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Spectrophotometric Determination of Glutathione Based Inhibitory Effect on Hemoglobin

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A high sensitive and simple catalytic spectrophotometric method for the determination of reduced glutathione based on its inhibitory effect on the hemoglobin-cayalyzed the reaction of H_2O_2 and acid chrome blue K(ACBK) was developed. The concentration of glutathione is linear with the percentage inhibition (I %) of system under the optimal experimental conditions. The calibration graph is linear in range 1.82×10^{-7} to 4.55×10^{-5} mol L^{-1} with the detection limit of 3.49×10^{-8} mol L^{-1}. The relative standard deviation was 3.97 % for 11 determinations of 1.82×10^{-5} mol L^{-1}. This method can be used for the determination of glutathione in pharmaceuticals and vegetables with satisfactory results.

Key Words: Catalytic spectrophotometry, Glutathione, Hemoglobin.

INTRODUCTION

Reduced glutathione (GSH, γ-L-glutamyl-L-cysteinyl-glycine) is the major non-protein thiol present in elevated amouts in most plants, animal and microbes, which acts as a redox buffer to prevent oxidative damage due to its producing and nucleopilic properties[1,2]. Moreover, glutathione is essential for the regulation of cell prolioferation and maintains the thiol redox protential in the cells, keeping sulphydril groups of proteins in the reduced form[3,4]. Several methods have already been reported for the quantitative determination of glutathione, including spectro-fluorimetry[5,6], chemiluminescence[7,8], electrochemical detection[9,10] and capillary electrophoresis[11]. However, some methods lack sensitivity and selectivity, others are laborious and time-consuming.

Enzyme-catalyzed analytical kinetic methods have been extensively used for substrate, enzyme, inhibitor and activator analysis in several areas of analytical chemistry such as in clinical, pharmaceutical, agricultural, industrial applications and process monitoring[12]. Horseradish peroxidase (HRP; EC 1.11.1.7) is one of the most important oxidases in biology. Having the function of active molecular oxygen, horseradish peroxidase can enhance the oxidation of H_2O_2 directly into H_2O. However, natural enzymes do have shortcomings in some aspects, for example, it is expensive and unstable in solution and has strict requirements for the experimental conditions

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and storage environment in order to retain its catalytic activity. Hemoglobin (Hb), a necessary vehicle for oxygen carriage in body, has the natural quaternary structure as enzymes. In a recent paper hemoglobin was used based on its similar catalytic function as horseradish peroxidase[13].

In this paper, a new sepectrophotometric method based on inhibitory effect of glutathione on the Hb-catalyzed the reaction of H_2O_2 and acid chrome blue K (ACBK) was proposed. The experimental conditions for the system were optimized and glutathione was detected by the decreased absorbance. This method is very simple, sensitive and the detection limit is 3.49×10^{-8} mol L^{-1}. The method has been applied to the determination of glutathione in pharmaceuticals and vegetables with satisfactory results.

EXPERIMENTAL

Hemoglobin (bovine erythrocytes) solution was prepared by dissolving certain amount of hemoglobin (Shanghai Institute of Biochemistry, Shanghai, China) in distilled water and stored below 4 ºC. Acid chrome blue K (ACBK) (Beijing Chemical Plant, Beijing, China) stock solution was prepared by dissolving 0.0586 g of ACBK in 100 mL of water, which was 10^{-3} mol L^{-1} in ACBK and diluted appropriately before use. Hydrogen peroxide solution was prepared by appropriately diluting 0.01 mL of 30 % H_2O_2 (standardized by titration with $KMnO_4$) to 100 mL. It was stored in a brown bottle in a refrigerator. Glutathione (Shanghai Institute of Biochemistry, Shanghai, China) solution was prepared in the concentration of 1.82×10^{-2} mol L^{-1}. Working solution was diluted appropriately before use with distilled water daily.

Doubly distilled water was used throughout. All other chemicals were of analytical-reagent grade.

The spectrophotometric detection was carried out on a V-530 UV-Vis spectrophotometer (Jasco). The pH values were measured with a pHS-3C precision pH meter (Shanghai, China).

