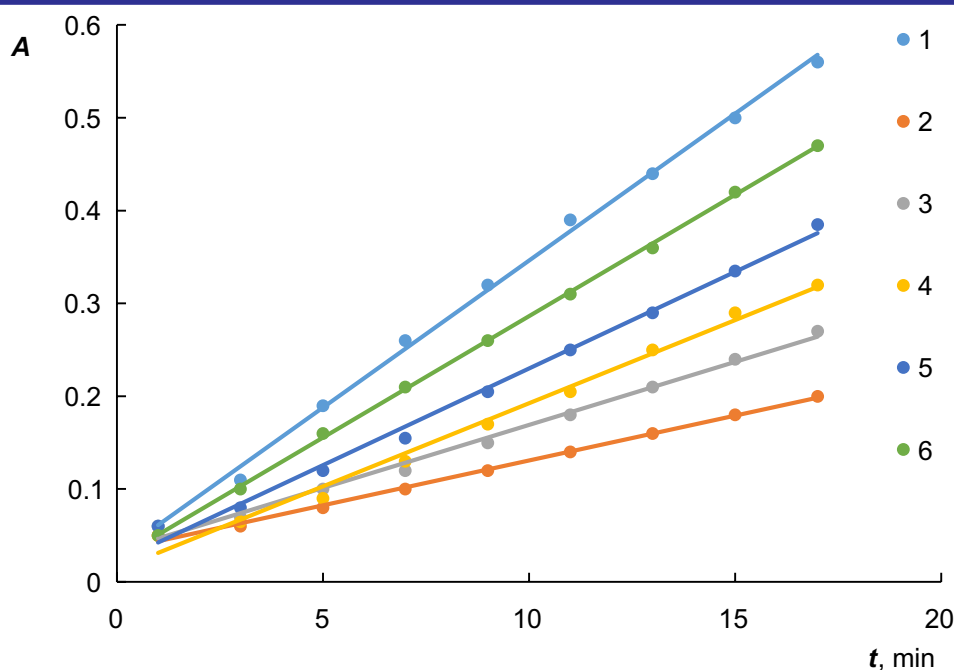
Figure 2. Kinetic curves of the conjugated oxidation of *p*-Phenetidine with hydrogen peroxide in the presence of: ACh (**1**), mixtures ACh + ChE (**2**) and ACh + ChE + Inh (**3–6**). *c* (ACh) = 3.3×10⁻⁴ mol L⁻¹; *w* (H₂O₂) = 1 %; *w* (AChE) = 0.24 mg mL⁻¹. *w* (ET): 17 ng mL⁻¹ (**3**); 34 ng mL⁻¹ (**4**); 68 ng mL⁻¹ (**5**); 102 ng mL⁻¹ (**6**); *w* (*p*-Ph) = 0.3 %
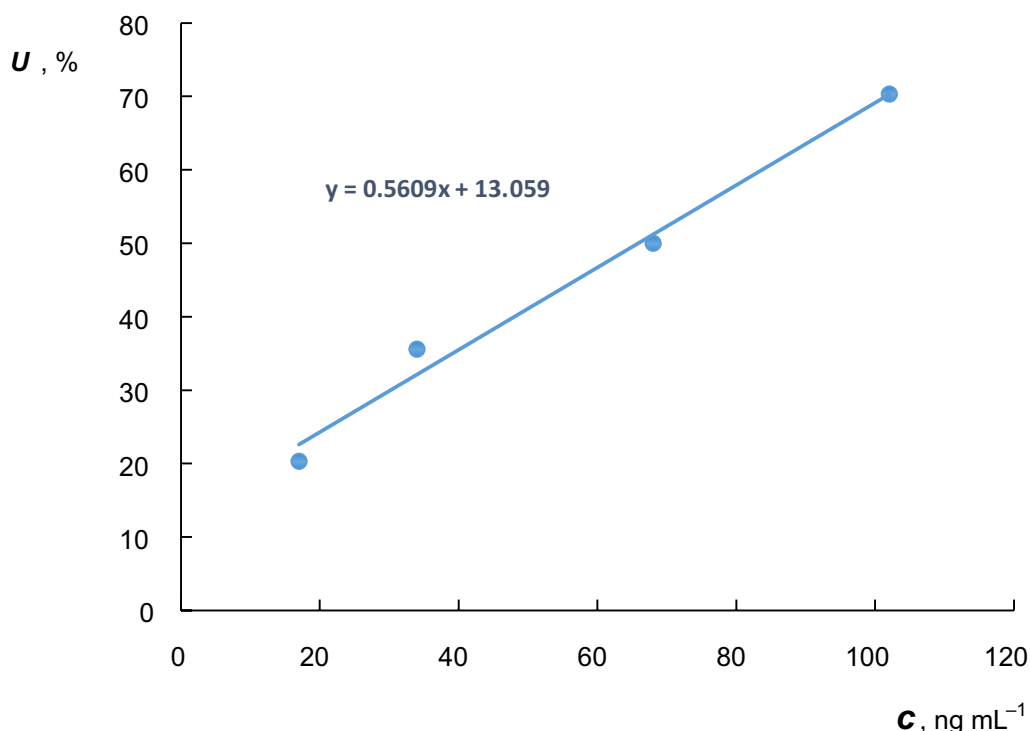
Figure 3. The calibration graph of the dependence of the inhibition degree on the concentration of ethonium in the mixture analyzed [ACh + (ChE + ET)] determined by the indicator reaction of *p*-Ph oxidation by hydrogen peroxide in the presence of residual ACh

