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The quantitative determination of glutathione by the effect of the chemiluminescence inhibition in the catalytic oxidation reaction of luminol with hydrogen peroxide in the presence of hemoglobin

Abstract

Aim. To develop a method for the quantitative determination of reduced glutathione in a lyophilized powder for the preparation of "Hepaval®" solution for intravenous and intramuscular administration by the effect of inhibiting chemiluminescence in the luminol (H_2L)–H_2O_2–hemoglobin (Hb) system.

Materials and methods. The study object was the reduced glutathione substance and lyophilized powder for the preparation of "Hepaval®" solution for injection, ampoules of 4 mL No. 10 manufactured by "Valartin pharma" (Italy). The glutathione content in powder was determined using the chemiluminescence method by the effect of inhibiting the luminol oxidation reaction with hydrogen peroxide in the presence of Hb as a catalyst. The analysis was performed by the standard addition method.

Results and discussion. As a result of the studies, it has been found that under optimal conditions glutathione shows a noticeable inhibitory effect on chemiluminescence in the H_2L–H_2O_2–Hb system. This phenomenon was used to develop a new procedure for the quantitative determination of glutathione in the substance and lyophilized powder for the preparation of "Hepaval®" aqueous solution for injection. The linear dependence of the integral chemiluminescence intencity (S) on the molar concentration of glutathione was maintained in the concentration range of $(2-20) \times 10^{-7}$ mol L^{-1}. The graph equation was $S = (-1.6 \pm 0.2) \times 10^7 \times c + (198.9 \pm 2.0)$, (r = 0.999). The relative standard deviation (RSD) was ±1.82 % (n = 7, P = 0.95).

Conclusions. The method has been developed, and the possibility of the quantitative determination of glutathione in powder for the preparation of "Hepaval®" solution for injection by the method of the chemiluminescence inhibition of the H_2L–H_2O_2–Hb system has been shown. The content of glutathione in powder calculated with reference to dried substance was 91.49 % (against 90.8 % by the certificate). The accuracy was +0.76 %.

Keywords: glutathione; chemiluminescence inhibition; quantification; luminol

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Кількісне визначення глутатіону за ефектом інгібування хемілюмінесценції в реакції каталітичного окиснення люмінолу гідроген пероксидом у присутності гемоглобіну

Анотація

Мета. Розробити методику кількісного визначення відновленого глутатіону в ліофілізованому порошку для приготування розчину для внутрішньовенного та внутрішньом'язового введення «Гепавал®» за ефектом інгібування хемілюмінесценції системи люмінол (H_2L)–H_2O_2–гемоглобін (Hb).

Матеріали та методи. Об'єктом дослідження були відновлений глутатіон та ліофілізований порошок для приготування розчину для ін'єкцій «Гепавал®», ампули по 4 мл № 10 виробництва «Валартин фарма» (Італія). Визначення вмісту глутатіону в порошку здійснювали методом хемілюмінесценції за ефектом інгібування реакції окиснення люмінолу гідроген пероксидом у присутності Hb як каталізатора процесу. Аналіз проводили методом стандартних добавок.

Результати та їх обговорення. У результаті дослідження з'ясовано, що в оптимальних умовах глутатіон виявляє помітну інгібувальну дію на хемілюмінесценцію в системі H_2L–H_2O_2–Hb. Це явище було використано для опрацювання нової методики кількісного визначення глутатіону в субстанції та ліофілізованому порошку для приготування водного

розчину для ін'єкцій «Гепавал®». Лінійна залежність сумарного світіння (*S*) від молярної концентрації глутатіону зберігалася в інтервалі концентрацій $(2-20) \times 10^{-7}$ моль л$^{-1}$. Рівняння графіка має вигляд $S = (-1,6 \pm 0,2) \times 10^7 c + (198,9 \pm 2,0)$, (r = 0,999). Відносне стандартне відхилення (RSD) становило ±1,82 % (*n* = 7, *P* = 0,95).

Висновки. Розроблено методику та продемонстровано можливість кількісного визначення глутатіону в порошку для приготування розчину для ін'єкцій «Гепавал®» методом інгібування хемілюмінесценції системи $H_2L - H_2O_2 - Hb$. Вміст глутатіону в порошку в перерахунку на суху речовину становив 91,49 % (проти 90,8 % за сертифікатом). Правильність становила +0,76 %.

