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DETERMINATION OF CYTOCHROME C BY A CHEMILUMINESCENCE METHOD WITH LUMINOL IN BIOLOGICAL OBJECTS

A sensitive, rapid, and simple chemiluminescence (CL) procedure was proposed for the assay of Cytochrome *c* (Cyt *c*). The determination of Cytochrome *c* was achieved by monitoring the chemiluminescence emission from the luminol-hydrogen peroxide reaction in the presence of Cyt *c*. Under optimum conditions, the linearity of the results was examined by measuring a series of standard solutions. The maximum CL intensity was found to be proportional to Cyt *c* concentration, and the response to the concentration was linear over the range from 0.18 to 1.8 μg ml^{-1} with the detection limit of 0.06 μg ml^{-1} (3σ, *n*=5). The regression equation was, I_{CL} = (0.59±0.03) × C, r^2=*0.9986*; LOQ (10S)=0.18 μg mL^{-1}. The recoveries for the different concentration levels varied from 98.0% to 109.0% with a relative standard deviation of less than 3.0%. A result determination of the concentration of Cyt *c* in serum of mice in norm (*n*=5; *P*=0.95) was 5.95±0.2 μmol·L^{-1} (RSD=2.5%).

Key words: Chemiluminescence, Cytochrome *c*, Determination, mice serum.

Для кількісного визначення Цитохрому *c* (Cyt *c*) була запропонована чутлива, швидка та проста хемілюмінесцентна (CL) методика. Визначення Цитохрому *c* було досягнуто за допомогою моніторингу хемілюмінесцентного віпромінювання в реакції гідроген перекису з люмінолом в присутності Cyt *c*. За оптимальних умова лінійність концентраційної залежності була досліджена шляхом вимірювання серії стандартних розчинів. Було виявлено, що максимальна інтенсивність CL пропорційна концентрації Cyt *c*, а відгук залишався лінійним в інтервалі концентрацій від 0,18 до 1,8 мкг $мл^{-1}$ з межею виявлення 0,06 мкг $мл^{-1}$ (3S, *n* = 5). Рівняння регресії мало вигляд I_{CL} = (0,59 ± 0,03) × *C*, r^2 = 0,9986; *LOQ* (10S) = 0,18 мкг $мл^{-1}$. Знайдене значення для різних рівнів концентрації коливалося від 98,0% до 109,0% при RSD < 3,0%. Результат визначення концентрації Cyt *c* у сироватці крові мишей в нормі (*n* = 5; *P* = 0,95) становив 5,95±0,2 мкмоль × L^{-1} (RSD = 2,5%).

Ключові слова: хемілюмінесценція, Цитохром *С*, визначення, сироватка мишей.

Чувствительная, быстрая и простая хемилюминесцентная (ХЛ) методика была предложена для количественного определения Цитохрома *c* (Cyt *c*). Определение Cyt *c* было достигнуто путем мониторинга эмиссии хемилюминесценции в реакции перекиси водорода с люминолом в присутствии Cyt *c*. В оптимальных условиях линейность результатов была исследована путем измерения серии стандартных растворов. Было обнаружено, что максимальная интенсивность ХЛ была пропорциональна концентрации Cyt *c*, и зависимость оставалась линейной в диапазоне от 0,18 до 1,8 мкг $мл^{-1}$ с пределом обнаружения 0,06 мкг $мл^{-1}$ (3S, *n* = 5). Уравнение регрессии имело вид: I_{CL} = (0,59 ± 0,03) × *C*, r^2 = 0,9986; LOQ (10S) = 0,18 мкг $мл^{-1}$. Величина найденного среднего значения для разных уровней концентрации варьировались от 98,0% до 109,0% с относительным стандартным отклонением менее 3,0%. Результат определения концентрации Cyt *c* в сыворотке мышей в норме (*n* = 5; P = 0,95) составил 5,95±0.2 мкмоль L^{-1} (RSD = 2,5%).

Ключевые слова: хемилюминесценция, Цитохром *c*, определение, сыворотка мышей.