2.5 mL of the gel solution, and 0.5 mL of the ChE solution were successively added to a 20 mL graduated test tube, the mixture was thoroughly shaken and incubated at +38 °C for 10 min. After that, 1.0 mL of the ACh solution was added, and the content was thoroughly shaken and incubated for another 10 min at +38 °C. Further, 1.6 mL of the hydrogen peroxide solution was added, and the test tube was incubated again at +38 °C for 10 min. After that, 1.0 mL of the *p*-Ph solution was added, diluted to the volume with double-distilled water, and the optical density of the solution was measured at 358 nm in a 1 cm cuvette with the aid of a spectrophotometer for 15 min. According to the plot of the dependence of optical density on time (kinetic curve), the tangent of the slope angle 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 and another was performed without the use of cholinesterase

enzyme. Both of the experiments were guided by the procedure for constructing the kinetic curves described in the previous section. As a result, the 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:

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

where 0.00059574 – is the mass of ethonium contained 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 (Inh) – is the relative reaction rate in the working experiment with the sample solution of the drug studied, min⁻¹;

*tg*α (ACh + ChE) – is the relative reaction rate in the absence of the inhibitor, min⁻¹;

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

The substance analyzed	ET found ($\bar{x} \pm \Delta x$), %[a]	*RSD*, %	The quality certificate data, %	Accuracy (δ, %)[b]
Ethonium gel 0.5 % (APTEKA PAVLOVA Ltd, Odesa, Ukraine)	0.446 ± 0.015	2.7	0.445	+ 0.23
Ethonium solution 0.1 % (prepared *ex tempore*)	0.101 ± 0.003	2.5	0.100	+1.00

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

$tg\alpha$ (ACh) – is the relative reaction rate (the tangent of the slope angle of the kinetic curve), in the working experiment without the use of the inhibitor and cholinesterase, min^{-1};

For the quantitative determination of the inhibitor in a 0.1% ethonium solution, 1.0 g (accurate weight) of the ethonium substance was dissolved in 1000 mL of double-distilled water. Next, the analysis was performed similarly to the procedure for the 0.5% gel formulation.

The results of the quantitative determination of ethonium in a 0.5% ethonium gel and a 0.1% solution according to the kinetic-spectrophotometric enzyme method proposed are presented in **Table**. They indicate that the relative standard deviation $(RSD) \leq 2.7\%$, with the correctness $(\delta) = +0.23 - 1.00\%$, $\delta < RSD$.

■ Conclusions

Thus, a new enzymatic kinetic-spectrophotometric method for determining the quaternary ammonium surface-active substance – ethonium in a gel preparation has been developed. The approach is based on the inhibition of the cholinesterase enzyme activity assessed by the residual acetylcholine substrate using the indicator reaction of *p*-phenetidine oxidation. The relative standard deviation of the procedure does not exceed 2.7%. The limit of quantitation is 17 ng mL^{-1}.

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