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Application of the enzymatic method for the quantitative determination of dequalinium chloride in lozenges

Abstract

Aim. To develop a new kinetic spectrophotometric enzymatic method suitable for the quantitative determination of dequalinium chloride in lozenges.

Materials and methods. An enzymatic kinetic spectrophotometric method for the quantitative determination of dequalinium chloride in lozenges has been proposed. It is based on the ability of dequalinium chloride to inhibit the enzymatic hydrolysis reaction of acetylcholine by cholinesterase in the presence of the acetylcholine (ACh) excess and H_2O_2. The degree of inhibition was determined by the kinetic method using two conjugated reactions: ACh perhydrolysis (interaction with an excess of hydrogen peroxide) followed by oxidation with the peroxyacid formed. Peracetic acid formed *in situ* by the reaction between unreacted ACh and H_2O_2 interacts with *p*-phenetidine forming a product, which absorbs at λ_{max} = 358 nm, in the phosphate buffer solution with pH 8.3 at room temperature.

Results and discussion. The linear dependence of the calibration graph for the quantitative determination of dequalinium chloride was in the concentration range of 0.2–0.8 μg mL^{-1} (r = 0.999). LOD and LOQ were 0.01×10^{-6} and 0.03×10^{-6} mol L^{-1}, respectively. For the quantitative determination of dequalinium chloride in lozenges, RSD ≤ 2.65 % (accuracy, δ = -1.10...+1.78 %).

Conclusions. A new enzymatic kinetic spectrophotometric method has been developed, and its applicability to the quantitative determination of dequalinium chloride in lozenges has been shown. It does not require a complicated treatment of the analyte and a tedious extraction procedure. The method proposed is sensitive enough to determine a small amount of the active pharmaceutical ingredient. These advantages encourage the application of the method proposed in routine quality control of the drugs studied in analytical laboratories.

Keywords: dequalinium chloride; cholinesterase; acetylcholine; quantification

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Застосування ензимного методу для кількісного визначення декваліній хлориду в пігулках для розсмоктування

Анотація

Мета. Розробити новий ензимний кінетико-спектрофотометричний метод, придатний для кількісного визначення декваліній хлориду в пігулках для розсмоктування.

Матеріали та методи. Було запропоновано ензимний кінетико-спектрофотометричний метод кількісного визначення декваліній хлориду в пігулках для розсмоктування. Метод заснований на здатності декваліній хлориду інгібувати реакцію ензимного гідролізу ацетилхоліну холінестеразою з наступним визначенням ступеня інгібування кінетичним методом з використанням двох спряжених реакцій: пергідролізу ацетилхоліну (реакція з надлишком гідроген пероксиду) та наступного пероксикислотного окиснення. Пероцтова кислота, утворена *in situ* в результаті реакції між H_2O_2 та непрореагованим ACh, реагує з *n*-фенетидином за кімнатної температури у фосфатному буферному розчині з pH 8,3 з утворенням продукту з максимумом світлопоглинання λ_{max} = 358 нм.

Результати та їх обговорення. Лінійна залежність градуювального графіка для кількісного визначення декваліній хлориду зберігається в інтервалі концентрацій 0,2–0,8 мкг $мл^{-1}$ (r = 0,999). LOD та LOQ були $0{,}01 \times 10^{-6}$ та $0{,}03 \times 10^{-6}$ моль $л^{-1}$ відповідно. За кількісного визначення декваліній хлориду в пігулках для розсмоктування RSD ≤ 2.65 % (точність, δ = -1.10...+1.78 %).

Висновки. Розроблено новий ензимний кінетико-спектрофотометричний метод, який легко можна застосувати до визначення декваліній хлориду в пігулках для розсмоктування. Він не вимагає складного обробляння аналіту та виснажливої

процедури екстрагування. Запропонований метод є достатньо чутливим, щоб дозволити визначати малу кількість активного фармацевтичного інгредієнта. Ці переваги спонукають до застосування запропонованого методу в рутинному контролі якості досліджуваних препаратів у аналітичних лабораторіях.

