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Determination of glutathione in hemolysed erythrocyte by flow injection analysis with chemiluminescence detection

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ABSTRACT

In this work, a new sensitive method is introduced for analysis of glutathione at trace levels in blood samples. The method is based on the effect of glutathione on the chemiluminescence signal of the oxidation of luminol by sodium periodate in basic solution. The influence of chemical and manifold variables on the sensitivity was studied. At the optimized conditions, the linear range for the determination of glutathione was 1.0×10^{-8} to 1.0×10^{-5} mol L^{-1} with the detection limit (3σ) of 8×10^{-9} mol L^{-1}. The relative standard deviation for 10 repeated measurements of 1.0×10^{-6} mol L^{-1} of glutathione was 4%. The results of the method were compared with the Ellman reference method and no significant difference was found. The influence of potential interference substances on the determination of glutathione was studied. The proposed method was applied successfully for the determination of glutathione in real samples such as erythrocyte hemolysed in normal subjects and diabetes.

1. Introduction

Glutathione (GSH) is the major intracellular tripeptide thiol in mammals. The role of glutathione in the human metabolism includes protection against oxidative stress and detoxification of xenobiotics. In addition, it plays an essential role in many important biological phenomena, including the synthesis of proteins and DNA and protection of cells against free radicals [1]. Changes in its concentration in biological fluids or tissues may be a useful marker in certain disorders such as leukemia [2], diabetes [3,4], DNA base damages [5,6] and in the investigation of some kinds of cancer [7,8].

A number of methods for the quantification of glutathione in biological samples have been reported, such as electrochemical [9], spectrophotometric [10,11], HPLC [12,13], colorimetric [14,15] and enzymatic method [16]. The enzymatic methods are laborious and complicated, rendering them not suitable for routine analysis. HPLC–colorimetric methods suffer from difficulties in sample preparation, the necessity of derivatization and lack of sufficient sensitivity. Electrochemical methods for determination of GSH have been receiving more and more attention in recent years. However, it should be noted that the hydrosulfide group of GSH is very reactive and it is easily oxidized by oxygen and peroxide. The electrochemical oxidation of GSH at unmodified electrodes is usually a very slow process except for at a mercury electrode [17,18] which is not an ideal sensor due to its toxicity. Chemiluminescence (CL) methods, in comparison to the other techniques, have the advantages of simplicity, low detection limit, wide linear dynamic range, inexpensive, and high sensitivity. The response characteristics of the proposed method are compared with recently reported methods for determination of glutathione [25–28] and the results are given in Table 1. As the data show, one of them [25] has a wider dynamic range and better sensitivity, but methionine and tryptophan act as interference. The second method [26] was not applied for analysis of any real samples.

In this work, we suggest a CL method for the determination of low level of glutathione concentration in blood samples based on its effect to enhance the chemiluminescence signal of the oxidation of luminol by sodium periodate in basic solution. The experimental conditions for the reaction were optimized, and the final procedure allowed the successful determination of trace levels of glutathione in erythrocyte hemolysed in normal subjects and diabetes with satisfactory results.

2. Experimental

2.1. Apparatus

The schematic diagram of the flow system is shown in Fig. 1. A 12-channel peristaltic pump (Desaga, Wiesloch, Germany), Model PLG, with three silicon rubber tubes (1.0 mm i.d., Desaga, Wiesloch, Germany) was used to deliver solutions at a flow rate of 2.6 mL min^{-1}. Sample solutions were injected into the carrier

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Table 1
Comparison of recently published CL methods and the propose method for determination of glutathione