Ключові слова: глутатіон; інгібування хемілюмінесценції; кількісний аналіз; люмінол

■ Introduction

Glutathione (GSH, *L*-γ-glutamyl-*L*-cysteinyl-glycine) is a biologically active tripeptide found in all organisms. It consists of γ-glutamic acid, cysteine, and glycine residues and can exist in both oxidized (GSSG) and reduced (GSH) forms (Figure 1). The reduced form of GSH protects SH-groups of proteins from oxidation by various oxidants [1].

The protection mechanism consists in the oxidation of the SH-group of GSH with the formation of the oxidized form (disulfide) and the retention of the SH-groups of proteins in the active reduced form. GSH acts as a cofactor of some oxidoreductases like glyoxalase system [2], and glutathione peroxidase [3]. An important role of GSH is binding of free radicals, and the reduction of hydrogen peroxide and other peroxides; it prevents the development of chain free radical processes [4]. Moreover, glutathione facilitates the metabolism of xenobiotics with support of glutathione S-transferase enzymes catalyzing its conjugation to lipophilic xenobiotics [5].

In laboratory practice, glutathione is needed to be determined both in biological fluids (saliva, urine, blood serum) and in pharmaceutical or cosmetic preparations. For this purpose, a variety of instrumental methods of analysis, such as spectrophotometry based on the interaction of glutathione with 5,5'-dithiobis-2-nitrobenzoate acid (Ellman's reagent) [6], high-performance liquid chromatography (HPLC) with various detectors and a precolumn derivative [7], electroanalytical methods [8], as well as the kinetic method of chemiluminescence [9–12], are widely used. The last one is characterized by simplicity of implementation, low cost, and the possibility of miniaturization of the instrument base. According to the British Pharmacopoeia, the reduced glutathione content in a pure substance is determined by iodimetry [13].

The aim of the study was to develop a method for the quantitative determination of reduced glutathione in a lyophilized powder for the preparation of "Hepaval®" solution for intravenous and intramuscular administration by the effect of inhibiting chemiluminescence in the luminol (H_2L)–H_2O_2–hemoglobin (*Hb*) system.

■ Materials and methods

The study object was the reduced glutathione substance and lyophilized powder for the preparation of "Hepaval®" solution for injection, ampoules of 4 mL No. 10 manufactured by "Valartin pharma" (Italy) (1 ampoule contains 643 mg of glutathione (reduced) sodium salt, which is equivalent to 600 mg of glutathione).

The glutathione content in powder was determined using the chemiluminescence method by the effect of inhibiting the luminol oxidation reaction with hydrogen peroxide in the presence of *Hb* as a catalyst. The analysis was performed by the standard addition method.
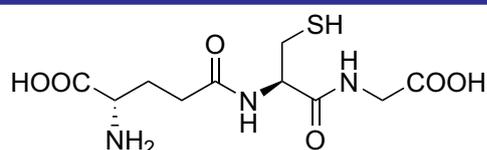

Figure 1. The structure of reduced glutathione

The substance of reduced *L*-glutathione produced by *Bio*Chemica (AppliChem GmbH, Germany) was used in the study. The certificate of analysis: HPLC 98.7%.

The standard $1{\times}10^{-3}$ mol L^{-1} solution of luminol (5-amino-2,3-dihydrophthalazine-1,4-dione, H_2L, RPF "Synbias", Ukraine): 0.217 g of luminol was placed in a 100 mL volumetric flask, dissolved in 10 mL of 0.01 mol L^{-1} sodium hydroxide solution, and diluted to the volume with double distilled water. The solution was kept in a dark place.

For the medium pH correction, 0.1 mol L^{-1} solution of sodium hydroxide was used; pH of the solutions was controlled by an "Ionomer I-130" laboratory potentiometer with an ESL-43-07 glass electrode, a silver-chloride electrode and an I-130 laboratory ionomer (ZIP, Gomel, Belarus). All solutions were prepared using double distilled water.

Hydrogen peroxide 5.8% solution was prepared from 58% high pure H_2O_2 solution (produced by LTD "Inter-Syntes", Boryslav, Ukraine) by its 10 times dilution with double distilled water: 10 mL of 58% H_2O_2 was transferred into a 100 mL volumetric flask and diluted to the volume at 293 K. This solution was stored at a temperature of +8–10°C. 0.29% working solution of H_2O_2 ($8.5{\times}10^{-2}$ mol L^{-1}) was obtained by diluting 5.8% solution with double distilled water 20 times: 5 mL of the original solution was transferred into a 100 mL volumetric flask and diluted to the volume at 293 K. The working solution can be used throughout the day.

