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Development of an Enzymatic Method for the Determination of Decamethoxine in Liquid Dosage Forms

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ABSTRACT

The Covid-19 pandemic has triggered scientists around the world to focus on the development of antiviral agents and the impovement of methods for their analysis. Decamethoxine (DM) is a quaternary ammonium compound. Its mechanism of action is to disrupt the permeability of the cytoplasmic membrane of bacteria and fungi by binding to the phosphatide groups of membrane lipids. In addition, DM is capable of inhibiting the enzyme acetylcholinesterase (AChE). Based on this property, we have developed, a new simple and sensitive enzymatic kinetic-spectrophotometric method for quantitative determination of DM in ear and eye drops, as well as in antiseptic formulations,. Changes in the activity of AChE alters the amount of unreacted acetylcholine (ACh) in the enzymatic hydrolysis reaction. Addition of hydrogen peroxide to the unreacted ACh leads to the formation of an equivalent amount of peracetic acid. Peracetic acid can react with p-phenetidine giving a colored product, allowing the quantitative estimation of peracetic acid and subsequently the amount of ACh. The method involves determination of the absorption of the product at λmax = 358 nm (logε = 4.2) over time. The rates of biochemical reactions are calulated from the the slopes of the linear portions of the kinetic curves in experiments with the inhibitor, as well as in two additional control experiments in the absence of the inhibitor, (control 1), and in the absence of the AChE (control 2), The degree of inhibition (U, %) is calculated from these reaction rates. The dependence of the U, % on the concentration of the inhibitor is used as a calibration curve. Linearity (r=0.996) is maintained in the range 1 to 6 ng/ml. The LOQ, evaluated as the concentration corresponding to 20% inhibition, is 1,0 ng/mL. The potential of quantitative determination of DM in factory-produced pharmaceuticals in the presence of various excipients has been demonstrated.

1. Introduction

The development of modern analytical methods that are rapid, cost-effective, and accurate, while meeting the principles of green chemistry, remains a pressing challenge in cmodern analytical chemistry. The relevance of such research is closely determined by the choice of the subject.

Decamethoxine (**DM)**, (1,10-decanediamine, N,N,N',N'-tetramethyl-N,N-bis((2-methyl-5-(1-methylethyl)cyclohexyl)methyl)-, dichloride) is a synthetic antiseptic and antifungal drug for local use. It is a bisquaternary ammonium compound. In terms of chemical structure and antimicrobial action, it is close to etonium (Figure 1).

Decamethoxine is used for purulent and fungal lesions of the skin (abscesses, purulent wounds, candidiasis, etc.), for proctitis, gingivitis, periodontitis, tonsillitis, otitis, and other purulent processes. It is used for lung diseases endobronchially and for conjunctivitis and blepharoconjunctivitis in ophthalmology, in the form of eye drops. Decamethoxine is also used for sterilization of medical instruments, devices, suture material, rubber gloves and for chemical sterilization and preservation of bone-tendon grafts also.[1-3].

Decamethoxine is available in several formulations: 0.2 mg tablets to dissolve in the mouth, 0 0.2% aqueous solution as eye drops and as the ear drops in the form of 0.05% alcohol solution in 5 and 10 mL[4-5].

"Dekasan"® is a 0.02% solution of decamethoxine for inhalation. This medicine has a powerful antibacterial, antiviral and fungicidal action. It has an antispasmodic, anti-inflammatory and desensitizing effect also. Decamrtoxine is an indispensable component of the complex treatment of infectious diseases of the respiratory tract in patients of all age groups. It shows no toxic effect on the human body. Decamethoxine was found to exert a pronounced virucidal effect on the SARS-CoV-2 virus (coronavirus), which causes the coronavirus disease COVID-19, decreasing the the viral load on the surfaces of the mucous membrane of the respiratory tract[6]

These data have recently been included in the instructions for medical use of the drug Dekasan.

In medicinal preparations, **DM** is usually contained in relatively small quantities and is part of multicomponent medicinal forms [7]. This creates certain difficulties during its quantitative determination. At present, the method of acid-base titration in a non-aqueous environment is used for the quantitative analysis of the **DM**[8-12].