CL system	Linear dynamic range (mol L^{-1})	Detection limit (mol L^{-1})	R.S.D.%	Reference
Ce(IV) + quinine	4.0×10^{-9} to 4.0×10^{-5}	5×10^{-10}	4% ($n = 11$)	[25]
Luminol–H_2O_2	6.5×10^{-9} to 2.6×10^{-6}	3×10^{-9}	2.5% ($n = 11$)	[26]
$Ru(phen)_3^{2+}$–$KMnO_4$	1.5×10^{-7} to 1.0×10^{-5}	5.8×10^{-8}	2.2% ($n = 11$)	[27]
Cu^{2+}–O_2–luminol	7.5×10^{-7} to 3.0×10^{-5}	7.5×10^{-7}	1.6% ($n = 5$)	[28]
Luminol–$NaIO_4$	2.0×10^{-8} to 1.0×10^{-5}	8.0×10^{-9}	3.8% ($n = 10$)	Proposed method

stream using a six positions rotary Supelco valve (Bellefonte, USA) with a 250 μL sample loop. PTFE tube (1.0 mm i.d.) was used for the reaction coil. The chemiluminescence signal was measured with a Hamamatsu photo multiplier tube (PMT, Tokyo, Japan), mode R_{212}, and a low pass filter, whose output was connected to a data processing system. Spectrometric measurements were performed with a UV–vis spectrophotometer JASCO (Tokyo, Japan), Model V-570.

2.2. Reagents and solutions

All the solutions were prepared using reagent grade chemicals and doubly distilled water was used throughout. Sodium hydroxide, sodium hydrogen carbonate, sodium carbonate, boric acid, sodium phosphate, and hydrochloric acid were purchase from Aldrich (Milwaukee, USA).

A stock standard solution of glutathione (1.0×10^{-3} mol L^{-1}) was prepared by dissolving 0.0760 g of glutathione (Sigma, St. Louis, USA) in water and diluting to 250 mL in a 250-mL volumetric flask. More dilute solutions were prepared daily by diluting the standard solution with water.

$NaIO_4$ stock solution, 5.0×10^{-3} mol L^{-1}, was prepared by dissolving 0.27 g of sodium metaperiodate (Merck, Darmstadt, Germany) in doubly distilled water and diluting to 250 mL in a 250-mL volumetric flask. This solution was prepared daily. More dilute solutions were prepared by diluting the stock solution with water.

Luminol stock solution, 1.0×10^{-2} mol L^{-1} (Aldrich, Milwaukee, USA), was prepared in 0.1 mol L^{-1} Na_2CO_3 solution and the working solutions were made by serial dilutions of the stock solution with 0.10 mol L^{-1} of the buffer.

5-Sulfosalysilic acid solution was prepared (10%, m/v) by dissolving 10.0 g of this reagent (Sigma, St. Louis, USA) in 100 mL water in a 100-mL volumetric flask.

Stock solution of 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) was prepared by dissolving 0.0396 g of this reagent (Sigma, St. Louis, USA) in 10 mL of 0.10 mol L^{-1} phosphate buffer (pH 7.8).

Various basic buffers solutions such as 0.10 mol L^{-1} NaOH–$NaHCO_3$, 0.10 mol L^{-1} NaOH–H_3BO_3, 0.10 mol L^{-1} $NaHCO_3$–Na_2CO_3, and 0.10 mol L^{-1} Na_3PO_4–NaOH were used to control the pH of the CL reaction medium.

The buffer solution (pH 10.5) was prepared by adding an appropriate amount of 0.1 mol L^{-1} HCl (to 0.1 mol L^{-1} Na_2CO_3) solution.

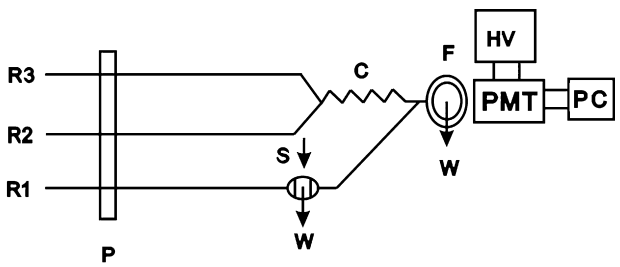


Fig. 1. Schematic diagram of the FIA system. R_1, H_2O; R_2, basic luminol; R_3, sodium periodate; P, peristaltic pump; S, injection valve; C, reaction coil; F, flow cell; W, waste.

