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A novel luminol-based chemiluminescence method for detecting acetylcholine

Aim. To develop a new simple non-enzymatic method for the determination of acetylcholine (ACh) by the chemiluminescent reaction of luminol under conditions of the enzymatic hydrolysis of acetylcholine (pH 8.5).

Experimental part. The method proposed is based on the perhydrolysis reaction of ACh by the excess of hydrogen peroxide with the formation of peracetic acid. The latter was further determined by the activation effect of the luminol chemiluminescent oxidation reaction in the presence of hydrogen peroxide. The analytical signal was the summary luminescence (Σ) registered within certain time.

Results and discussion. The pH range of the analytically applicable system was from 8.2 to 8.5. The effect of ACh + H₂O₂ incubation period on the reaction progress was also studied. The increase of the incubation period enhanced the sensitivity of the method (the limit of detection (LOD)), but because of practical reasons (especially the detection speed) and practical experience the incubation period was set to 30 min. The linear dependence was observed in the acetylcholine chloride concentration range of $(0.8-2.8) \times 10^{-4}$ mol/L. While determining acetylcholine chloride in the concentration range of $(1.1-2.2) \times 10^{-4}$ mol/L the relative standard deviation (RSD) did not exceed 3% ($(\bar{X} - \mu) \times 100\% / \mu = -0.5...+0.5\%$). The Limit of Quantitation (LOQ, 10S) was 7.7×10^{-5} mol/L.

Conclusions. A new non-enzymatic kinetic method for the chemiluminescent determination of ACh in aqueous solutions and the pharmaceutical formulation Acetylcholinchlorid Injeel® has been proposed. This method is simple, fast, inexpensive, and thus appropriate for the routine ACh quality control in the laboratories of hospitals, pharmaceutical industries and research institutions.

Key words: acetylcholine; chemiluminescence method

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Новий хемілюмінесцентний метод визначення ацетилхоліну з використанням люмінолу

Мета. Розробити новий простий неферментативний метод визначення ацетилхоліну (ACh) за допомогою хемілюмінесцентної реакції люмінолу за умов ферментативного гідролізу ацетилхоліну (pH 8,5).

Експериментальна частина. Запропонований спосіб засновано на реакції пергідролізу ацетилхоліну надлишком перексиду водню з утворенням пероцтової кислоти. Пероцтову кислоту далі визначали за допомогою ефекту активації реакції хемілюмінесцентного окиснення люмінолу в присутності перексиду водню. Аналітичним сигналом була сумарна люмінесценція (Σ) за певний час.

Результати та їх обговорення. Діапазон рН аналітично застосовної системи становив від 8,2 до 8,5. Додатково досліджено вплив інкубаційного періоду ACh+H₂O₂ на перебіг реакції. Збільшення інкубаційного періоду підвищило чутливість методу (межу виявлення (LOD)), але з практичних причин (особливо швидкості визначення) та практичного досвіду інкубаційний період було зафіксовано на рівні 30 хв. Лінійна залежність спостерігалась у діапазоні концентрацій ацетилхолін хлориду $(0,8-2,8) \times 10^{-4}$ моль/л. За визначення ацетилхолін хлориду в модельному розчині в межах концентрацій $(1,1-2,2) \times 10^{-4}$ моль/л відносно стандартне відхилення RSD не перевищувало 3% ($(\bar{X} - \mu) \times 100\% / \mu = -0,5...+0,5\%$). Межа кількісного визначення (LOQ, 10S) становила $7,7 \times 10^{-5}$ моль/л.

Висновки. Запропоновано новий неферментативний кінетичний метод для хемілюмінесцентного визначення ацетилхоліну у водних розчинах та фармацевтичному засобі Acetylcholinchlorid Injeel®. Цей метод є простим, швидким, недорогим і придатним для рутинного контролю якості ацетилхоліну в лабораторіях лікарень, фармацевтичної промисловості та науково-дослідних установ.

Ключові слова: ацетилхолін; хемілюмінесцентний метод

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Acetylcholine (ACh) (Fig. 1) is a neurohormone of the autonomic nervous system playing an important role in maintaining the vital functions of a living organism.