Human blood hemoglobin (*Hb*) produced by "Simko Ltd" (Lviv, Ukraine) was used as a catalyst. 100 μg mL^{-1} hemoglobin solution was prepared by dissolving 10 mg of hemoglobin in 50 mL of double distilled water in a 100 mL volumetric flask under heating and subsequent adding of 1 mL of 1.0 mol L^{-1} sodium hydroxide solution. The volume was diluted with double distilled water at 293 K and stirred. The working solution of hemoglobin was prepared by diluting the initial one with double distilled water 100 times. The working solution can be used throughout a day.

The intensity of chemiluminescence was measured in relative units on a device with a FEU-84-A photoelectric multiplier, using an IMT-0.5 and quick-acting (time constant 0.1 s) automatic potentiometer for measuring low currents.

The reaction accompanied by chemiluminescence was performed in a cylindrical 30 mm diameter quartz cell with a working volume of 10 mL. The following order of mixing the reagents was used in the experiment: to the mixture of the luminol indicator in the alkali solution and H_2O_2, with the presence or absence of the glutathione solution (in the control experiment), 0.50 mL of the *Hb* solution was added using a volumetric pipette. The kinetic curve of the chemiluminescence intensity in relative units against time was registered. The volumetric pipette was built into the removable holder isolating a photocathode of the photoelectric multiplier from outside light, and allowing it to work in the daylight. All experiments were performed at 293 K. The integral chemiluminescence intensity was registered using a "Digital automatic integrator I-02" (Micron, Russia) for 40 seconds.

All other chemicals were of analytical grade. Solutions were prepared with double distilled water produced in a quartz water distiller.

The sample solution of glutathione

0.3618 g (accurate weight) of the glutathione powder was transferred into a 100.0 mL measuring flask, dissolved in double distilled water, and diluted to the volume with the same solvent. Using a pipette, 10.00 mL of the solution prepared was transferred to a 1000 mL volumetric flask, diluted to the volume with double distilled water, and mixed thoroughly.

The standard working solution of glutathione ($c_{st} = 1{\times}10^{-5}$ mol L^{-1})

It was prepared in the same way as the previous one with the difference that the glutathione powder with the known basic content was used as an addition substance, and then the solution was prepared with the known concentration of glutathione ($C_{10}H_{17}N_3O_6S$) calculated with reference to dried substance. 0.31137 g (accurate weight) of the glutathione powder was transferred into a 100.0 mL measuring flask, dissolved in double distilled water at 393 K, and diluted to the volume with the same solvent. Using a pipette, 10.00 mL of the solution prepared was transferred to a 1000 mL volumetric flask, diluted to the volume with double distilled water, and mixed thoroughly.

The procedure for determining the content of glutathione in "Hepaval®" (by the addition method)

The solutions were sequentially introduced into the quartz cuvette of the chemiluminometer in the following order: 1.00 mL of $1{\times}10^{-3}$ M solution of H_2L, 5.00 mL of 0.1 M solution of sodium hydroxide, $(10 - x)$ mL of double distilled water (where x is the total volume of all reagents with the sample solution (or sample and addition in another experiment with an addition), in mL), 0.50 mL of $8.5{\times}10^{-2}$ M of H_2O_2 solution and

0.50 mL of the sample solution (or with 0.50 mL of the addition solution). The cuvette with the resulting mixture was placed in a chemiluminometer light-resistant chamber and using a dispenser, 0.50 mL of 1 µg mL^{-1} working solution of *Hb* was added.

The molar concentration of glutathione in the sample solution c_x (mol L^{-1}) was calculated by the formula:

$$c_x = \frac{c_{st} \times S_x}{S_{x+a} - S_X}$$

where c_{st} – is the molar concentration of glutathione in the standard working solution of glutathione, $C_{10}H_{17}N_3O_6S$, mol L^{-1};
S_x – is the analytical signal of the integral chemiluminescence intensity in the experiment with the sample solution of glutathione for 40 s, rel. units;
S_{x+a} – is the analytical signal of the integral chemiluminescence intencity in the experiment with the sample solution of glutathione and addition for 40 s, rel. units;

The content of glutathione in powder calculated with reference to dried substance X (%) was determined by the formula:

$$X = \frac{c_x \times M \times 1000 \times K \times 100 \times 100 \, \%}{m \times (100 - w)}$$

where c_x – is the molar concentration of glutathione in the sample solution, mol L^{-1};

M – is the molar mass of glutathione (reduced) sodium salt, 329.307 g mol^{-1};
K – is the dilution factor of the sample solution, 100;
w – is the mass fraction in loss on drying, % (1.0% by the certificate);
m – is the weight of the glutathione sample taken for analysis, g.