2.3. Procedure

The flow injection system is shown in Fig. 1 with the optimum conditions as stated. The flow lines were connected with a carrier H_2O (R_1), 2.0×10^{-4} mol L^{-1} luminol solution in Na_2CO_3 buffer (pH 10.5) (R_2), and 1.5×10^{-3} mol L^{-1} sodium periodate solution (R_3). Luminol solution firstly was mixed with $NaIO_4$ solution through 20-cm silicon tubing (1.0 mm i.d.) to give a stable baseline. Then 250 μL solution of standard GSH was injected into the carrier stream via the sample injection valve which pumped continuously. The mixture was passed through the CL cell, while the CL signal was recorded with the PMT. A typical CL signal for three different concentrations of GSH is shown in Fig. 2.

2.4. Real sample analysis

For the determination of GSH in human erythrocyte, human whole blood was obtained from the Isfahan University Hospital and erythrocytes were separated from whole blood by removing the plasma. Human whole blood (2.0 mL) was firstly centrifuged for 10 min at 3000 rpm. The supernatant (plasma) was discarded and the rest was mixed with 5 mL 0.9% NaCl solution. The solution was centrifuged for another 5 min at 3000 rpm and the supernatant (diluted plasma) was again discarded. The washing procedure with NaCl solution was repeated three times in order to remove the plasma almost completely.

The erythrocyte pellets were hemolysed with water (1:1, v/v). For protein precipitation, the hemolysate was mixed with 5-sulfosalysilic acid (10%, m/v) in the ratio of 2:1 (v/v). This mixture was centrifuged in the same condition described above. Then, the supernatant was divided to two parts one for spectrophotometric determination and another for the proposed chemiluminescence method.

For spectrophotometric measurements of the Ellman, a reference method [19] was performed which is based on the reaction of glutathione and DTNB (Ellman's reagent), generating 2-nitro-5-mercapto-benzoic acid. The absorbance was monitored

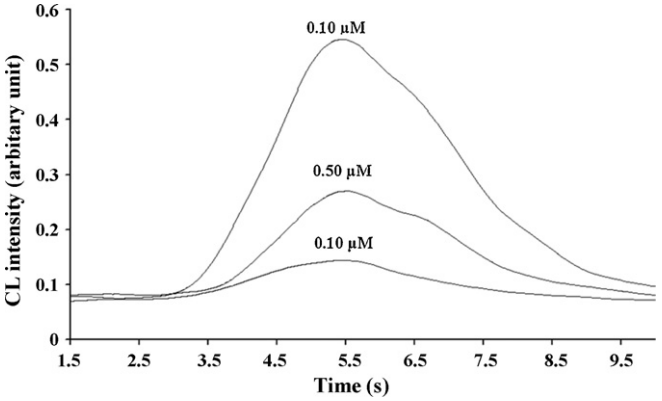


Fig. 2. Typical CL signal for three different concentration of GSH at 1.0×10^{-4} mol L^{-1} luminol; 1.0×10^{-3} mol L^{-1} IO_4^-; pH 10.5.

spectrophotometrically at 412 nm. For CL measurement, 100 μL of the supernatant was diluted to 25 mL with water and the previous procedure was carried out.

3. Result and discussion

Chemiluminescence based on the oxidation of luminol is one of most extensively studied and best-known CL system. The oxidation is usually carried out in an alkaline solution. In batch CL mode, strong CL signals were recorded when 200 μL alkaline luminol solutions (2.0×10^{-4} mol L^{-1}) were injected into 200 μL of sodium periodate (1.5×10^{-3} mol L^{-1}). The signal came back to the baseline after 5 s. Subsequently injecting 200 μL (1.0×10^{-6} mol L^{-1}) glutathione to the above mixture resulted a new CL emission and after 8 s from the injection time, the chemiluminescence reaction terminated and the CL signal declined to a baseline. We realized that CL intensity was inhibited by the addition of 5% DMSO which is generally used as a hydroxyl radical scavenger. Moreover when the dissolved oxygen was removed from the solution by purging the solution with nitrogen gas, the CL intensity increased. These results may indicate that the superoxide does not play a major role in the CL reaction but the hydroxyl radical that is formed in this reaction affected the CL emission. Due to the structural similarity between cysteine and glutathione the mechanism could be the same as reported for cysteine [20]. GSH is composed of three amino acids, L-cysteine, L-glutamate and glycine; from them only cysteine has a –SH group which can be oxidized in the presence of IO_4^-. Lu et al. [20] have reported the mechanism of the CL for the cysteine. Since the active site of cysteine and GSH in the CL reaction is the –SH group, it could be concluded that the mechanism of CL for these two compounds are the same. The proposed CL reaction mechanism of luminol–$NaIO_4$–GSH and the structure of GSH are shown in Scheme 1.