Cytochrome *c* (Cyt *c*) is a small heme protein that is localized in the compartment between the inner and outer mitochondrial membranes where it functions to transfer electrons between complex III and complex IV of the respiratory chain. It can also form an intimate association with the mitochondrion-specific phospholipid cardiolipin that induces a conformational change in the protein enabling it to act as a peroxidase catalyzing the oxidation of cardiolipin and thereby instigating a chain of events that leads to apoptosis.

Cyt *c* can catalyze the oxidation of various electron donors by hydrogen peroxide (H_2O_2), including Luminol. With ferrocytochromes with oxidation reactions, there is a phase delay corresponding to H_2O_2 mediated cytochrome c oxidation to the iron state; no phase delay with ferricytochrome *c* is observed (Fig. 1). However, short pre-incubation of ferricytochrome with H_2O_2 increases its catalytic activity to progressive inactivation and degradation. Superoxide (O^{2-}) and hydroxyl radical (·OH) are not involved in this catalytic activity because it was not sensitive to superoxide dismutase (SOD) or mannitol [1].

Currently obtained results are consistent with an initial activation of Cyt *c* by H_2O_2 to a catalytically more active species in which a high oxidation state of an oxo-heme complex mediates the oxidative reactions. The lack of SOD effect on cytochrome c-catalyzed, H_2O_2-dependent luminol chemiluminescence supports a mechanism of chemiexcitation whereby a luminol endoperoxide is formed by direct reaction of H_2O_2 with an oxidized luminol molecule, either luminol radical or luminol diazoquinone [1].

High-performance liquid chromatography [2] and capillary electrophoresis (CE) [3] were reported for the determination of Cyt c. Compared with other methods for the assay of Cyt c, CL method offers the advantages

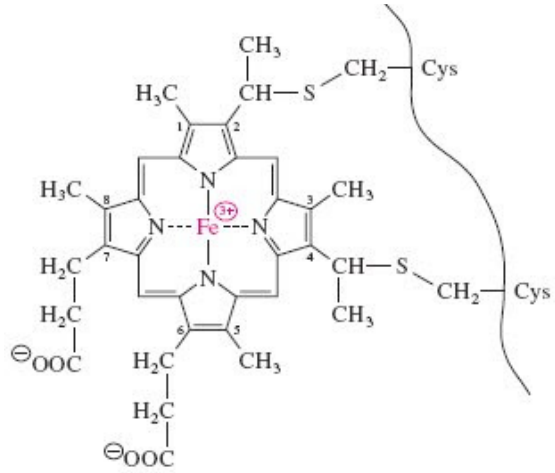

Fig. 1. Cyt *c* heme group

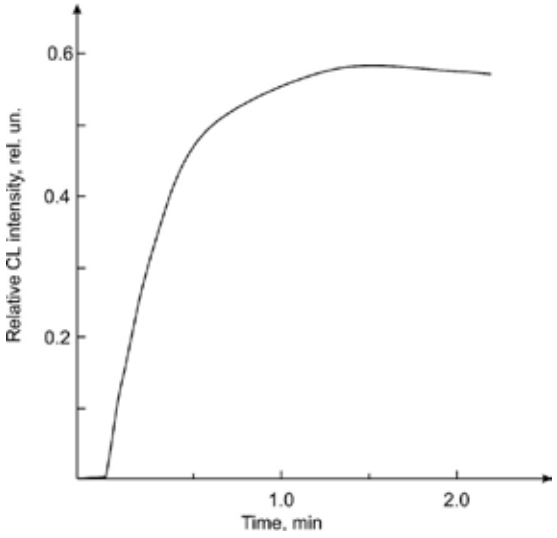

Fig. 2. Kinetic CL intensity–time profile in Luminol–H_2O_2–Cyt *c* system (for 1.5 μg ml^{-1} Cyt *c*). *c*(NaOH) = 0.02 mol·L^{-1}, *c*(H_2L) = 1·10^{-4} mol·L^{-1}, *c*(H_2O_2) =0.85·10^{-3} mol·L^{-1}