Ключові слова: декваліній хлорид; холінестераза; ацетилхолін; кількісне визначення

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■ Introduction

For more than 60 years dequalinium chloride (DQ) has been used as an anti-infective drug, mainly to treat local infections. Nowadays, it is a common ingredient of sore-throat lozenges. As a lipophilic *bis*-quaternary ammonium salt (Figure 1) the drug exhibits membrane effects and selectively targets mitochondria to deplete DNA and block energy production in cells. Beyond its mitochondriotropic property, DQ can interfere with the correct functioning of diverse proteins. A dozen of DQ protein targets have been identified; their implication for the antibacterial, antiviral, antifungal, antiparasitic, and anticancer properties of the drug has been discussed [1]. The clinical efficacy and potential attractiveness of this substance have been confirmed by numerous publications [2–5]. Today, the Ukrainian pharmaceutical industry produces a number of local antiseptics with DQ, which are used to treat diseases of the upper respiratory tract. Thus, the question of choosing the method of analysis for lozenges with DQ is relevant.

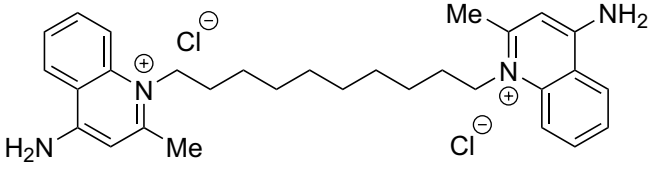
To quantify DQ, the European Pharmacopoeia proposes the method of non-aqueous titration. Acetic anhydride and formic acid are used as solvents, 0.1 M perchloric acid solution is applied as a titrant, and the end point of the titration is recorded potentiometrically [6]. The British Pharmacopoeia also recommends non-aqueous titration to quantify DQ with the perchloric acid solution in a mixture of dioxane/glacial acetic acid with addition of mercury(II) acetate, and crystalline violet is used as an indicator [7]. Additionally, the method of high-performance liquid chromatography is also widely used in the analysis of DQ [8–11]. Several works proposed the method of spectrophotometry for the quantitative determination of DQ [12–14]. All the methods mentioned above have certain advantages; however, they require specific equipment and trained staff.

Thereby, the aim of the current study was to develop a simple, fast, sensitive and affordable method for the quantitative analysis of DQ, particularly in lozenges for treating throat diseases. Previously, we developed a simple, fast, sensitive kinetic-enzymatic spectrophotometric method for the determination of another quaternary ammonium salt – benzalkonium chloride in various dosage forms [15].

■ Materials and methods

Reagents and equipment

Dequalinium chloride (pp. 1410001632, OLON, Italy) with the active substance content of 94.8% was used in the work.



INN: Dequalinium dichloride
Chemical name: 1,1'-(decane-1,10-diyl)bis(4-amino-2-methylquinolin-1-ium)
Molecular weight: 527.6

Figure 1. The structure of dequalinium chloride

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

Acetylcholine chloride, 0.2 g of the substance in ampoules, was of pharmaceutical grade (the State Science Center of Virology and Biotechnology "Vector", Russia).

Disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$), puriss. p.a. ("ReaChem", Kharkiv, Ukraine), and a dry cholinesterase from horse serum (SMU "Biomed", Russia), 80 mg (VI class, activity 22 AU mg^{-1}), were used in the study. The catalytic activity of 1 activity unit (AU) is manifested by such an amount of this enzyme preparation that converts 1 μmol of the substrate in 1 min under given reaction conditions.

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

High purity double-distilled water was used during our experiments.

"Amilar IC" – orange-flavored lozenges. Active ingredients (in 1 tablet) are: dequalinium chloride, 0.25 mg, dibucaine hydrochloride, 0.03 mg, manufactured by "InterChem SLC" (Ukraine), batch number s.02501118.

"Dekvadol" – white tablets. Active ingredients (in 1 tablet) are: dequalinium chloride, 0.25 mg, dibucaine hydrochloride, 0.03 mg, manufactured by JSC "Kyiv Vitamin Plant" (Ukraine), batch number VM 491219.

"Lizak" – grey to brown tablets. Active ingredients (in 1 tablet) are: dequalinium chloride, 0.25 mg, lysozyme hydrochloride, 10 mg, manufactured by JSC "Farmak", Ukraine, batch number s. 150920.

The absorbance measurements were performed on a colorimeter (CFC-2) (Zagorsky optical and mechanical plant, Russia) using quartz cells with a width of 1 cm.