ACh was the first neurotransmitter to be characterized. It is the primary neurotransmitter in muscular synapses. In the central nervous system (CNS) it is associated with attention, learning, memory, consciousness, sleeping, and control of voluntary move-

ments [1]. Disruption of normal ACh signaling is implicated in Huntington's disease, Alzheimer's disease, schizophrenia, and Parkinson's disease [2], endarteritis, intermittent claudication, spasms of retinal arteries, intestinal and bladder atony. It is used to facilitate radiographic diagnosis of esophagus achalasia [3].

When the nerve impulse reaches the end of an axon, ACh is released transmitting the impulse further. This action of ACh is subsequently

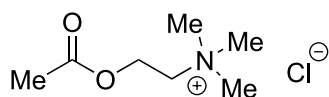


Fig. 1. Acetylcholine chloride

discontinued as a result of its hydrolysis catalyzed by the cholinesterase enzyme presented in living tissues (Scheme 1).

In order to study various disorders mentioned above and develop new medicines, it is important to measure the ACh concentration with simple, fast, inexpensive, and accurate methods.

For the quantitative determination of acetylcholine chloride the European Pharmacopoeia and US Pharmacopoeia use the method of acidimetry [4, 5]. According to the current analytical normative documentation, the content of the main substance is argentometrically determined by Folgard or LC [4]. The titrimetric procedures are highly accurate, but are not specific to ACh. A colorimetric method widely used in the practice of biological researches for determining ACh concentration was proposed by Hestrin [6].

Common procedures for ACh detection include the high-performance liquid chromatography (HPLC) method on microdialysis samples [7], electrochemical methods using potentiometric and amperometric sensors [8] and ion-sensitive field-effect transistors (ISFETs) [9].

HPLC on microdialysis samples has very high resolution in the pM range. However, this method has disadvantages, namely the tedious sample pretreatment and high cost (a microdialysis probe costs about \$200).

A simple, rapid and sensitive optical biosensor for detection of choline and ACh based on the hydrogen peroxide (H_2O_2)-sensitive quantum dots (QDs) was constructed. The detection limit for ACh was found to be $10\ \mu M$, and the linear range was $10\text{--}5000\ \mu M$. The wide linear ranges were shown to be suitable for routine analyses of choline and ACh. The detection linear range of ACh in the serum was $10\text{--}140\ \mu M$. The excellent performance of this biosensor showed that the method can be used in practice detection of choline and ACh. The performance of the device exceeds most reported ACh microsensors. However, the characteristic curve is not linear, and calibration is required for this device [10].

Ternaux and Chamoin described an enhanced chemiluminescence assay method for the determination of ACh [11]. In some methods ACh was hydrolyzed by acetylcholinesterase before the analysis [12]. Electron transfer and oxidation by hydrogen peroxide were

facilitated by immobilizing the enzyme on a redox polymer [13].

ACh is not electroactive, thus, it cannot be analyzed by electrochemical methods. Therefore, methods recently published for monitoring ACh involve either biosensor or MS detection. Biosensors were used for the direct detection of ACh or preceded by LC. A common biosensor scheme requires co-immobilization of acetylcholinesterase and choline oxidase. ACh is converted to choline, and choline is further oxidized by choline oxidase to produce hydrogen peroxide, which is detected. Since choline is a normal metabolite of ACh *in vivo*, another biosensor coated only with choline oxidase is often used together with the ACh biosensor to measure and subtract out the signal due to endogenously occurring choline. As with all biosensors or microelectrodes, major concerns are their selectivity and sensitivity in relation to a target molecule. Therefore, interfering electroactive species were excluded from ACh electrodes with permselective membranes composed of over oxidized poly(pyrrole)-poly(2-naphthol) films [14]. Carballo *et al* demonstrated liquid chromatography with the electrochemical detection (LC-EC) system using an electrode for the detection of ACh [15, 16].