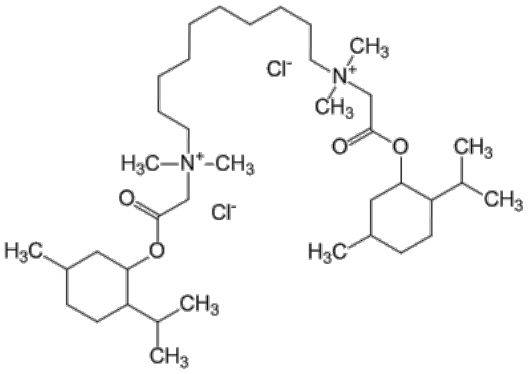


Figure1. *Structural formula of Decamethoxine*

Furthermore, the photocolorimetry method is also used for the analysis of medicinal forms of **DM**[13], extraction photometry[14], as well as spectrophotometry in the form of a **DM** complex with eosin at 540 nm[9,10, 15-18]. However, the known method of photometric determination of DM using eosin Y does not allow obtaining correct results, since $\delta < RSD$[19].

A film decamethoxine selective electrode is described in the literature[20], as well as solid-contact ion-selective electrode (ISE) for ionometric determination of decamethoxine[21]. Additionally, a method for the detection and quantitative determination of dental formulations containing decamethoxine and lidocaine hydrochloride in gel form has been described using HPLC[22].

A review article has been published on the analytical methods for the quantitative determination of decamethoxine in biomedical research. It discusses from a comparative aspect, the advantages and disadvantages of known analytical methods for the quantitative determination of quaternary ammonium compounds, in particular decamethoxine in pharmaceutical formulations[19]. It is shown that a very promising method for the pharmaceutical analysis of dosage forms of quaternary ammonium compounds is the kinetic-spectrophotometric meth-

od based on the inhibition of the biochemical reaction of acetylcholine decomposition by the enzyme cholinesterase. Optimal conditions of the method were developed in our laboratory[23] and its potential ton be applied for quantitative determination of decamethoxine, in model solutions[24] and biological material[25] using the oxidation reaction of *p*-phenetidine as an indicator.

In this article, we present the results of the methodology for the quantitative determination of decamethoxine in commercially manufactured pharmaceutical preparations in the presence of various excipients.

2. Experimental part

Materials and Methods

1). The chemical name of the antiseptic drug «Dekamethoxine®» is 1,10-Decamethylene-bis (N,N-dimethylmenthoxycarbonylmethyl) - ammonium dichloride. CAS: 61192-62-9. Molecular weight: 693.9112 g/mol. Research substance DM® «Research production of the Institute of Organic Chemistry of the National Academy of Sciences of Ukraine» (Ukraine). White fine crystalline powder with a weak specific smell, easily soluble in water and 96% alcohol. The pH of the studied sample of **DM** ® was found to range from 5.5 to 7.5. The sample exhibited a specific optical rotation from 48 to 51°, which corresponds to the standard of this medicinal product according to the State Pharmacopoeia of Ukraine. The melting point of **DM** ® was in the range of 163–168 °C with decomposition.

The mass loss during drying was determined out in accordance with the requirements of the Federal State of Ukraine. An amount of 0.5000 g was dried at a temperature from 100 to 105 °C to a constant mass[26].

The following products were used in the study:

«Auridexan» ear drops 0.5 mg/ mL in a 5 ml bottle with krish-drop Storage:active substance: decamethoxine; 1 mL of solution contains 0.5 mg of decamethoxine in 100%; excipient: ethanol (70%). Series No. 20222. Manufacturer: «Experimental Plant State Scientific Center for Medicines» Kharkiv, Ukraine. Limited Liability Company «Pharmaks group» (Kyiv, Ukraine). Data of Quality Certificate No. 8: «quantification» indicator of decamethoxine (from 0.45 mg to 0.55 mg decamethoxine in 1 ml of the drug) 0.540 mg/ mL.

«Oftalmodek», eye drops 0.2 mg/mL, 5 mL in a bottle. active substance: decamethoxine; Composition: 1 mL of solution contains: Decamethoxine (calculated as 100% substance) - 0.2 mg; excipients: sodium chloride, purified water. Series No. 0030222. Manufacturer: Limited Liability Company «Research Plant «State Scientific Center for Medicinal Products» (Kharkiv, Ukraine). Limited Liability Company «PHARMAKS GROUP» (Kyiv, Ukraine). Data of the Quality Certificate of the medicinal product series: «Quantitative determination» indicator of decamethoxine (tolerances: from 0.18 mg to 0.22 mg in 1 mL of the drug) 0.204 mg/ml; sodium chloride (from 8.7 mg to 9.3 mg in 1 mL of the drug) 9.2 mg/ mL.