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

Preparation of 0.2 M phosphate buffer solution (pH 8.3)

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

Preparation of 10% hydrogen peroxide solution

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

Preparation of 1% *p*-phenetidine hydrochloride solution

1.00 g of *p*-phenetidine hydrochloride was dissolved in 80 mL of double-distilled water in a 100 mL volumetric flask and diluted to the volume with the same solvent.

Preparation of cholinesterase (ChE) solution

In a flask containing a dry powder of cholinesterase (80 mg) double-distilled water (10 mL) was added, and the flask was moved to a thermostat for 10 min at a temperature of 37–40°C.

Preparation of acetylcholine chloride (ACh) solution

The ampoule content (0.2 g of ACh) was dissolved in 200 mL of double-distilled water. For this purpose, an ampoule was opened, 4.0 mL of water was pipetted and added to the ampoule and then shaken until ACh was completely dissolved. Then the ACh solution was transferred into a 200 mL volumetric flask and diluted to the volume with double-distilled water [17].

Preparation of the stock solution

0.05277 g (accurate weight) of the DQ substance (the active substance content $w = 94.8\%$) was dissolved in double-distilled water in a 100.0 mL volumetric flask, and the solution was diluted to the volume with the same solvent (with the concentration of 0.5 mg mL^{-1}) at 20°C. The content of the flask was mixed thoroughly.

10.00 mL of the initial solution was pipetted into a 100.0 mL volumetric flask, and the solution was diluted to the volume with double-distilled water at 20°C. The content of the flask was mixed thoroughly (with the concentration of 50 μg mL^{-1}).

The general procedure for the quantitative determination

The first part: 10.0 mL of 0.2 M phosphate buffer solution (pH = 8.3) was transferred into a 20 mL graduated test tube with a ground stopper, 1% ACh solution (1.0 mL) and 10% hydrogen peroxide solution (2.0 mL) were consecutively added, and the stopwatch was started. After that, the solution was shaken thoroughly and thermostated for 10 min at a temperature of 37°C. Then 1% *p*-phenetidine hydrochloride solution (1.0 mL) was added to the test tube, and

the solution was diluted to the volume with double-distilled water. The stopwatch was started again, and the solution was scanned photometrically every minute over the period of 15 min on a photoelectric colorimeter, the color filter No.2 (λ_{max} = 358 nm) and a 1.0 cm cuvette were used. The solution containing only 0.2 M phosphate buffer (10.0 mL, pH = 8.3) was used as a reference solution. The relative rate of the reaction [(ACh + H_2O_2) + *p*-Ph] ($tg\alpha_{V_{max}}$, min^{-1}) was determined as a slope of the the optical density (*A*) *vs* time (*t*, min) kinetic curve.

The second part: 0.2 M phosphate buffer solution (10.0 mL, pH = 8.3), 1% ACh solution (1.0 mL) and the ChE solution (2.0 mL) were transferred into a 20 mL graduated test tube with a ground stopper. After that, the solution was shaken thoroughly and thermostated for 10 min at a temperature of 37°C. Then 10% hydrogen peroxide solution (2.0 mL) was introduced into the test tube while stirring. The mixture was shaken thoroughly and kept for 10 min in a thermostat at the same temperature. Then 1% *p*-phenetidine hydrochloride solution (1.0 mL) was added, and the solution was diluted to the volume with double-distilled water. The stopwatch was started, and every minute the solution was scanned photometrically over the period of 15 min on a photoelectric colorimeter; the color filter No. 2 (λ_{max} = 358 nm) and a 1.0 cm cuvette were used. The solution containing only 0.2 M phosphate buffer (10.0 mL, pH = 8.3) was used as a reference solution. The relative rate of the reaction [(ChE + ACh) + H_2O_2 + *p*-Ph] ($tg\alpha_{min}$, min^{-1}) was determined as a slope of the optical (*A*) *vs* time (*t*, min) kinetic curve.