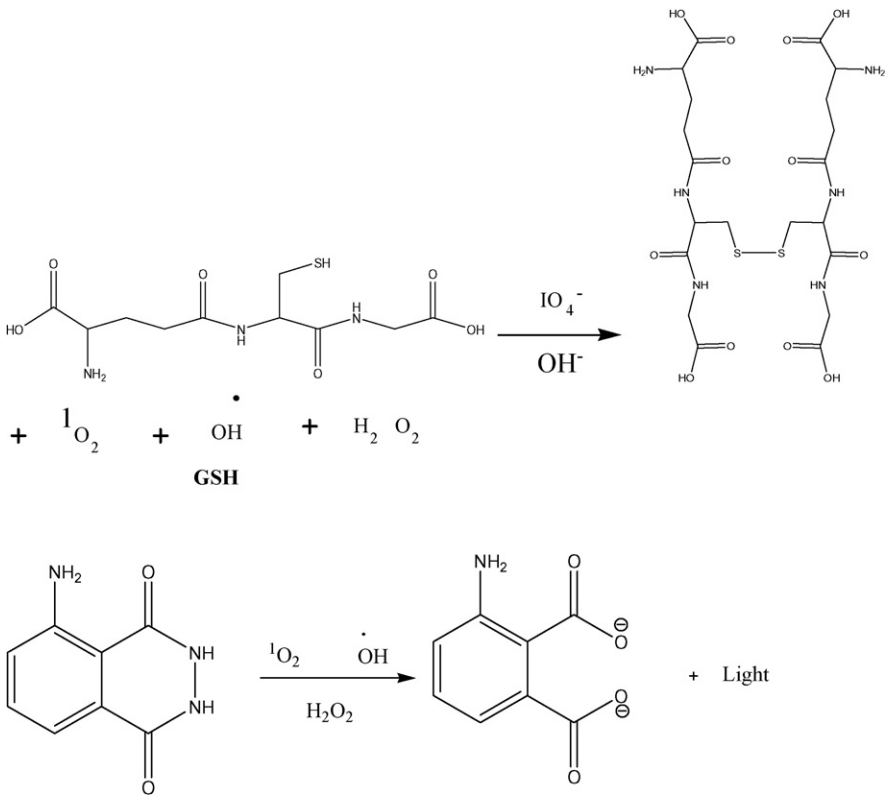
3.1. Effect of variables

The effect of chemical variables such as type of buffer and its pH, concentration of luminol, periodate concentration, and the manifold variables such as pump flow rate, and length of the reaction coil were studied to get the maximum sensitivity.

Chemiluminescence based on the oxidation of luminol is one of the most extensively studied and best-known CL systems. The oxidation is usually carried out in an alkaline solution. The R.S.D. and sensitivity was investigated for three measurements. In Na_2CO_3 buffer, the CL emission was the most stable resulting in the lowest R.S.D. It was observed that higher chemiluminescence signals and better precision could be obtained with the Na_2CO_3–$NaHCO_3$ buffer. The effect of pH of the $NaHCO_3$–Na_2CO_3 buffer on the CL reaction was further examined in the pH range of 9.0–11.5 with 1.0×10^{-3} mol L^{-1} IO_4^-, 1.0×10^{-4} mol L^{-1} luminol, 250 μL 1.0×10^{-6} mol L^{-1} GSH, flow rate of 3.6 mL min^{-1}, and reaction coil of 40 cm. The results showed that increasing the pH values up to 10.5 caused increases in the CL signal, beyond which they decreased slightly. Thus, the optimum pH value was 10.5. Therefore, pH 10.5 ($NaHCO_3$–Na_2CO_3, 0.1 mol L^{-1}) was selected for the study.