«Dekasan»® (Decasanum) Composition: active substance: decamethoxine; 1 mL of solution contains 0.2 mg of decamethoxine; excipients: sodium chloride, water for injections. Producer «Yuriya-Pharm» (Ukraine). Pharmacotherapeutic group. Antiseptic and disinfectants. ATX code D08A. 2 ml each in a single-dose container; 12 containers in a pack. Series No.: API 501/1. Quantitative determination (from 0.190 mg/ mL to 0.210 mg/ mL) 0.195 mg/ mL.

Reagents.

The foolowing reagents were used:

1). *p*-Phenetidine (4 – ethoxyaniline, *w*= 98%) (SIGMA - ALDRICH); A0281408 series, New Jersey, USA; *p* - phenetidine hydrochloride (Ph), obtained by precipitation of the free the base by hydrogen chloride in a chloroform solution.

2). Pharmacopoeial acetylcholine chloride - 0.2 g per amp/5 mL (manufactured by "VECTOR" – State Science Center of virology and biotechnology in Russian Federation" (Russia).

3). Dry protein drug of cholinesterase from horse serum - 80 mg / fL (VI class), 27 AU / mg (manufactured by SMU "Biomed").

4). "Stabilized Hydrogen Peroxide 30-40%" (LLC

"Inter - Synthes", Boryslav, Ukraine); The content of hydrogen peroxide was determined by SPU according to the monograph "High-test hydrogen peroxide solution 27.5-31.0%[27].

Preparation of the solution of acetylcholine chloride (ACh).

To the ampoule, add 4.0 mL of water with pipette, and shake until acetylcholine is completely dissolved. Then tranfer the acetylcholine solution quantitatively into 200 ml volumetric flask and dilute to the mark with double-distilled water.

Cholinesterase solution preparation (ChE). Add 10.0 mL double - distilled water in a flask, containing 80 mg of dry cholinesterase drug, shake up and incubate at 38 °C for 10 min.

Phosphate buffer solution preparation (pH 8.35). Bring 35.75 g of disodium hydrogen phosphate in a 500 mL flask, add 300 ml double-distilled water, dissolve it, add 19 mL of 0.1 mol/L solution of hydrochloric acid, stir and dilute with double distilled water to 500.0 ml. The pH of the final solution is potentiometrically controlled.

Hydrogen peroxide solution 10%. It is prepared by diluting high-purity hydrogen peroxide with double-distilled water. The concentration of hydrogen peroxide in the solution was controlled according to the recommended permanganatometry method[27].

p-phenetidine hydrochloride solution preparation 0,5%. 0.50 g *p*-phenetidine hydrochloride is dissolved in 80 ml of double-distilled water into a 100 mL volumetric flask and diluted to the mark.

Methods

For analyzing ear drops «Auridexan» (0.5 mg/ mL) 1.00 mL of the drug solution was transferred using a pipette into a 1 liter volumetric flask and diluted with double distilled water to the mark at 20 ºC and thoroughly mixed. The resulting solution is precisely diluted 10 times with double-distilled water. The final solution is used for further analysis.

Preparation of a solution of a working standard sample (WSS) *of* Decamethoxine 50 ng/ mL.

0.0500 g of Decamethoxine powder is dissolved in 1000 mL of double distilled water. An aliquot of 10 ml of the resulting solution was transfered using a pipette and to a 1-liter volumetric flask, bring the volume up to the mark and mix thoroughly. Then, using a pipette, take 10 mL of this solution and transfer it to a 100 mL volumetric flask, bring the volume up to the mark and mix thoroughly.

For analyzing eye drops «Oftalmodek» (0.2 mg/ mL), as well as «Dekasan»® solution, 1.00 mL of the drug solution is transferred with a pipette into a 1-liter volumetric flask and diluted with double-distilled water to the mark at 20 ºC and carefully stirred. The resulting solution is precisely diluted 4 times with double-distilled water. The final solution is used for further analysis.

Preparation of a solution of a working standard sample (WSS) *of* Decamethoxine 20 (μg/ mL. 0.0400 g of Decamethoxin powder is dissolved in 2000.00 mL of double-distilled water. Take an aliquot of 10 mL of the resulting solution using a pipette and transfer it to a 1-liter volumetric flask, bring the volume up to the mark and mix thoroughly. Then, using a pipette, take 10 mL of this solution and transfer it to a 100 mL flask, bring the volume up to the mark and mix thoroughly.