Each colour comparison tube was filled with 2.00 mL of pH 8.9 NH_3-NH_4Cl buffer solutions, 3.00 mL of 1.0×10^{-4} mol L^{-1} ACBK, 0.80 mL of 1.0×10^{-3} mol L^{-1} H_2O_2, a proper amount of glutathione solutions and 2.50 mL of 5.0×10^{-6} mol L^{-1} hemoglobin and then diluted with water to 10 mL. After 15 min, absorbance was monitored at the selected maximum absorption wavelength of 563 nm. The percentage inhibition (I %) was calculated on the base of the following equation:

$$I\ \% = 100[(A_s-A_e)-(A_s-A_i)]/(A_s-A_e)=100[(A_i-A_e)/(A_s-A_e)]$$

where A_s, substrate absorbance alone; A_i, substrate absorbance in presence of hemoglobin and inhibitor and A_e, substrate absorbance in the presence hemoglobin only.

RESULTS AND DISCUSSION

The hemoglobin-catalyzed reaction is shown below:

$$H_2O_2 + ACBK \xrightarrow{\text{hemoglobin [O]}} ACBK^+ + H_2O$$

In this redox reaction between H_2O_2 and ACBK, different amounts of glutathione had inhibitory effects on hemoglobin-catalyzed reaction. In addition, there was a good linearity between the amounts of glutathione and I %, on which a new method was based. The absorbance spectra of hemoglobin-catalyzed reaction were obtained and shown in Fig. 1. It is noted that both in the absence of glutathione and in the presence of glutathione, the spectral shapes of the Hb-catalyzed reaction were identical and were consistent with that of the case in the absence of hemoglobin. They were similar in profile but different in size. The addition of glutathione resulted in the inhibition of glutathione on hemoglobin activity.

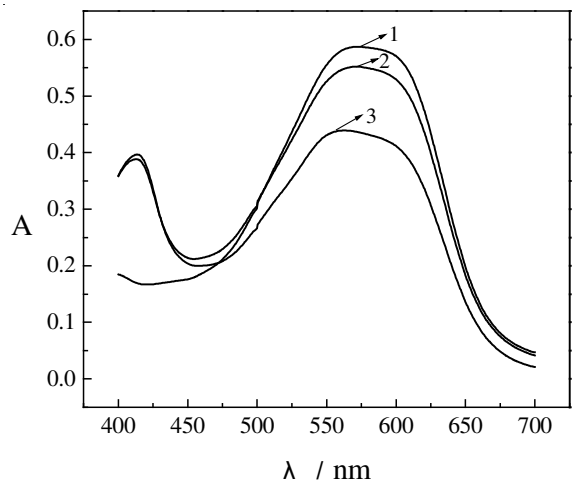

Fig. 1. Absorbance spectra of the system; 1 = In the absence of hemoglobin and glutathione; 2 = In the presence of hemoglobin only; 3 = In the presence of hemoglobin and glutathione. 8.00×10^{-5} mol L^{-1} H_2O_2, 3.00×10^{-5} mol L^{-1} ACBK, 7.50×10^{-7} mol L^{-1} hemoglobin and 3.64×10^{-5} mol L^{-1} glutathione

The variable and ranges studied and the consequent recommended values are summarized in Table-1.

TABLE-1
OPTIMIZATION STUDY FOR GLUTATHIONE
DETERMINATION BY INHIBITION OF HEMOGLOBIN

Variable	Range studied	Recommended value
pH	8.3-9.8	8.9
Hemoglobin (mol L^{-1})	$3.00\text{-}9.00 \times 10^{-7}$	7.50×10^{-7}
H_2O_2 (mol L^{-1})	$0.10\text{-}1.50 \times 10^{-4}$	8.00×10^{-5}
ACBK (mol L^{-1})	$1.50\text{-}3.50 \times 10^{-5}$	3.00×10^{-5}
Time (min)	1-25	15

It is noted that glutathione has less effect in assay involving higher concentrations of hemoglobin. The per cent inhibition increased with increase in hemoglobin

concentration at first, but decreased over 7.50×10^{-7} mol L^{-1}. So 7.50×10^{-7} mol L^{-1} of hemoglobin was selected for further work.