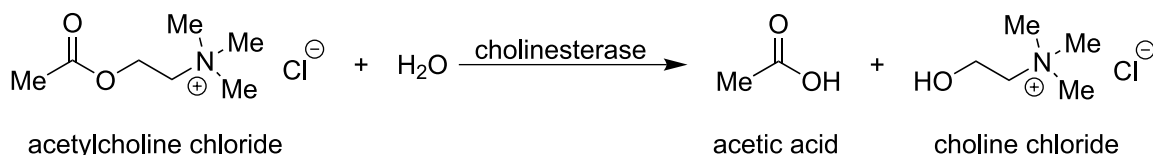
Those methods published for the determination of ACh by LC-MS in dialysate or cell culture samples sought rapid separations and a sensitive detection with the minimal ion suppression during ESI [17–19].

In some new enzymatic methods based on the use of two sequential reactions involving acetylcholinesterase and choline oxidase, the control of the content of hydrogen peroxide is carried out by the chemiluminescent method following the catalytic oxidation reaction of the chemiluminescent luminol indicator in the presence of the enzyme peroxidase [10, 12].

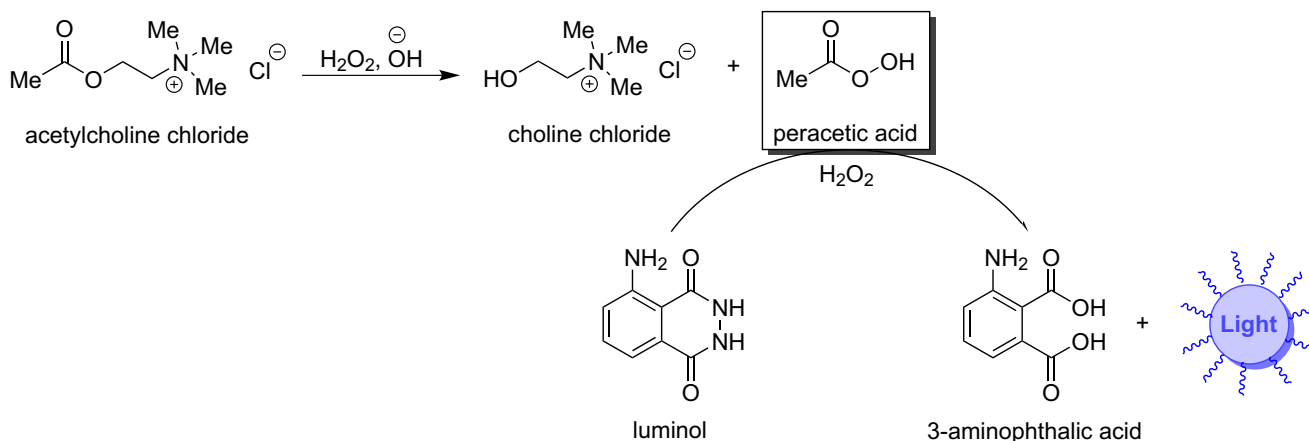
In this article a new kinetic-chemiluminescent method for the determination of acetylcholine chloride has been proposed. It is based on the application of a conjugated system of two successive reactions – ACh perhydrolysis and oxidation reaction of the chemiluminescence indicator luminol (H_2L) induced by peracetic acid formed at the first stage.

Peracetic acid formed as the result of ACh perhydrolysis reacts with luminol in the presence of hydrogen peroxide with the generation of chemiluminescence measured by the chemiluminescence method of the fixed time. The scheme of luminol oxidation is given in Scheme 2.

Based on the experimental data it was found that within the pH range of 8.2–8.5 the intensity of chemi-



Scheme 1. Acetylcholine hydrolysis



Scheme 2. The reaction scheme of ACh perhydrolysis and chemiluminescent oxidation of luminol

luminescence (emitted by oxidation of luminol with peracetic acid in the presence of hydrogen peroxide) was directly proportional to the concentration of ACh. The initial stage – ACh perhydrolysis – determined the rate of the whole process. The kinetic curves of chemiluminescence produced in the reaction of luminol with hydrogen peroxide in the presence of ACh are shown in Fig. 2.

Experimental Part

Acetylcholine chloride – 99% (Acros), argentometric titration – $\geq 98.5\%$ (on dried base); the loss on drying – $\leq 1.0\%$ (105°C, 3 h); the molecular formula – C₇H₁₆ClNO₂; the molecular weight – 181.66 g/mol.

