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The enzymatic method for the quantitative determination of benzalkonium chloride in the antiseptic solution "CUTASEPT® F"

Abstract

Aim. To develop an alternative method for the quantitative determination of the benzalkonium chloride content as an active pharmaceutical ingredient in the disinfectant solution "CUTASEPT® F".

Materials and methods. The method is based on the ability of benzalkonium chloride to inhibit the enzymatic hydrolysis of acetylcholine by acetylcholinesterase. The reaction rate is assessed by the non-hydrolyzed acetylcholine residue, which is determined by the amount of peracetic acid produced during the interaction with the excess of the hydrogen peroxide solution. The indicator reaction is the interaction of *p*-phenetidine with peracetic acid that leads to the formation of 4,4'-azoxyphenetole with λ_{max} = 358 nm ($\log_{10} \varepsilon$ = 4.2).

Results and discussion. As a result of the research conducted the linear dependence of the degree of inhibition of the enzymatic hydrolysis of acetylcholine (*U*, %) on the concentration of benzalkonium chloride was determined in the concentration range of (0.5 – 7.0) × 10^{-6} mol L^{-1} with the correlation coefficient of 0.999. The limit of quantitation was 1.9 × 10^{-6} mol L^{-1}.

Conclusions. As a result of the research conducted the kinetic enzymatic method for the quantitative determination of benzalkonium chloride has been developed by its inhibitory effect in the biochemical reaction of acetylcholine hydrolysis. This method is fast, cheap and easy to perform, does not require expensive equipment, and available for use in the field.

Keywords: benzalkonium chloride; CUTASEPT® F; cholinesterase; acetylcholine

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Ензимний метод кількісного визначення бензалконій хлориду в антисептичному розчині «КУТАСЕПТ® Ф»

Анотація

Мета. Розробити альтернативний метод кількісного визначення вмісту бензалконій хлориду як активного фармацевтичного інгредієнта в дезінфекційному розчині «КУТАСЕПТ® Ф».

Матеріали та методи. Метод заснований на здатності бензалконій хлориду пригнічувати гідроліз ацетилхоліну за посередництва ензиму холінестерази. Швидкість реакції оцінюють за залишком негідролізованого ацетилхоліну, який визначають за кількістю пероцтової кислоти, утвореної в результаті взаємодії ацетилхоліну та надлишку гідроген пероксиду. Індикаторною реакцією є взаємодія *п*-фенетидину з пероцтовою кислотою, що призводить до утворення 4,4'-азоксифенетолу з λ_{max} = 358 нм ($\log_{10} \varepsilon$ = 4,2).

Результати та їх обговорення. У результаті проведених досліджень отримано лінійну залежність ступеня інгібування швидкості ензимного гідролізу ацетилхоліну (*U*,%) від концентрації бензалконій хлориду в межах концентрацій (0,5–7,0) × 10^{-6} моль $л^{-1}$ з коефіцієнтом кореляції 0,999. Межа кількісного визначення становила 1,9 × 10^{-6} моль $л^{-1}$.

Висновки. У результаті проведених досліджень розроблено кінетико-спектрофотометричну методику кількісного визначення бензалконій хлориду за його інгібіторною дією в біохімічній реакції гідролізу ацетилхоліну. Цей метод швидкий, дешевий та простий у виконанні, не потребує коштовного обладнання, доступний для використання в «польових» умовах.

Ключові слова: бензалконій хлорид; КУТАСЕПТ® Ф; холінестераза; ацетилхолін

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■ Introduction

A rapid spread of coronavirus infections (SARS-CoV-2) in the world stimulated mass efforts by governments, local authorities and healthcare institutions on conducting disinfection campaigns of state and public institutions [1]. The World Health Organization recommended the extensive use of disinfectants and antiseptics. Therefore, an effective drug "CUTASEPT® F" (BODE Chemie GmbH, Germany) has become especially widespread. It is based on alkyldimethylbenzylammonium chloride. "CUTASEPT® F" according to methodical recommendations [2] is a high-performance fast-acting solution with the prolonged action, it guarantees a long-term protection of a patient at a long-term operation, does not irritate the skin, provides degreasing and cleaning, promotes effective fixing of a film after drying.