1). Control experiment: 10.00 mL of a buffer solution with a pH of 8.35, 2.00 mL of a decamethoxine sample solution and 0.50 mL of an acetylcholinesterase solution are successively added to a graduated test tube with a polished cork, thoroughly shaken and kept at 38ºC for 10 min. After that, add 1.00 mL of acetylcholine solution, shake thoroughly and incubate again for 10 min at 38ºC; then add 1.60 mL of hydrogen peroxide solution and incubate again for 10 min at 38ºC, after which add 1.00 mL of *p*-Ph solution and 2.00 mL of distilled water, stopper the test tube, shake the solution thoroughly and record the increase in the optical density of the solution at 358 nm in a cuvette by 1 cm on a spectrophotometer for 15 min (control 1). From the plot of optical density versus time (kinetic curve), the tangent of the linear portion tg α (X) was found, in min^{-1}.

Add 10.00 mL of a buffer solution with a pH of 8.35, 0.50 mL of distilled water, 0.50 mL of acetyl-

cholinesterase solution and 1.00 mL of acetylcholine solution to another test tube with a polished cork, stopper the test tube, shake thoroughly and keep at +38ºC within 10 min. Next, add 1.60 mL of hydrogen peroxide solution and shake thoroughly, and then thermostat for 10 min at 38ºC, after which add 1.00 mL of p-phenetidine solution and 2.0 mL of distilled water, stopper the test tube, shake the solution and immediately measure the growth optical density of the solution for 15 min (control 2). From the plot of optical density on time (kinetic curve), the tangent of the linear portion tg α (2) was found, in min^{-1}.

In parallel, a working standard solution sample (WSS) of decamethoxine was analysed in the same way used for the test sample of decamethoxine.

The tangent of the angle of the slope of the linear portion was determined from the plot of optical density versus time [Fig. 2] (kinetic curve) tg α (st), y min^{-1} (Working experience with WSS) «Ach+ChE+Inh-Standard»).

The content of decamethoxine, *X*, mg/mL is calculated according to the formula:

$$X = \frac{Cst\ [tg\alpha\ (X) - tg\alpha\ (2)]}{tg\alpha\ (St) - tg\alpha\ (2)\}} \times k,$$

C_{st} is concentration of decamethoxine WSS solution, ng/mL.

k is dilution factor (for «Auridexan» ear drops 0.5 mg/ mL k=10000; for «Oftalmodek», eye drops 0.2 mg/ mL, and «Dekasan»® solution k=4000).

Equipment.

A single-beam spectrophotometer SF-26 LOMO) was used to measure light absorption with 1 cm quartz cuvettes.

The solutions were thermostated using an air thermostat TS-80M-2.

pH value was measured using an Ionomer I - 160M laboratory (Belarus) with an EGL 43-07 glass pH electrode together and an auxiliary EAL-1M3.1 type silver chloride electrode, saturated with potassium chloride.

Analysis procedure.

All solutions are thermostated for 20 min in an air thermostat at 38ºC before the analysis.

Calibration curves.