Luminol (5-amino-2,3-dihydro-1,4-phthalazine-dione, H₂L, Sigma-Aldrich, 97%) was additionally recrystallized from a saturated solution of sodium hydroxide. All other chemicals were of analytical grade. Solutions were prepared with double distilled water produced in a quartz water distiller.

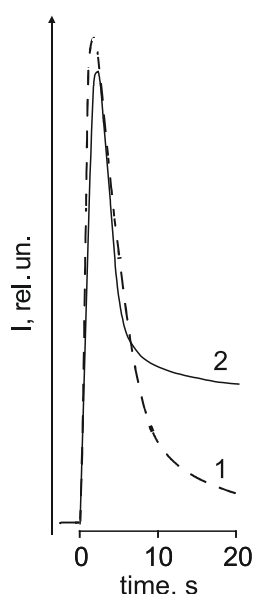


Fig. 2. The effect of ACh (ACh-luminol-H₂O₂, 2) on the intensity of chemiluminescence in the luminol-H₂O₂ (1) system for the reaction time of 20 sec, *w* (ACh): 1 – 0%; 2 – $1.5 \cdot 10^{-3}\%$; *c* (H₂L) = $5.0 \cdot 10^{-5}$ mol/L; *w* (H₂O₂) = $5.0 \cdot 10^{-2}\%$; pH 8.5

Acetylcholinchlorid Injeel® (Biologische Heilmittel Heel GmbH) – composition: 1 ampoule of 1.1 mL contains as an active ingredient acetylcholine chloratum 0.367 g (on dried base), ethanol 43% (w/w). Excipient: sodium chloride.

The stock solution of acetylcholine chloride ($2.75 \cdot 10^{-2}$ mol/L) was prepared daily and stored at 4°C. The analyte was weighed using a calibrated scale (model AG204, Mettler-Toledo, Greifensee, Switzerland).

The experiment conditions used in the research were selected based on the literature sources [21–23].

Preparation of the Working Standard Solution of acetylcholine chloride, 5.07 mg/mL. Dissolve 0.50717 g of acetylcholine chloride reference standard *ex tempore* in degassed double distilled water to make 100.00 mL of solution. Store in a high-density polyethylene or polypropylene bottle at 4°C.

The content of ACh in the solution was controlled by argentometric titration [6]. Working solutions with a smaller amount of acetylcholine chloride were prepared daily by diluting accurately the stock solution.

Preparation of phosphate buffer, pH 8.5

To 250.0 ml of 0.2 mol/L solution of disodium phosphate add 8.0 mL of 0.1 mol/L of hydrogen chloride solution. The pH control was carried out potentiometrically using a glass electrode ELS-43-07 and I-130 ionomer.

Preparation of hydrogen peroxide solution, 5% (1.5 mol/L). Dissolve 16.5 g of 30% hydrogen peroxide in water in a 100 mL volumetric flask and dilute the solution to the volume with water. The content of hydrogen peroxide in the solution was controlled by the method of permanganometry.

Preparation of 0.01 mol/L stock solution of luminol in 0.01 mol/L solution of sodium hydroxide. Dissolve 0.1772 g of luminol in 100.0 mL of 0.01 mol/L solution of sodium hydroxide. Dilute the resulting solution of luminol 10 times with double distilled water.

The chemiluminescence intensity was measured using an assembled chemiluminescence installation (Chemiluminometer-01), including a FEU-84-A