A disinfectant and at the same time an antiseptic "CUTASEPT® F" was designed for the pre- and postoperative treatment of the skin and surgical sutures; disinfection of the patients' skin before invasive procedures: injections, punctures, blood sampling; disinfection of the skin with minor injuries; hygienic and surgical disinfection of the skin of the hands of the medical staff of treatment and prevention institutions in emergency situations; rapid disinfection of surfaces, medical devices, including tools and gloves in emergency situations.

The drug has the bactericidal, tuberculocidal, fungicidal and virucidal action. This makes it especially popular during coronavirus infection (SARS-CoV-2) in the world. "CUTASEPT® F" is active against coronaviruses in a concentration of ≤ 1% when exposed to less than for 1 min [2, 3].

According to the guidelines, the quantitative content of benzalkonium chloride is determined by the method of titration with sodium tetraphenylborate in the aqueous medium at an approximate pH value of 3.4. The potentiometric determination of the titration endpoint is used [2, 4]. The disadvantage of this method is that even though the amalgam electrode can withstand many hundreds of titrations, but, if necessary, one needs to repeat the amalgamation [2].

As an alternative to the existing method of quantifying the content and in addition to the valuable chromatographic methods described in the literature [5–11] we have proposed a relatively cheap, affordable, fast kinetic photometric method for determining the quantitative content of benzalkonium chloride in the disinfectant solution "CUTASEPT® F".

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

■ Materials and methods

Reagents and equipment

Benzalkonium chloride (BAC), 50% water solution (Akzo Nobele, Surface Chemistry AS, Sweden) is a mixture of benzyl(dodecyl)dimethyl ammonium chloride (approx. 65%) and benzyl(tetradecyl)dimethyl ammonium chloride (approx. 35%).

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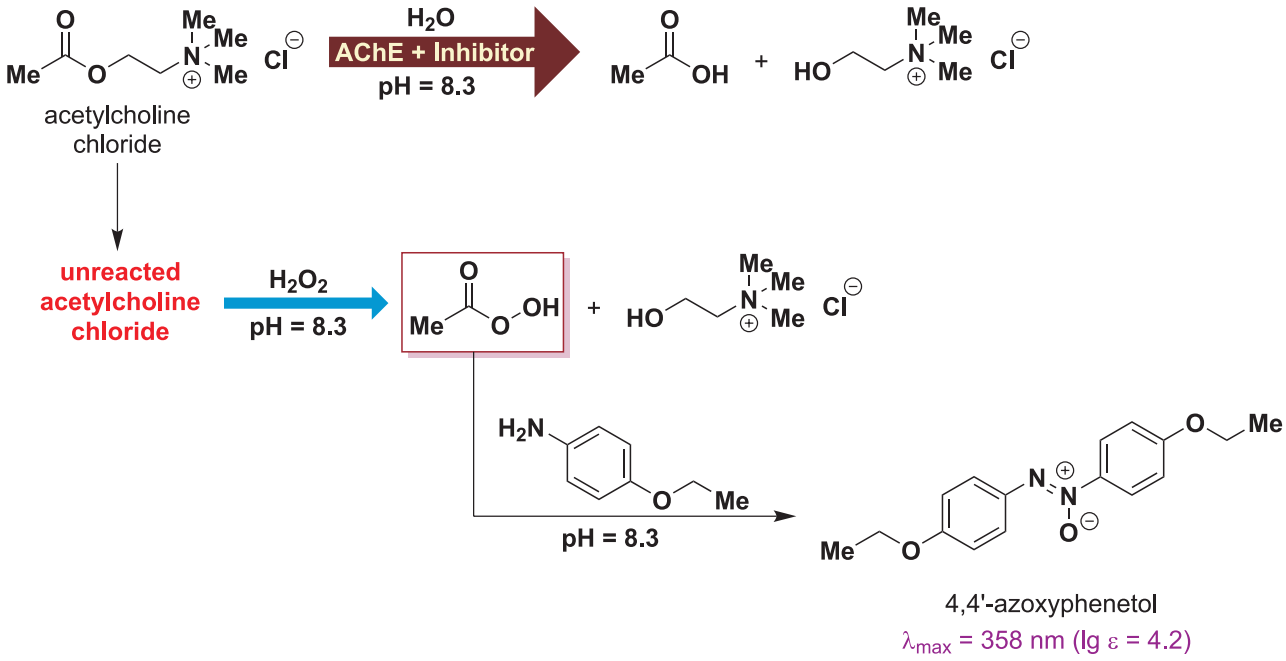