■ Results and discussion

The dependence of the *chemiluminescence intensity* (I_{CL}) on *time* (s) is shown on the kinetic graph (Figure 2). The experiment was repeated five times. The desired signal was the area under the curve – the integral chemiluminescence intensity over the time period (40 s) (*S*, rel. units) obtained by averaging the values of five experiments.

The highest intensity of chemiluminescence in the H_2L–H_2O_2–Hb system was observed when the *Hb* solution was added last. The optimal conditions of the experiment were determined earlier [14]: $c(NaOH) = 0.05$ mol L^{-1}, $c(H_2O_2) = 4.25 \times 10^{-3}$ mol L^{-1}, $c(H_2L) = 1 \times 10^{-4}$ mol L^{-1}, $c(Hb) = 5 \times 10^{-2}$ µg mL^{-1}.

The presence of glutathione in the H_2L–H_2O_2–Hb system leads to a decrease in the maximum intensity (Figure 2) and the integral chemiluminescence intensity (*S*), indicating the chemiluminescent reaction inhibition (Figure 3). This effect increases with the increasing concentration of the inhibitor.
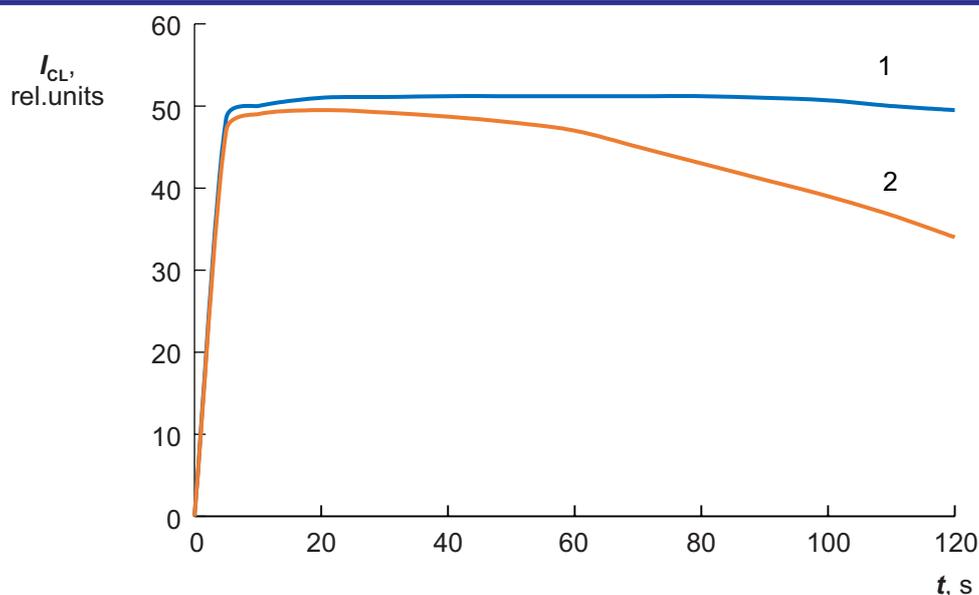