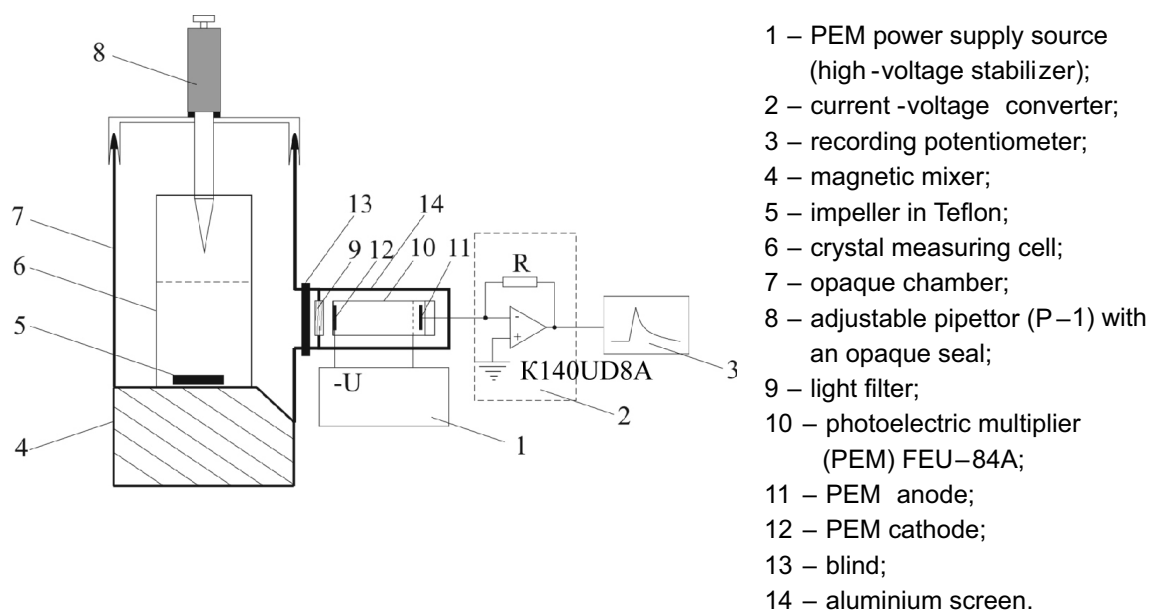


Fig. 3. The installation for measuring chemiluminescence

photoelectric-multiplier (PEM), a IMT-0.5 weak current meter and a LINE RECORDER TZ 4620 fast-operating recording potentiometer (Laboratorni pristroje, Czech Republic) (Fig. 3).

The procedure for the quantitative determination of acetylcholine by the chemiluminescence method. When studying the impact of the ACh concentration on chemiluminescence in the (ACh + buffer + H₂O₂) + H₂L system, an order of operations was as follows. A sample of the test solution of ACh (from 0.10 to 1.00 mL), 1.00 mL of 5% solution of hydrogen peroxide and (10 - Y) mL of 0.2 mol/L of the phosphate buffer solution (pH 8.5) (where «Y» is the total volume of all other components in the solution) were added into a flask with a lapped stopper, and all components were mixed thoroughly. The flask was left in the thermostat at 40°C for 15 min. Then 1.00 mL of the resulting mixture was taken and placed in a quartz cuvette, with stirring, 8.5 mL of phosphate buffer solution (pH 8.5) was added, and the cuvette was placed in a measuring cell of a chemiluminescent photometer. After that the curtain was opened and 0.5 mL of 1 × 10⁻³ mol/L of luminol solution was added to the cuvette. The total luminescence was recorded using an I-02 digital automatic integrator for 20 sec. The dependence of the chemiluminescence intensity on time (sec) was registered on the kinetic graph (Fig. 2). The experiment was repeated five times. The desired signal was the area under the curve – the integral of the chemiluminescence over time period (20 sec) (Σ, relative units (rel. un.)) obtained by averaging the values of five experiments. The sensitivity was 2 mV, and the chart speed was $v = 0.6$ cm/min. All experiments were conducted at 18 to 20°C. The content of acetylcholine was found on the calibration graph.

The control experiment was performed as follows: 9.0 mL of 0.2 mol/L phosphate buffer solution (pH 8.5)

and 1.0 mL of 5% solution of hydrogen peroxide were added to a 10 mL tube with a lapped stopper with stirring. 1.00 mL of the solution was introduced into the cuvette, stirred with 8.5 mL of phosphate buffer (pH 8.5), and the cell was placed in a chemiluminescent photometer. The curtain was opened, and the value of integral chemiluminescence was registered using an I-02 digital automatic integrator for 20 sec (Σ, rel. un.).

The method of obtaining the data for the calibration curve. In a tube with a lapped stopper, (10 - Y) mL of 0.2 mol/L phosphate buffer solution (pH 8.5) (where «Y» is the total volume of all other components in the solution), 1.00 mL of 5% solution of hydrogen peroxide and a sample of the test solution of ACh (0.10; 0.20; 0.40; 0.50; 0.70 and 1.00 mL) were added. The analysis was performed in the same way as when testing model solutions (see the procedure above).