The effect of luminol concentration on the CL intensity was examined from 2.0×10^{-5} to 1.0×10^{-3} mol L^{-1} with 1.0×10^{-3} mol L^{-1} IO_4^-, 1.0×10^{-4} mol L^{-1} luminol, 250 μL of 1.0×10^{-6} mol L^{-1} GSH, flow rate of 3.6 mL min^{-1}, and reaction coil of 40 cm. Maximum CL intensity was obtained at 2.0×10^{-4} mol L^{-1} luminol concentration. Therefore, 2.0×10^{-4} mol L^{-1} luminol was used as an optimum concentration.

The influence of $NaIO_4$ concentration on the sensitivity was studied in the range of 1.0×10^{-4} to 5.0×10^{-3} mol L^{-1} sodium periodate with 2.0×10^{-4} mol L^{-1} luminol, pH 10.5, 250 μL of 1.0×10^{-6} mol L^{-1} GSH, flow rate of 3.6 mL min^{-1}, and reaction coil of 40 cm. The results showed that increasing periodate concentra-



Scheme 1. Proposed CL reaction mechanism of luminol–$NaIO_4$–GSH system.

Table 2
Concentration values obtained from the proposed and the reference method for glutathione analysis in human erythrocyte for seven normal subjects

Sample	CL (mmol L^{-1})	Spectrophotometric (mmol L^{-1})	$F_{exp.}$	$F_{tab.(0.05);2,2}$	S_{pooled}	$t_{exp.}$	$t_{tab.(98\%)}$
1	0.89 ± 0.05	1.01 ± 0.09	3.24	19	0.073	3.5	3.8
2	1.18 ± 0.1	1.23 ± 0.07	2.04	19	0.086	2.13	3.8
3	1.00 ± 0.07	1.07 ± 0.12	2.94	19	0.098	0.87	3.8
4	0.98 ± 0.08	1.13 ± 0.06	1.78	19	0.071	2.59	3.8
5	1.30 ± 0.1	1.22 ± 0.07	2.04	19	0.086	1.14	3.8
6	1.23 ± 0.11	1.1 ± 0.07	2.47	19	0.092	1.73	3.8
7	1.05 ± 0.08	1.11 ± 0.06	1.78	19	0.071	1.03	3.8

± shows the standard deviation with three replicates determination.

tion up to 1.5×10^{-3} mol L^{-1} cause increasing CL sensitivity, then it decreased slightly. Therefore, 1.5×10^{-3} mol L^{-1} sodium periodate was selected for further study.

The influence of flow rate on sensitivity was studied in the range of 0.8–4.1 mL min^{-1} with the optimized luminol and iodate concentration at pH 10.5. Higher flow rates gave higher CL signals. This may be because at low flow rate the GSH emit CL at the initial section of the cell and not exposed in front of the detector, whereas at high flow rate the whole GSH emission occurs in the front of the detector. For achieving fast analysis with lower consumption of materials, a flow rate of 3.6 mL min^{-1} was selected.

The effect of length of the reaction coil on the sensitivity was also studied under the optimum chemical variables with flow rate of 3.6 mL min^{-1}. The results showed that the CL sensitivity is dependent on the residence time of the sample zone in the reaction coil. Reaction coil between 10 and 60 cm were tasted. The results showed that the CL intensity was the highest and almost constant between 50 and 60 cm length of the reaction coil. Therefore, a 55 cm reaction coil length was selected for the study.

4. Calibration graph

At the optimum conditions as pH 10.5 ($NaHCO_3$–Na_2CO_3, 0.1 mol L^{-1}), 2.0×10^{-4} mol L^{-1} luminol, 1.5×10^{-3} mol L^{-1} sodium periodate, with a flow rate of 3.6 mL min^{-1}, and 55 cm reaction coil length, the results showed linear correlation between the CL signal and GSH concentration in the range of 1.0×10^{-5} to 2.0×10^{-8} mol L^{-1} with a regression equation of $I = 0.82286\ C_{GSH} + 0.0409$ ($r^2 = 0.9911$), where C_{GSH} is the concentration of GSH (μmol L^{-1}).