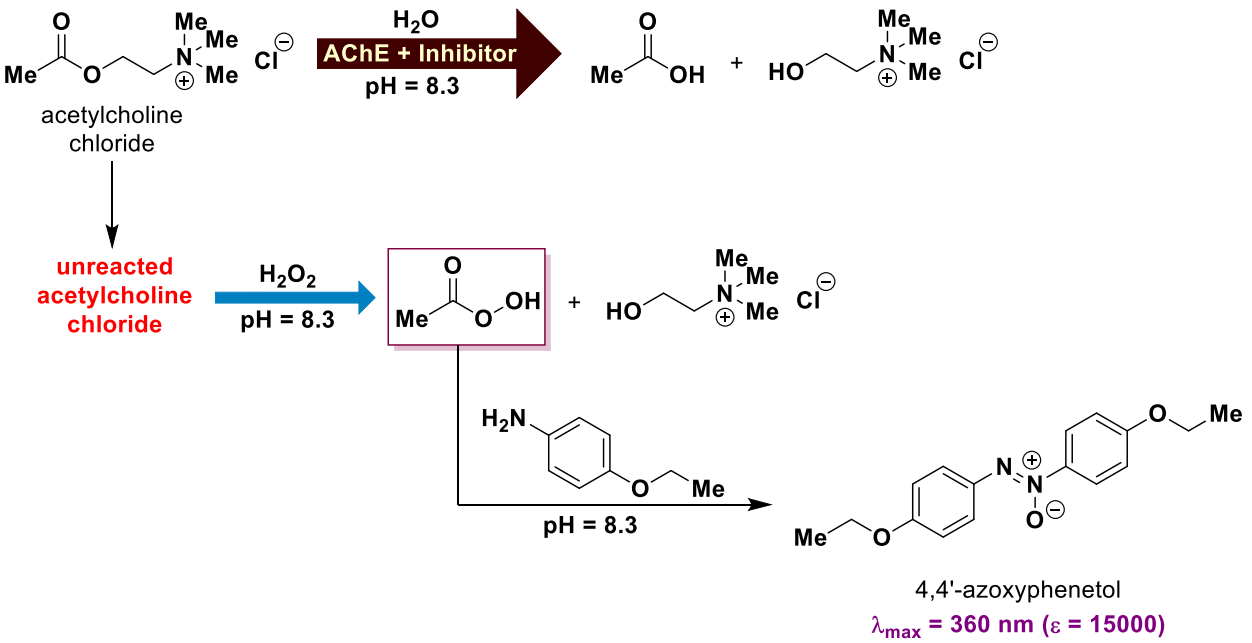
Preparation of a working standard sample solution (WSS) *of* DM $1 \cdot 10^{-7}$ mol/L. 0.0694 g of Decamethoxin powder is dissolved in 1000.00 mL of double distilled water. Take an aliquot of 10 mL of the resulting solution using a pipette and transfer it to a 1-liter volumetric flask, bring the volume up to the mark and mix thoroughly. Then, using a pipette, take 10 mL of this solution and transfer it to a 100 mL flask, bring the volume up to the mark and mix thoroughly.

1) Working experiments (Ach+ChE+Inh).

10.00 mL of a buffer solution with a pH of 8.35, were placed in three test-tubers. Then 0.50 mL of the working standard solution of decamethoxine (WSS) was added to the 1-st test-tube, 1.00 mL– to the second, and 1.50 mL to the third. Then 0.50 mL of an acetylcholinesterase solution was transferred to each test tube, shaken thoroughly and kept at 38ºC for 10 min. Then, the 1.00 mL acetylcholine's solution was added, shaken thoroughly and incubated again for 10 min at 38ºC; then 1.60 mL of hydrogen peroxide solution was added and incubated again for 10 min at 38ºC. After that, 1.00 mL of *p*-Phenetidin's solution was added to each test tube. 2.00, 1.50, 0.50 mL of distilled water were added to the 1-st, 2-nd, 3d test tube, The tubes were stoppered and the solutions thoroughly shaken. The increase in optical density was recorded at 358 nm in a 1 cm cuvette for 15 min. The tangents of the slope of the linear section tg α (X) in min^{-1} was determined from the plot of optical density versus time.

2) *Control experiment with Ach 1*.

Control experiment with Ach 11 is carried out similarly to Working experiments (Ach+ChE+Inh) but without adding the inhibitor and ChE to the test tube. Volumes are adjusted to the mark with a larg-



Scheme 1. *Method of the quantitative determination of quaternary ammonium compounds*