Results and discussion

A series of experiments allowed us to determine the dependence of integral chemiluminescence (ΔΣ, rel. un.) on the concentration of ACh in the cell (c , mol/L) (Fig. 4). The linear dependence was observed on the acetylcholine chloride concentration range of $(0.8-2.8) \times 10^{-4}$ mol/L (Table 1).

The concentration of ACh was calculated using the following formula:

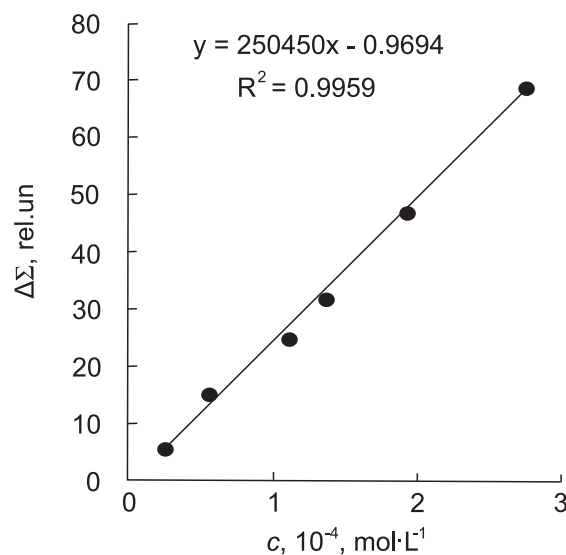
$$c = \frac{(\Delta\Sigma - a) \times 10 \times 10}{b \times V_a \times 1},$$

where: a – is the axis intersection of the calibration curve equation ($\text{tg}\alpha = bc + a$), rel. un.; b – is the slope of the calibration curve equation ($\text{tg}\alpha = bc + a$) – the coefficient of instrumental sensitivity, L/mol; ΔΣ – is the difference in values of integral chemiluminescence

Table 1

Regression output

Parameter	Data
Regression equation $y = a + bc$	$y = (250.4 \pm 31.2) \times 10^3 c$
Correlation coefficient (r)	0.998
S_a	1.931073
S_b	11273.28
a	-0.9694
b	250448
$\Delta a = S_a \times t_a$	5.36152
$\Delta b = S_b \times t_a$	31230
$LOD = 3S_d/b$	2.3×10^{-5} mol/L
$LOQ = 3.3 \times LOD$	7.7×10^{-5} mol/L

Fig. 4. The dependence of integral chemiluminescence ($\Delta\Sigma$, rel. un.) on the concentration of ACh in the cell (c , mol/L)

in the working and control (without ACh) experiments, in relative light units; V_a – is the volume of the sample solution taken for testing, mL; 10, 10 – is the dilution.

The results of determining acetylcholine chloride in model solutions by the chemiluminescence method using the reaction with hydrogen peroxide and luminol is presented in Table 2.

The procedure of the ACh assay in Acetylcholinchlorid Injeel® (Biologische Heilmittel Heel GmbH). The procedure was as follows: 1.00 mL solution contained in an ampoule of Acetylcholinchlorid Injeel® (Biologische Heilmittel Heel GmbH) (0.367 g (on dried base) in 1.1 mL) was quantitatively transferred into a 100 mL volumetric flask, and double distilled water was added to make 100 mL solution. The micropipettes were used to introduce a sample of the test solution of ACh prepared (1.00 mL), 1.00 mL of 5% solution of hydrogen peroxide and (10 – Y) mL of 0.2 mol/L phosphate buffer solution (pH 8.5) (where “Y” is the total volume of all other components of the solution) into a tube with a lapped stopper, and the content was mixed thoroughly. The analysis was performed in the same way

as when testing model solutions (see the procedure above “The procedure for the quantitative determination of acetylcholine by the chemiluminescence method”).