Scheme. The method of the quantitative determination of benzalkonium chloride

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 an ampoule, is of pharmaceutical grade (State Science Center of Virology and Biotechnology "Vector", Russia).

"CUTASEPT® F", 50 mL (BODE Chemie GmbH, Humburg) is an antiseptic and disinfectant solution. The composition of the drug, *wt.*%, is as follows: 2-propanol – 60.0–66.0; benzalkonium chloride – 0.020–0.030; dyes – orange-yellow S (E110), quinolone yellow (E104), diamond black BN (E151); water – up to 100.0 mL.

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 were applied. The catalytic activity of 1 activity unit (AU) is manifested by such an amount of this enzyme preparation that converts 1 μmole of this 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 according to the State Pharmacopoeia of Ukraine [12].

High purity double distilled water was used during our experiments.

The absorbance measurements were performed on a colorimeter (CFC-2) (Zagorsky Optical and Mechanical plant, Russia) using quartz cells with the 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 the test solution

4.00 mL of "CUTASEPT® F" was transferred into a 1000.00 mL volumetric glass flask, and double-distilled water (10 mL) was added, the content was then mixed and diluted to the volume with the same solvent. The concentration was 2.8×10^{-5} mol L^{-1}.

Preparation of the work standard solution

The work standard solution was prepared using double-distilled water. 0.09910 g of the BAC standard solution (50.0%) was quantitatively transferred into a 500.00 mL volumetric flask and diluted to the volume with water. 1.00 mL of this solution was transferred into a 10 mL volumetric flask and was diluted to the volume with water.

Preparation of 0.2 M phosphate buffer solution (pH 8.35)

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

Preparation of 10% hydrogen peroxide solution

The solution was prepared by dilution of hydrogen peroxide with the required amount of double-distilled water. The content of hydrogen peroxide

in 10% work solution was determined permanganatometrically [12].

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, the flask was transferred to a thermostat for 10 min at a temperature of 37–40°C.

Preparation of acetylcholine chloride solution (ACh)

The ampoule content (0.2 g of ACh) was dissolved in 20 mL of double-distilled water. An 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 ACh solution was transferred into a 200 mL volumetric flask and diluted to the volume with double-distilled water.

The general procedure for the quantitative determination of benzalkonium chloride in the antiseptic solution "CUTASEPT® F"

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. Then 1% *p*-phenetidine hydrochloride solution (1.0 mL) was added to the test tube and 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; a color filter No. 2 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 rate of the reaction [(ACh + H_2O_2) + *p*-Ph] (tg$\alpha_{V_{max}}$, min^{-1}) was determined as a slope of the kinetic curve optical density (*A*) *vs* time (*t*, min).