The third part: 0.2 M phosphate buffer solution (10.0 mL, pH = 8.3) was transferred into a 20 mL graduated test tube with a ground stopper. The accurate volumes of the working standard solutions of DQ or test solutions of the drugs (see below) were added into the test tube. The ChE solution (2.0 mL) was added while stirring, a stopwatch was started, and every solution was shaken thoroughly and thermostated at 37°C for 10 min. Then 1% acetylcholine solution (1.0 mL) was quickly added to the test tube, the stopwatch was started, and the mixture was shaken thoroughly and thermostated for 10 min again. After that, 10% hydrogen peroxide solution (2.0 mL) was added, the mixture was kept in the thermostat for 10 min; further 1% *p*-phenetidine hydrochloride solution (1.0 mL) was added, and the solution was diluted to the volume with double-distilled water. The stopwatch was started, and the solution was scanned photometrically on a photoelectric colorimeter every minute over a period of 15 min; the color filter No. 2 (λ_{max} = 358 nm) and a 1.0 cm cuvette were used. The solution containing 0.2 M phosphate buffer (10.0 mL, pH = 8.3) was used as a reference solution. The relative rate of the reaction [[(ChE + DQ) + ACh] +H_2O_2 + *p*-Ph] ($tg\alpha_{c_i}$, min^{-1}) was determined as a slope of the optical density (*A*) *vs* time (*t*, min) kinetic curve.

The calibration graph procedure

Working standard solutions 1-7 used for calibration

4.00, 6.00, 8.00, 10.00, 12.00, 14.00, 16.00 mL of the stock solution were pipetted into a series of 50.0 mL volumetric flasks, and the solutions were diluted to the volume with double-distilled water at 20°C (final concentrations were 4.0; 6.0; 8.0; 10.0; 12.0; 14.0; 16.0 μg mL^{-1}, respectively).

The performance of the method proposed was verified on the sample concentrations of 0.2; 0.3; 0.4; 0.5; 0.6; 0.7; 0.8 μg mL^{-1} according to the *General procedure*.

The inhibition degree of the enzymatic hydrolysis of acetylcholine (*U*, %), in the presence of DQ was calculated by the following formula:

$$U(\%) = \frac{tg\alpha_{c_i} - tg\alpha_{min}}{tg\alpha_{V_{max}} - tg\alpha_{min}} \times 100\ \%$$

where $tg\alpha_{V_{max}}$ (min^{-1}) – is the relative rate of the *p*-phenetidine oxidation reaction with peroxyacetic acid formed in the reaction of ACh perhydrolysis (in the absence of ChE and DQ);
$tg\alpha_{min}$ (min^{-1}) – is the relative rate of the *p*-phenetidine oxidation reaction with peroxyacetic acid formed in the reaction of perhydrolysis of unreacted ACh (in the presence of ChE and in the absence of DQ);
$tg\alpha_{c_i}$ (min^{-1}) – is the relative rate of the *p*-phenetidine oxidation reaction with peroxyacetic acid formed in the reaction of perhydrolysis of unreacted ACh (in the presence of ChE and DQ with the concentration c_i).

■ Results and discussion

Parameters that can affect the performance of the method proposed were studied to reach the optimum working conditions and reagent concentrations [18].

The measurement velocity of changing the absorption *vs* time ($\Delta A/\Delta t$, min^{-1}) made it possible

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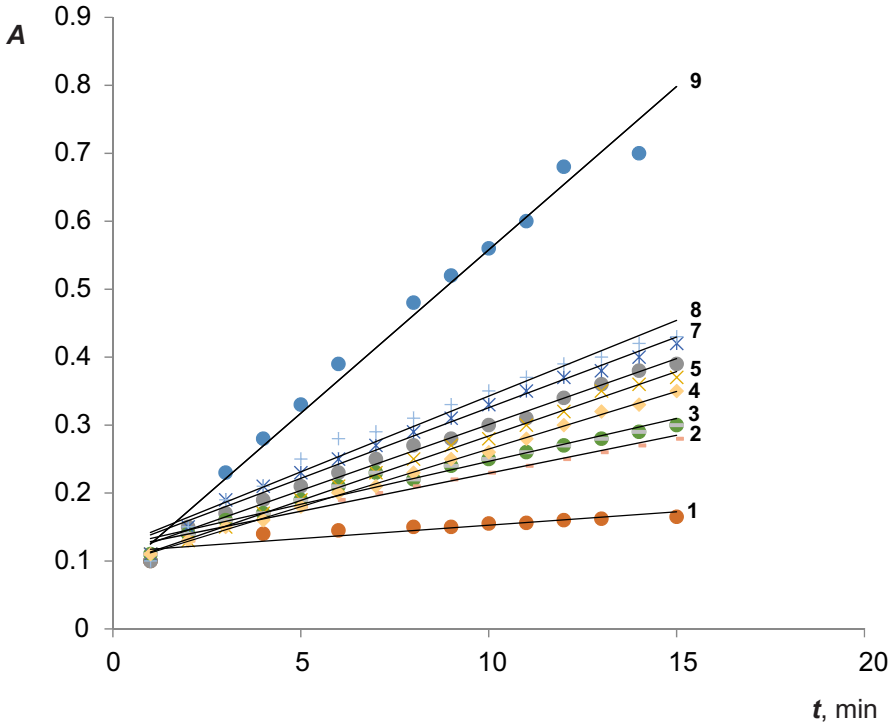