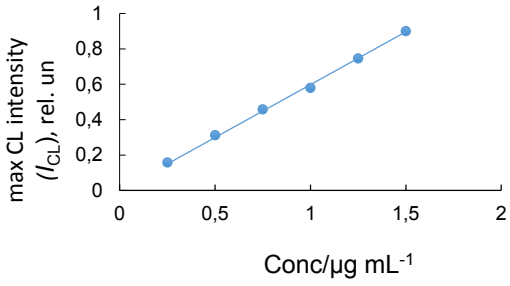

Fig. 3. Effect of Cyt *c* concentration on max CL intensity in Luminol–H_2O_2–Cyt *c* system

of simplicity of apparatus, low reagent consumption, higher sensitivities, and higher sample throughput [4].

A sensitive and simple CL procedure was proposed for the determination of Cyt c in a flow injection (FI) system, giving a linear ranging from 5 to 700 ng ml^{-1} [5]. The proposed method was applied to the determination of Cyt *c* in pharmaceutical injections and human serum samples.

In this work, the chemiluminescent activity of Cyt *c* was studied in order to quantitative determination of enzyme in serum of mice in a static system. A discrete portions of the Cl reagent and the sample are mixed rapidly in reaction cell. A final reagent (containing H_2O_2 solution) that initiated the CL was added with a semi-automatic injector. The whole CL intensity-versus-reaction-time profile was monitored. As an analytical signal was taken the maximum CL intensity (peak height). By measuring the increment of the CL intensity, the concentration of Cyt *c* could be quantitatively determined, viz. $I_{CL} = I_s - I_o$, where I_s and I_o are the maximum CL intensity in the presence and absence of Cyt *c*, respectively (Fig. 2).

The intensity of chemiluminescence was measured using an FEU-84-A photomultiplier tube with Low current meter IMT-0.5. The signal was recorded with a LINE RECORDER TZ 4620 recorder (LABORATORNI PŘISROJE PRAHA). Solutions were rapidly injected (0.1 s) into a hemispherical quartz cuvette mounted on the front of the phototube. The standard concentrations of substrates in the reaction mixture were as follows: 10 mM H_20_2, 20 mM NaOH, 0.125-1.5 μg cytochrome *c* and water up to 10 cm^3. The temperature was 22-25°C. The reproducibility of chemiluminescence peak heights was about 5-10% and each measurement was repeated five to eight times. All results are expressed as $\bar{x} \pm ts/\sqrt{n}$ in which $\bar{x}$ is the mean and *s* the standard deviation of *n* determinations; *t* is a factor dependent on *n* and the confidence level. In our studies, *n* varied between 5 and 7, and or the confidence level we chose 95%.

Reagents and solutions. Bovine heart Cyt *c* (Samson-Med production, Peterburg, Russia), molecular weight 12800 g mol^{-1}; $\varepsilon_{550}/\varepsilon_{280}$ =1.2; [Fe]=0.435%, 0.25% solution was used as standard solution. All reagents were analytical reagent grade and double distilled water was used throughout these experiments. Luminol (5-amino-2,3-dihydro-1, 4-phthalazinedione, 99%) was purchased from the Corporation of Sigma (USA).

The 0.01 mol·L^{-1} luminol stock solution was prepared by dissolving 0.1772 g luminol with 2 mL 1 mol·L^{-1} NaOH solution and diluted with distilled water to 100 ml. Working standard solutions of luminol were freshly prepared from the stock solution by appropriate dilutions before use and adjusted its pH with 0.1 mol·L^{-1}NaOH.

The serum samples were supplied by the Central Research Laboratory of National University of Pharmacy. To prepare the spiked samples, known quantities of Cyt *c* were spiked into 1.0 ml of serum. After homogenization, the spiked samples were diluted, and the influence of foreign species existing in serum could be eliminated. In order to evaluate the validity of the proposed method, recovery studies were carried out on samples to which known amounts of Cyt *c* were added.