The second 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. After that, the cholinesterase solution (2.0 mL) was added followed by 1.0 mL of 1% acetylcholine solution. The mixture was shaken thoroughly and thermostated for 10 min. 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. Then 1% *p*-phenetidine hydrochloride solution (1.0 mL) was added and diluted to the volume with double-distilled water. The stopwatch was started, and every minute the solution was scanned photometrically for 15 min on a photoelectric colorimeter; a color filter No. 2 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 rate of the reaction [(ChE + ACh) + H_2O_2 + *p*-Ph] (tgα_{min}, min^{-1}). was determined as a slope of the kinetic curve optical density (*A*) *vs* time (*t*, min).

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 test solution of BAC were added to the test tube. The cholinesterase solution (2.0 mL) was added while stirring, a stopwatch was started; each solution was shaken thoroughly and thermostated for 10 min. Then 1% acetylcholine solution (1.0 mL) was quickly added, and a stopwatch was started, 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, and then 1% *p*-phenetidine hydrochloride solution (1.0 mL) was added and diluted to the volume with double-distilled water. The stopwatch was started, and the solution was scanned photometrically on a photoelectric colorimeter every minute for 15 min; a color filter No. 2 and a 1.0 cm cuvette were used. Solution containing 0.2 M phosphate buffer (10.0 mL, pH = 8.3) was used as a reference solution. The rate of the reaction [[(ChE + BAC) + ACh] + H_2O_2 + *p*-Ph] (tgα_{c_i}, min^{-1}) was determined as a slope of the kinetic curve optical density (*A*) *vs* time (*t*, min).

The calibration graph procedure

The performance of the method proposed was verified on samples containing from 1.00 mL to 5.00 mL of the BAC work standard solution according to the *General procedure*.

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

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

where tg$\alpha_{V_{max}}$ (min^{-1}) – is the rate of the *p*-phenetidine oxidation reaction with peroxyacetic acid

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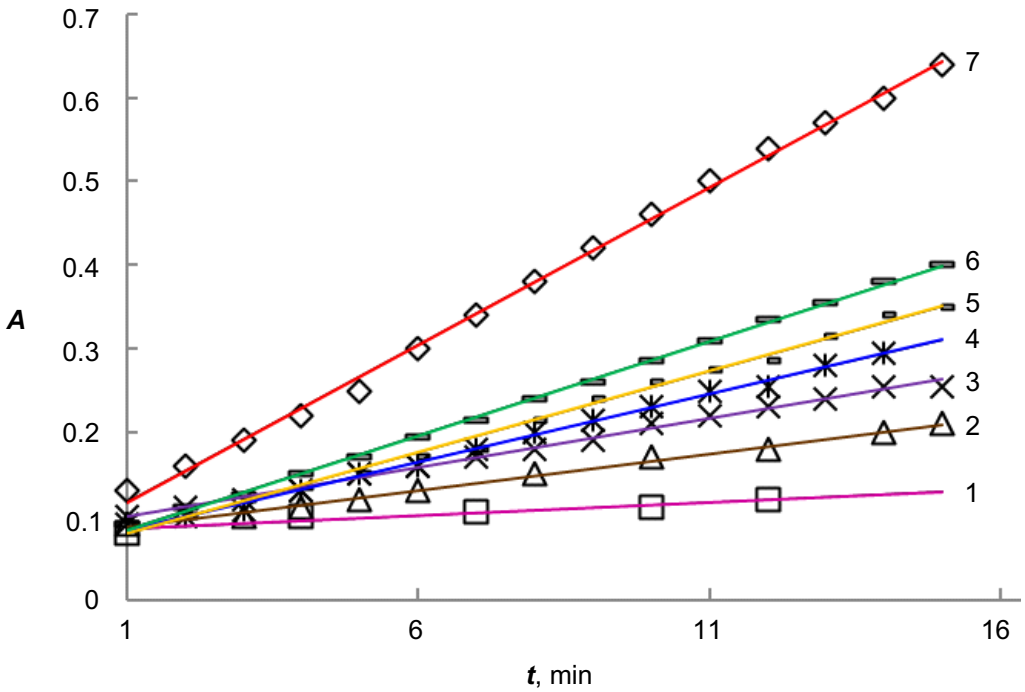