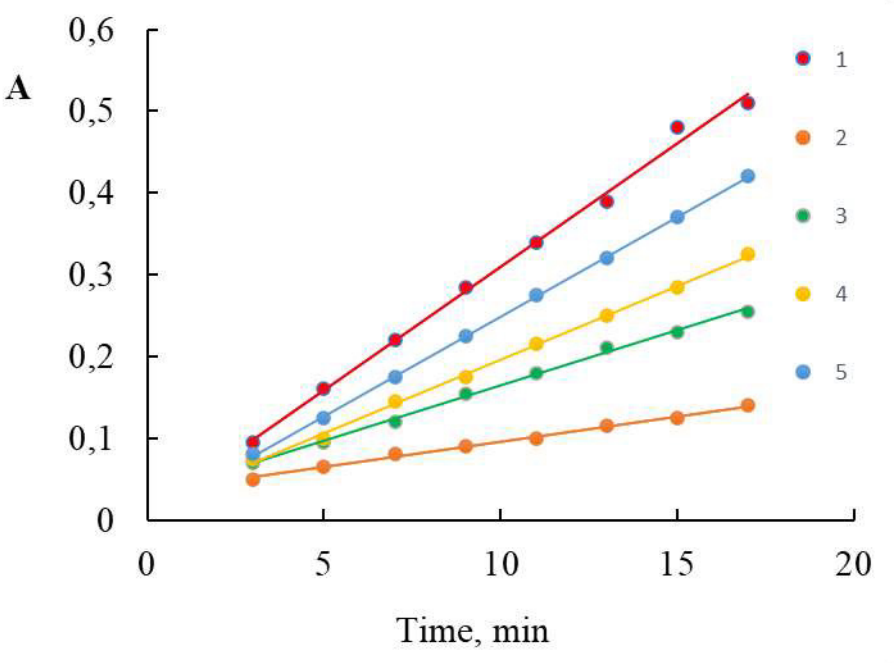


Figure 2. *Kinetic curves of the conjugated oxidation of p-phenetidine with hydrogen peroxide in the presence of: Ach (1), mixtures Ach+ChE (2) and Ach+ChE+Inh (3-5). c (Ach)=3.3·10^{-4} mol L^{-1}); w(H_2O_2)=1%; w(AchE)= 0.24 mg/ mL; C(DM): 2.0 ng/ mL (3); 4.0 ng/ mL (4); 6.0 ng/ mL (5); w (p-phen) = 0.03%.*

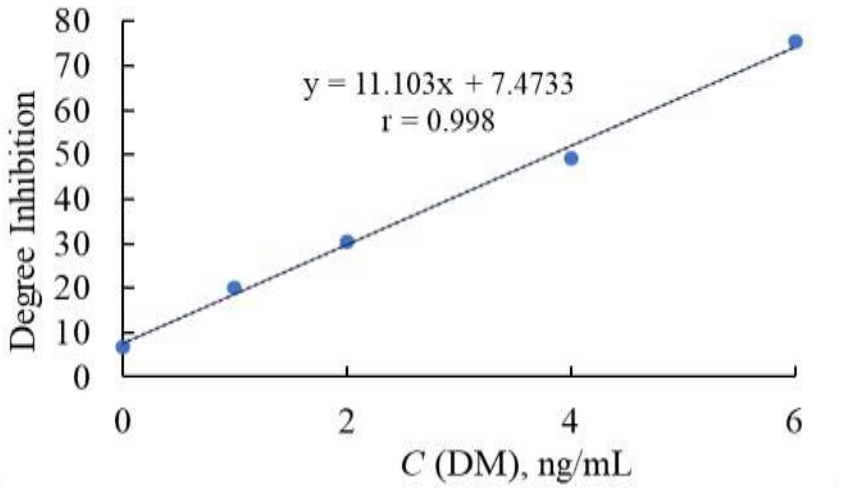


Figure 3. *Dependence of the degree of inhibition on the inhibitor concentration (DM) in the ACh-(ChE+Inh) system*

er amount of double-distilled water. From the plot of optical density versus time (kinetic curve), the tangent of the slope of the linear portion tg α (1) was determined, in min^{-1}.

3). *Control experiment 2* (*Ach+ChE*).

Control experiment 2 is carried out similarly to Working experiments (Ach+ChE+Inh) but without adding the inhibitor) to the test tube. Volumes are adjusted to the mark of test-tube with a larger amount of double-distilled water.

3. Results and Discussion

DM is a quaternary ammonium compound, which is capable of inhibiting the enzyme acetylcholinesterase (AChE). In turn, a change in activity of AChE causes a change in the amount of unreacted acetylcholine (ACh) in the enzymatic hydrolysis reaction (Scheme 1). Addition of hydrogen peroxide to the unreacted ACh leads to the formation of equivalent amount of peracetic acid. The latter can react with *p*-phenetidine giving a coloured product which makes it possible to estimate the quantitative content of peracetic acid and subsequently amount of ACh. The method involves photometric detection of the coloured product at λ_{max} = 358 нм ($\log_{10}$ ε =4.2)[21].

Figure 2 shows the 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 (3-5).