Figure 2. Kinetic curves of *p*-phenetidine oxidation by hydrogen peroxide in the presence of the system: 1 – ACh + ChE, 2–8 –ACh + (ChE + DQ), 9 – ACh; *w* (ACh) = 1%; ChE = 22 AU; *c*(DQ), μg mL^{-1}: 2 – 0.2, 3 – 0.3, 4 – 0.4, 5 – 0.5, 6 – 0.6, 7 – 0.7, 8 – 0.8

Table 1. Metrological characteristics of the linear dependence of the inhibition degree of the enzymatic hydrolysis of acetylcholine (*U*, %) on the concentration of the dequalinium chloride solution

Linearity parameters	$U = b_1X + a_1$	$Y = b_2X + a_2$
b	97.1×10^6	1.45
S_b	0.9×10^6	0.01
a	-14.1	-0.42
S_a	±0.5	1.50
R	0.99997	0.9997
RSD_0	0.49	1.47%
LOD	0.01×10^{-6} mol L^{-1}	–
LOQ	0.03×10^{-6} mol L^{-1}	–

to quantify DQ in the substance. Figure 2 shows the kinetic curves of the reaction of *p*-phenetidine oxidation by hydrogen peroxide in the presence of different concentrations of DQ. They have a linear character at the initial stage; it allows us to use the slope (tgα) of kinetic lines as the value of an analytic signal that corresponds to a certain content DQ in the sample.

The range of application of this procedure was 0.2–0.8 mg mL^{-1}. The calibration graph (Figure 3) was constructed in the coordinates of the concentration (mol L^{-1}) *vs* the inhibition degree (%).

According to the data obtained on the inhibition degree of ChE by various concentrations of DQ, the calibration dependence was constructed in absolute and normalized coordinates (normalization was performed according to the nominal

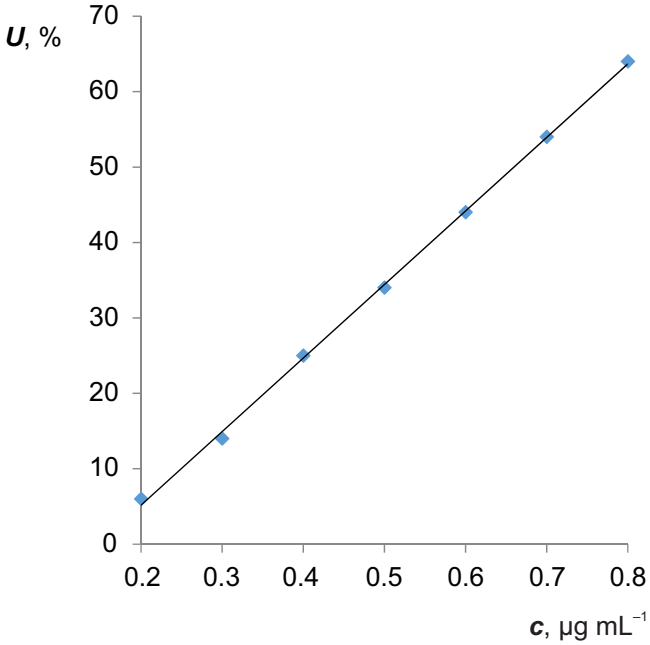

Figure 3. The calibration graph of the dependence of the inhibition degree of the enzymatic hydrolysis of acetylcholine (*U*, %) on the concentration of the dequalinium chloride solution

content in the drug): $U = b_1X + a_1$ and $Y = b_2X + a_2$, respectively (Tables 1 and 2). The least squares method was used to calculate the metrological characteristics of the calibration dependences obtained (angular coefficient *b* and its standard deviation S_b, free term *a*, and its standard deviation S_a), as well as correlation coefficient (R) and residual standard deviation RSD_0 (Table 1).