Figure 1. Kinetic curves of *p*-phenetidine oxidation by hydrogen peroxide in the presence of the system: 1 – ACh + ChE, 2–6 – ACh + (ChE + BAC), 7 – ACh; *w*(ACh) = 0.1 %; ChE = 22 AU/mg; *c*(BAC), 10^{-6} mol L^{-1}: 2 – 1.4, 3 – 2.8, 4 – 3.4, 5 – 5.6, 6 – 7.0

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

■ Results and discussion

Parameters affecting the performance of the method proposed were studied to reach the optimum working conditions and reagent concentrations [13]. Once the optimum working conditions were determined, we evaluated the enzymatic kinetic photometric method with respect to its linearity, limit of determination (LOD), accuracy, and precision.

Kinetic curves (Figure 1) of the indicator reaction of *p*-phenetidine oxidation by hydrogen peroxide in the presence of the systems mentioned above (ACh *or* ACh + ChE *or* ACh + (ChE + BAC)) were linear within the first 15 minutes of the optical density measurements.

This enables the use of angular coefficient of slope ($tg\alpha$, min^{-1}) of the derived kinetic lines, built in the optical density (*A*) *vs* time (*t*, min) coordinates, as a value of the analytical signal corresponding to the certain content of the inhibitor in a sample for assessing the reaction rate.

The calibration graph was constructed using the average of 5 values of the degree of inhibition of the enzymatic hydrolysis of acetylcholine (*U*,%) corresponded to a definite concentration of the BAC solution (Figure 2). The equation of the calibration dependence was found to be $U(\%) = 0.7434 \times 10^7 c + 5.54$ (where *c* is the BAC concentration, mol L^{-1}; $b = 0.7434 \times 10^7$; $a = 5.54$ mol L^{-1}).

The calibration curve was linear within the concentration range of $(0.5–7.0) \times 10^{-6}$ mol L^{-1} of BAC with a correlation coefficient of 0.999, LOD was 1.9×10^{-6} mol L^{-1}.

Table 1. The assessment of accuracy and precision of the BAC concentration determination in the standard solutions

BAC Taken	BAC Found[a]	*RSD*, %	Recovery, %	δ, %
$c \times 10^{-6}$, mol L^{-1}				
2.80	2.83 ± 0.10	2.95	101.0	+1.07
4.20	4.22 ± 0.08	1.57	100.5	+0.48
5.60	5.65 ± 0.06	0.86	100.9	+0.89
7.00	7.05 ± 0.60	0.71	100.7	+0.71