The limit of detection was 8×10^{-9} mol L^{-1} and the relative standard deviation for 10 replicate measurements of 1.0×10^{-7} and 1.0×10^{-6} mol L^{-1} glutathione was 3.8% and 4%, respectively.

5. Interference study

Generally, the study of interferences on the CL signal is useful for sample preparation, with the goal to minimize the effects of potential interfering substances on the analysis. In this work the interferences were considered to be common foreign species and compounds which were structurally related to GSH and present in blood samples. For the interference study, the signal of 1.0×10^{-6} mol L^{-1} GSH was recorded. The foreign species were considered not to be interferes if they caused a relative error of less than ±5% in the analytical signal of the GSH. The tolerance ratios were as follows: 1000-fold for K^+, Na^+, PO_4^{3-}, Ca^{2+}, Zn^{2+}, Mg^{2+}, NH_4^+, NO_3^-, SO_4^{2-}, Cl^-, glucose, glycine, tyrosine, valine, and 500-fold for phenylalanine, leucine, methionine, tyrosine, alanine, and 100-fold for tryptophan. However, equal amounts of cysteine or ascorbic acid interfered with the glutathione signal. In addition, captopril interfered at onefold level.

Although ascorbic acid, captopril and cysteine show interferences, they are not present at significant level in hemolysed erythrocyte samples. For investigation of the effect of these two interferences, a concentration of 1.0×10^{-6} mol L^{-1} GSH was recorded and the obtained signal was compared with the mixture of GSH and interfering compound in the ratio of 100:2 for cysteine and 25:1 for ascorbic acid. This ratio was chosen since these interferences are not found in blood at higher concentration [21,22]. In these ratios, the results showed no significant interference for both compounds. Moreover, the interference from ascorbic acid could be minimized by using ascorbic oxidize enzyme which exhibits a high selectivity for oxidation of ascorbic acid if necessary. It is necessary to point out that, in human blood more than 99.5% of GSH was localized in erythrocyte and 97% of cysteine was in plasma [23]. Furthermore, the GSH content is higher than 90% of the total thiol containing compound in blood; therefore thiol compounds in whole blood can be regarded as GSH [24]. Thus, despite significant interference of cysteine the amount of cysteine in hemolysed blood is low and does not affect the CL signal in the evaluated GSH concentration range.

6. Sample analysis

In order to evaluate the applicability of the proposed method, the proposed CL technique was used for determination of GSH in erythrocyte sample of humans and the results were compared with the spectrophotometric method which is a common method for glutathione determination. Seven different blood samples of normal subject (women and men) were determined. The results for analyses of different normal and diabetes hemolysed erythrocyte samples with both methods are given in Tables 2 and 3. The statistical results show that there are good agreements between the results of the proposed CL and standard spectrophotometric methods.

Table 3
Concentration values obtained from the proposed and reference method for glutathione analysis in diabetes

Sample	CL (mmol L^{-1})	Spectrophotometric (mmol L^{-1})	$F_{exp.}$	$F_{tab.(0.05);2,2}$	S_{pooled}	$t_{exp.}$	$t_{tab.(98\%)}$
1	0.70 ± 0.04	0.74 ± 0.06	2.25	19	0.051	0.96	3.8
2	0.69 ± 0.05	0.72 ± 0.09	3.24	19	0.07	1.22	3.8
3	0.66 ± 0.12	0.8 ± 0.06	4	19	0.095	1.8	3.8
4	0.72 ± 0.07	0.81 ± 0.05	1.96	19	0.061	1.8	3.8
5	0.76 ± 0.05	0.7 ± 0.1	4	19	0.08	0.92	3.8

± shows the standard deviation with three replicates determination.

7. Conclusions

The concentration of glutathione in red blood cells is a biomarker for some diseases. In this work, new flow injection CL detection for glutathione in erythrocyte was introduced based on luminol–$NaIO_4$–glutathione system. The proposed method is superior to the other methods, not only for its simplicity, but also for relatively high sensitivity to measure glutathione as low as 8×10^{-9} mol L^{-1}. Moreover some common amino acids did not have any significant interference on the determination of GSH, therefore this method is suitable for measuring glutathione in blood samples.

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