Figure 2. The kinetic curves of the dependence of the chemiluminescence intensity on time in the H_2L–H_2O_2–Hb system in the absence (1) and in the presence (2) of glutathione. $c(NaOH) = 0.05$ mol L^{-1}, $c(H_2O_2) = 4.25 \times 10^{-3}$ mol L^{-1}, $c(H_2L) = 1 \times 10^{-4}$ mol L^{-1}, $c(Hb) = 5 \times 10^{-2}$ µg mL^{-1}, $c(GSH) = 1 \times 10^{-6}$ mol L^{-1}
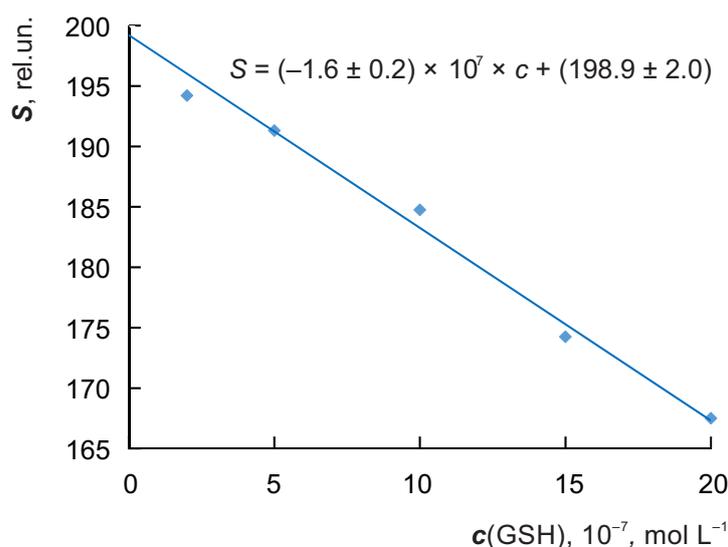
Figure 3. Dependence of *S* (in rel. units) on the molar concentration of glutathione

Table 1. Analytical characteristics of the calibration graph (y = bx + a) for the quantification of glutathione in "Hepaval®"

Characteristics	Parameters
Concentration range (μg mL^{-1})	0.2–30
Correlation coefficient (r)	0.999
Linear regression equation	$S = (-1.6 \pm 0.2) \times 10^7 \times c + (198.9 \pm 2.0)$
Slope (b$\pm\Delta$b)	$(-1.6 \pm 0.2) \times 10^7$
Intercept (a$\pm\Delta$a)	198.9 ± 2.0
S.D. of slope (S_b)	0.06×10^7
S.D. of intercept (S_a)	0.70
LOD (3S) (mol L^{-1})	1.5×10^{-7}
LOQ (10S) (mol L^{-1})	4.4×10^{-7}

Table 2. The results of the quantitative determination of glutathione in powder for the preparation of "Hepaval®" solution for injection

Taken	Found, %	Metrological characteristics (*n* = 7, *P* = 0.95)
0.31137 g A lyophilized powder for the preparation of "Hepaval®" solution for injection manufactured by "Valartin pharma" (Italy)	92.1 90.9 89.1 91.2 92.8 90.2 94.1	$\overline{x}$ = 91.49 *S* = 1.67 $\Delta\overline{x}$ = 1.55 *RSD* = ±1.82% $\delta^{[a]}$ =+0.76%

Note: [a] The calculation is based on the certificate of quality data of the powder for the preparation of "Hepaval®" solution for injection of glutathione ($C_{10}H_{17}N_3O_6S$) calculated with reference to dried substance by the method of Ph. Eur. – 90.8 % (μ). $\delta = (\overline{x} - \mu) \times 100\%/\mu$

The linear dependence of *S* on the molar concentration of glutathione was maintained in the concentration range of $(2-20)\times10^{-7}$ mol L^{-1}. The graph equation was $S = (-1.6\pm0.2)\times10^7\times c + (198.9\pm2.0)$, (r = 0.999) where *c* was the concentration of glutathione solution in mol L^{-1} (Figure 3). Analytical characteristics of the calibration graph are given in Table 1.

Precision and accuracy of the quantitative determination of glutathione in powder for the preparation of "Hepaval®" solution for injection by the method of the chemiluminescence inhibition of the *H₂L–H₂O₂–Hb* system were studied by analyzing seven replicates of the sample solutions. The precision of the method developed with reference to the relative standard deviation (RSD) was ±1.82% (*n* = 7, *P* = 0.95). The accuracy was

+0.76%. The results obtained are summarized in Table 2.

■ Conclusions

The method has been developed, and the possibility of the quantitative determination of glutathione in powder for the preparation of "Hepaval®" solution for injection by the method of the chemiluminescence inhibition of the *H₂L–H₂O₂–Hb* system has been shown. The content of glutathione in powder calculated with reference to dried substance was 91.49% (against 90.8% by the certificate). The accuracy was +0.76%. The method proposed is promising for the determination of glutathione in substances and drugs in the practice of control and analytical laboratories, as well as in the chemical and pharmaceutical industry.

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