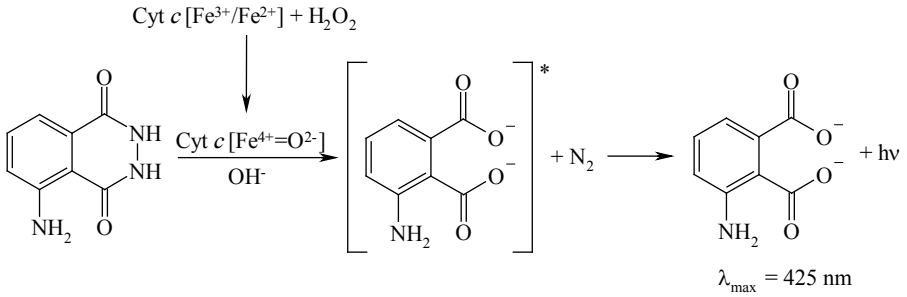

Fig. 4.

Experimental part. In the course of studies it was found that under optimum conditions (c(NaOH) = 0.02 mol·L^{-1}, $c(H_2O_2)$ =0.85·10^{-3} mol·L^{-1}, $c(H_2L)$ = 1·10^{-4} mol·L^{-1}, $C(Cyt\ c)$ = 0.25-1.5 μg·mL^{-1}) *Cyt c* exhibits an enhance effect on intensity of CL in H_2L – H_2O_2 system (Fig. 2).

Hydrogen peroxide (H_2O_2) 5.8% (wt.) solution was prepared from 58% high pure preparation (produced by LTD "Inter-Syntes", Boryslav, Ukraine) by its 10 times dilution with DDW: 10 mL was transferred into volumetric flask of 100 mL and volume was brought to the mark at 293 K. This solution was stored at reduced temperature of + 8 – 10 °C. The content of hydrogen peroxide in solution was controlled by permganatometric titration. Working solution of H_2O_2 0.058% (wt.) (1.7·10^{-2} mol·L^{-1}) was obtained by the appropriate dilution of the original solution exactly 100 times. The working solution can be used throughout the day.

Procedure of determination. Solutions were added to a chemiluminescent quartz cell consistently as follows: 1.0 mL of 1·10^{-3} mol·L^{-1} H_2L, 1.0 mL of 0.2 mol·L^{-1} sodium hydroxide solution, (10 – x) mL of double distilled water (also abbreviated ddH_2O), where x is the total volume of all reagents and samples, (mL), 0.25-1.75 mL of dilute working-standard solution of *Cyt c* (or solution of mice serum) and 0.50 mL of 1.7·10^{-2} mol·L^{-1} H_2O_2 (final solution). Cell with the mixture was placed in chemiluminometer and 0.5 mL of working solution of 1.7·10^{-2} mol·L^{-1} H_2O_2 was added.

The regression equation was, I_{CL} = (0.59±0.03)×C, r^2=*0.9986*; LOD (3S)=0.06 μg mL^{-1}; LOQ (10S) = 0.18 μg mL^{-1} (Fig. 3).

The mechanism of the enhanced effect of Cyt *c* on luminol–hydrogen peroxide CL reaction could be presented as following (Fig. 4).

In order to evaluate the validity of the proposed method for the determination of Cyt *c* in serum of mice, standard addition methodology was implemented to test the recovery. The recoveries for the different concentration levels varied from 98.0% to 109.0% with a relative standard deviation of less than 3.0%. A result determination of the concentration of Cyt *c* in serum of mice in norm (n=5; P=0.95) was 5.95±0.2 μmol·L^{-1} (RSD=2.5%).

Conclusions. A chemiluminescent activity of Cyt *c* was studied in order to quantitative determination of enzyme in serum of mice in a static Luminol–H_2O_2–Cyt *c* CL system. A sensitive, rapid, and simple chemiluminescence (CL) procedure was proposed for the assay of Cytochrome *c* (Cyt *c*). The proposed CL method for the assay of Cyt *c* offers the advantages of simplicity of apparatus, less time consumption, and higher sample throughput compared with the CL procedure reported by Robert Feissner. The satisfactory performance in an assay of Cyt *c* in mice serum demonstrated that the proposed CL method is practical for biological samples, confirming the promise for pharmacological and clinical research.

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