Note: [a] $n = 5$; $P = 0.95$ %

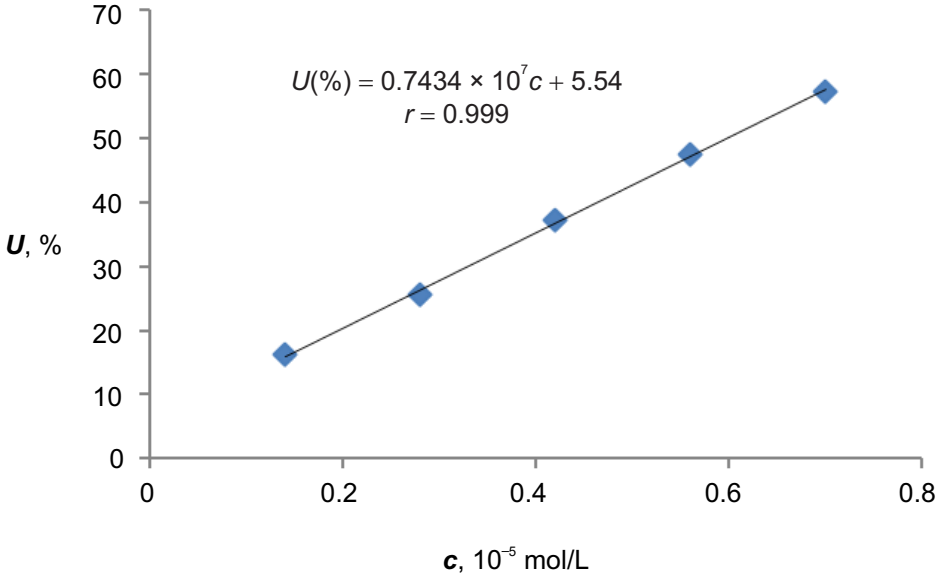

Figure 2. The calibration graph of the dependence of the degree of inhibition of the enzymatic hydrolysis of acetylcholine (*U*, %) on the concentration of the BAC solution

Table 2. The assessment of accuracy and precision of the BAC concentration determination in "CUTASEPT® F" solutions

BAC taken, $c \times 10^6$ mol L^{-1}	BAC added, $c \times 10^6$ mol L^{-1}	BAC found, $c \times 10^6$ mol L^{-1}	Found BAC added[a], $(\bar{X} \pm \Delta\bar{X}) \times 10^6$, mol L^{-1}	*RSD*, %	Recovery of BAC added, %	δ, %
2.80	–	2.81	–	–	–	–
2.80	2.80	5.72	(2.82 ± 0.10)	2.85	100.7	+0.71
2.80	4.20	7.30	(4.27 ± 0.06)	1.13	101.7	+1.67
2.80	5.60	8.35	(5.67 ± 0.08)	1.14	101.25	+1.25

Note: [a] the mean of five measurements (P = 0.95)

In order to estimate the accuracy and precision of the method proposed, BAC standard solutions with the concentrations of 2.8×10^{-6}; 4.2×10^{-6}; 5.8×10^{-6} and 7.0×10^{-6} mol L^{-1} were analyzed according to the *General procedure*. For this purpose, five replicate determinations of each concentration were carried out. In each of 4 graduated test tubes with a ground stopper 0.2 M phosphate buffer (8.0 mL) and 2.00 mL, 3.00 mL, 4.00 mL and 5.00 mL of the BAC solution, respectively, were added. The mixtures obtained were analyzed according to the *General procedure*. The results of the BAC concentration assay in the standard solutions are presented in Table 1. As one can see, the recovery percent ranged from 100.5 % to 101.0 % (δ < *RSD*), while the relative standard deviations ranged from 0.71 % to 2.95 %.

The procedure of the BAC concentration assay of "CUTASEPT® F" was as follows. 1.00 mL of "CUTASEPT® F" was transferred into a 5 mL volumetric calibrated flask and diluted to the volume with double-distilled water for the sample solutions preparation. The known volumes of the sample solution (2.00 mL) were analyzed by the kinetic-photometric method proposed (according to the *General procedure*). In each of 4 test tubes with a ground stopper 0.2 M phosphate buffer (8 mL), 2.00 mL of the "CUTASEPT® F" sample solution and 2.00 mL, 3.00 mL, 5.00 mL of the BAC work standard solution were added, respectively, one test-tube remained untouched. The solutions were analyzed according to the *General procedure*. Each test was repeated three times. The results compiled in Table 2 show that recovery was in the ran- ge of 100.7-101.25 %. It indicates that being a constituent of "CUTASEPT® F", 2-propanol did not interfere with the kinetic photometric deter-mination of BAC. Thus, the method proposed al-lows us to obtain accurate and reliable results: (δ < *RSD*) and *RSD* < 2.8 %.

■ Conclusions

The kinetic photometric method can be applied for the enzymatic determination of benzal konium chloride in the antiseptic and disinfection solution "CUTASEPT® F". The method proposed is inexpensive, rapid and sensitive. The analytical parameters, sensitivity, precision, and accuracy allow this method to be an alternative to chromatographic methods.

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