Accurately weighed 0.367 g of acetylcholine chloride reference standard was dissolved *ex tempore* in degassed double distilled water to make 100.00 mL of solution. The micropipettes were used to introduce a sample of standard solution of acetylcholine (1.00 mL), 1.00 mL of 5% solution of hydrogen peroxide and (10 – Y) mL of 0.2 mol/L phosphate buffer solution (pH 8.5) (where «Y» is the total volume of all other components of the solution) into a tube with a lapped stopper, and all the components were mixed thoroughly. The flask was left in the thermostat at 40°C for 15 min. 1.00 mL of the resulting mixture was collected and placed in a quartz cuvette, with stirring, 8.5 mL of phosphate buffer solution (pH 8.5) was added, and the cuvette was placed in a measuring cell of a chemiluminescent photometer. After the curtain opening 0.5 mL of 1×10^{-3} mol/L of luminol solution was added. The total luminescence was registered using an I-02 digital automatic integrator for 20 sec. The exper-

Table 2

The results of the acetylcholine chloride determination in model solutions by the chemiluminescence method using the reaction with hydrogen peroxide and luminol ($P = 0.95$, $n = 5$)

C taken, 10^{-4} mol/L	C found, 10^{-4} mol/L	Recovered, mol/L	RSD, %	δ , %
1.10	1.10; 1.06; 1.13; 1.07; 1.13	$(1.10 \pm 0.04) \times 10^{-4}$	3.0	0%
1.38	1.43; 1.41; 1.36; 1.35; 1.37	$(1.38 \pm 0.04) \times 10^{-4}$	2.5	0%
1.93	1.97; 1.93; 1.94; 1.89; 1.86	$(1.92 \pm 0.05) \times 10^{-4}$	2.3	-0.5%
2.20	2.15; 2.21; 2.26; 2.20; 2.25	$(2.21 \pm 0.055) \times 10^{-4}$	2.0	+0.45%

periment was repeated five times.

The control experiment was performed as it was mentioned above (see “Procedure for the quantitative determination of acetylcholine by the chemiluminescence method”).

The content of ACh (X (g) of a dried substance) in one ampoule was calculated by the following formula:

$$X = (\Delta\Sigma x \times m_{st} \times w_{st} \times V) / (\Delta\Sigma st \times 100 \times 1.00),$$

where: m_{st} – is the standard sample weight, g; w_{st} – is the content of anhydrous ACh, %; 1.00 – is the volume of the test solution taken for analysis, mL; V – is the average volume of the solution in ampoules, mL; $\Delta\Sigma x$ – is the difference in values of integral chemiluminescence in the working and control (without ACh) experiments in relative light units; $\Delta\Sigma st$ – is the difference in values of integral chemiluminescence in the standard solution and control (without ACh) experiment in relative light units.

The results of the ACh analysis in the pharmaceutical formulation Acetylcholinchlorid Injeel® (Biologische Heilmittel Heel GmbH) by the method proposed ($n = 7$, $P = 0.95$) are presented in Table 3.

From the results given above, we can conclude that this chemiluminescent method is a fast, simple and non-enzymatic approach for the quantitative determination of ACh in aqueous solutions; it is based on the reaction of perhydrolysis of ACh to peracetic acid and the subsequent chemiluminescent de-

Table 3

The results of ACh the analysis in the pharmaceutical formulation Acetylcholinchlorid Injeel® (Biologische Heilmittel Heel GmbH) by the method proposed ($n = 7$, $P = 0.95$)

Labelled amount, g/amp	Recovered \pm RSD, %	δ , %*
0.367	99.5 \pm 1.9%	-0.5%

Note: * $\delta = (\bar{X} - \mu) \times 100\% / \mu$

termination of its amount by the reaction of chemiluminescent oxidation of luminol. While determining acetylcholine chloride in the concentration range of $(1.1 - 2.2) \times 10^{-4}$ mol/L the relative standard deviation did not exceed 3% ($RSD \leq 3\%$, $((\bar{X} - \mu) \times 100\% / \mu = -0.5...+0.5\%)$). The limit of quantitation (LOQ, 10 S) was 7.7×10^{-5} mol/L.

Conclusions

A new non-enzymatic kinetic method for the chemiluminescent determination of acetylcholine in aqueous solutions and the pharmaceutical formulation Acetylcholinchlorid Injeel® has been proposed. This method is simple, fast, inexpensive, and thus appropriate for the routine acetylcholine quality control in the laboratories of hospitals, pharmaceutical industries and research institutions.

Conflict of interests: the authors have no conflict of interests to declare.

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