Table 2. The results of the analysis of the working standard solutions and their statistical processing

The actual concentration of DQ in the solutions (C_{crit} = 0.5 mg mL^{-1})		Degree of inhibition ChE U_i, %	Found (%) to $U_{reference}$ Y_i, % ($U_{reference}$ = 33.5%)	The calculated concentration of DQ in the solutions X_i^{calc}, %	RR_i, % = $\frac{X_i^{calc}}{X_i} \times 100$ %
C_i, mg mL^{-1}	X_i, %				
0.2	40	5.02	14.99	39.31	98.26
0.3	60	15.32	45.73	60.51	100.85
0.4	80	24.15	72.09	78.69	98.36
0.5	100	33.95	101.34	98.87	98.87
0.6	120	45.21	134.96	122.05	101.71
0.7	140	52.65	157.16	137.37	98.12
0.8	160	62.54	186.69	157.73	98.58
				$\overline{RR}$, %	99.25
				δ, % = |100 – $\overline{RR}$|	0.75
				δ, % ≤ 0.32 · $max\Delta_x$ = 0.96%	satisfied
				RSD_{RR}, %	1.43
				Δ_{RR}, % = RSD_{RR} · t(95%, n – 1)	2.76
				Δ_{RR}, % ≤ $max\Delta_x$ = 3.05%	satisfied

Table 3. The results of the quantitative determination of DQ in lozenges by the method proposed

Drug	Manufacturer, pharmaceutical factory	The content of DQ (mg)	DQ Found, ($\overline{X}$ ± ΔX), mg[a]	RSD, %	Accuracy (δ, %)[b]
Amilar IC	Ukraine, ALC "Interhem" s. 02501118	0.2422	0.246 ± 0.0081	2.65	+1.56
Dekvadol	Ukraine, PC "Kyiv Vitamin Plant", VM 491219	0.2513	0.2518 ± 0.0030	1.17	-1.10
Lizak	Ukraine, PC "Farmak" s. 150920	0.258	0.2534 ± 0.0058	1.86	+1.78

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

From Table 1 one can conclude that the parameters of the calibration linear dependence constructed in normalized coordinates are characterized by satisfactory linearity for the given range of concentrations.

The recommended procedure for the analysis of "Amilar IC", "Dekvadol" and "Lizak" drugs

Twenty (20) tablets of each "Amilar IC", "Dekvadol" and "Lizak" drugs were crushed to homogeneity, and 2.5097 g (accurate weight) of this powder was dissolved in double-distilled water in a 100 mL volumetric flask. The solution was filtered, the filter was washed, and diluted to the volume with double-distilled water. The analysis was performed according to the *General procedure*.

The quantitative content (x) of dequalinium chloride ($C_{30}H_{40}Cl_2N_2$) in one tablet (lozenge) was calculated by the following equation:

$$x\ (g) = \frac{m_{st} \times w\% \times U_{sample} \times 100 \times 20 \times \overline{m}_{tabl}}{m_s \times U_{st} \times 100\ \%}$$

where m_{st} – is the mass of the standard sample of the DQ substance, 0.05277 g;

w – is the active substance content in the working standard solutions of DQ, %;

U_{sample} – is the cholinesterase inhibition degree by the working sample, %;

U_{st} – is the cholinesterase inhibition degree by the standard sample, %;

$\overline{m}_{tabl}$ – is the average mass of the drug working samples, g;

100, 10, 20 – is dilution coefficients.

The degree of inhibition (U_{sample} and U_{st} in %) was calculated according to the procedure described in the literature [19].

The data obtained and presented in Table 3 indicate the possibility to use the method proposed for the quantitative determination of DQ in lozenges.

■ Conclusions

A new enzymatic kinetic spectrophotometric method has been developed, and the possibility of the quantitative determination of dequalinium chloride in lozenges has been shown. The linearity range for the quantification of dequalinium chloride by the enzymatic method was 0.2–0.8 μg mL^{-1}; RSD ≤ 2.65% (accuracy, δ = –1.10…+1.78%); LOQ = 0.03×10^{-6} mol L^{-1}.

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