Based on the linear portions of the kinetic curves, the tangents of the slope angles were calculated, representing the conditional values of the reaction rates, and thus a graded dependence of the degree of inhibition on the final concentration of decamethosine (an inhibitor of the cholinesterase enzyme in the biochemical reaction of the decomposition of acetylcholine) was constructed (Figure 3). The degree of inhibition was calculated according to the formula:

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

where: *tg*α (X-*Inh*) is conditional reaction rate in the working experiment in the Ach– (ChE+Inh) – H_2O_2 – *p*-Phen system at C_i concentration of the inhibitor (DM), min^{-1};

*tg*α (Ach) is conditional reaction rate in the absence of an inhibitor (DM) and cholinesterase enzyme in system Ach – H_2O_2 – *p*-Phen (*control experiment* 1), min^{-1};

*tg*α (Ach+ChE) is conditional reaction rate in the absence of an inhibitor (DM) in the system Ach – ChE –H_2O_2 – *p*-Phen (*control experiment 2*), min^{-1};

Table 1 Characteristics of linear regression equation curve calibration graph

Characteristics	**Parameters**
Y= bx+a	y = 11.103x + 7.47
Correlation coefficient (r)	0.996
Linear regression equation	lnh = 11.103 C + 7.47
Slope (b±Δb)	11.103±1.325
Intercept (a±Δa)	7.47±4.47
S.D. of slope (Sb)	0.4164
S.D. of intercept (Sa)	1.406
LOD (3 Sa/b), ng/mL	0.380
LOQ = 10 Sa/b	1.266

Note: S_a- standard deviation of the intercept of regression line; S_b, standard deviation of the slope of regression line.

Table 2. Results from the analysis of dosage forms of Decamethoxine by the kinetic-spectrophotometric enzyme method

(*n* = 5; *P* = 0.95)

Detected substance/ - analyzed drug	Found ($\overline{x} \pm \Delta\overline{x}$), mg/ml	RSD, %	Data of Quality Certificate: "quantification" (μ *)	δ= $\frac{(\overline{x} - \mu)}{\mu *} 100$ (%)
Decamethoxine / – "**Auridexan**" Auridexan ear drops 0.5 mg/ml bottle 5 mL, No. 1; Experimental Plant State Scientific Center for Medicines/Harkiv, Ukraine	0.521±0.014	2.17	0.540	– 1.10
Decamethoxine / "**Oftalmodek**", eye drops 0.2 mg/ mL, 5 mL in a bottle. Limited Liability Company "Research Plant" «State Scientific Center for Medicines/Harkiv, Ukraine	0.202±0.006	2.39	0.204	– 0.98
Decamethoxine / "**Dekasan**"® 0.2 mg/mL (Produced "Yuriya-Pharm" (Ukraine).	0.198±0.006	2.52	0.195	+1.59

* Data of the official method specified in the Certificate, μ".

As can be seen from figure 3, the linear dependence of the degree of inhibition on the concentration of the inhibitor in the solution is achieved in the range of 1-6 ng/ mL.

The characteristics of the calibration curve of the linear regression equation were as follows: *U*,%= (11.103±1.325)× *C* (DM) + (7.47±4.47) (Table 1).

The LOQ, defined as the concentration corresponding to 20% inhibition, is 1.0 ng/mL

The obtained research results are the basis for the quantitative determination of decamethoxine in pharmaceutical preparations using the standard method.

The results of the analysis of dosage forms of Decamethoxine according to the proposed method by the kinetic-spectrophotometric enzyme method are given in table 2. The relative standard deviation does not exceed 2.5% when accuracy (δ) – 1.1...+1.6%. $\delta = \left| \left(\overline{XX} - \mu \right) 100\% / \mu \right| < t_{\alpha} \times RSD / \sqrt{n}\sqrt{n}$. ($n$ = 5; P = 0.95).

4. Conclusions

Highly sensitive methods have been developed and the potential of quantitative determination of decamethoxine in ear and eye drops, as well as in antiseptic preparation has been shown.

The relative standard deviation did not exceed 2.5%. The *LOQ* is 1.0 ng/mL, evaluated as the concentration corresponding to 20% inhibition. The potential of quantitative determination of DM in commercial pharmaceuticals in presence of various excipients was demonstrated. The accuracy of the obtained results was assessed by comparing the average found ($\overline{X}$) $\overline{X}$) with that of the independent recommended method (μ).

The developed method is improved in regard to sensitivity and selectivity, compared other photometric methods. At the same time, the developed method does not require expensive equipment, specially trained personnel and complies with the principles of "green chemistry", since all substances are used in small quantities, do not require additional specific disposal and thet are not carcinogenic, while of the reagents are natural substrates

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