The effect of H_2O_2 concentration on inhibition was studied. The I % increased with the increase in H_2O_2 up to 8.00×10^{-5} mol L^{-1}, above which it had little effect. Thus 8.00×10^{-5} mol L^{-1} H_2O_2 was selected for further study. The I % was greatest at pH 8.9. Considering the absorbance intensity getting too weak at very low ACBK concentration, 3.00×10^{-5} mol L^{-1} ACBK was chosen for further study.

The effect of temperature on the system was investigated in a range from room up to 50 ºC. The time needed to reach equilibrium, not more than 15 min, was prolonged with the decreasing temperature. Given decomposition of H_2O_2 at high temperature, temperature was kept at room temperature and the measurements were carried out after 15 min.

From the results obtained under the recommended conditions (Table-1), it was found that the degree of inhibition of glutathione on the hemoglobin-catalyzed reaction was linear in the range 1.82×10^{-7} to 4.55×10^{-5} mol L^{-1}. The linear response can be fitted to an equation as follows:

$$I\ \% = (20.4381 \pm 2.3888) + (13.5553 \pm 1.0885)\left(\frac{c}{10^{-5}}\right) (r = 0.9782,\ n = 9)$$

'C' is the concentration of glutathione in mol L^{-1}. 'r' and 'n' are the linear correlation coefficient and the number of experiments, respectively. The detection limit, calculated according to the $3S_b/k$ criterion (in which 'k' is the slope over the range of linear used and 'S_b' is the standard deviation (n = 11) of the signal from the blank), was found to be 3.49×10^{-8} mol L^{-1}. The relative standard deviation for 11 replicate determination of 1.82×10^{-5} mol L^{-1} glutathione was 3.97 %. The existing methods for the determination of glutathione are summarized in Table-2. It can be seen that the proposed method has higher sensitivity.

TABLE-2
COMPARISON OF EXISTING METHOD FOR THE DETERMINATION OF GLUTATHIONE WITH PROPOSED METHOD[a]

Methods of determination	Detection limit (mol L^{-1})	Linear range (mol L^{-1})	Ref.
UV-Vis (EI)	3.49×10^{-8}	$1.82 \times 10^{-7} - 4.55 \times 10^{-5}$	Present work
FL	1.10×10^{-10}	$5.00 \times 10^{-6} - 2.00 \times 10^{-4}$	5
FL	6.38×10^{-7}	$3.00 \times 10^{-4} - 2.00 \times 10^{-2}$	6
CL	8.00×10^{-9}	$1.00 \times 10^{-8} - 1.00 \times 10^{-5}$	7
CL	6.80×10^{-8}	$3.00 \times 10^{-7} - 2.00 \times 10^{-5}$	8
ECL	1.80×10^{-5}	$3.20 \times 10^{-5} - 1.60 \times 10^{-3}$	9
ECL	5.00×10^{-7}	$1.00 \times 10^{-7} - 1.70 \times 10^{-6}$	10
CE	5.00×10^{-6}	$9.90 \times 10^{-6} - 9.90 \times 10^{-4}$	11

[a]UV-Vis: Ultraviolet spectrophotometry; EI: Enzymatic inhibition; FL: Fluorimetry; CL: chemiluminescence; ECL: Electrochemical analytical method; CE: Capillary electrophoresis.

Several common amino acids like glycine, alanine, serine and threonine, reducing compounds and vitamins were investigated for their interference for the determination of 1.82×10^{-5} mol L^{-1} glutathione. When the permitted relative deviation is larger than ± 5.0 %, the examined species may cause a significant alteration in the results. The results are shown in Table-3. Results show that the proposed method has good selectivity.

TABLE-3
EFFECT OF VARIOUS SPECIES ON HEMOGLOBIN ACTIVITY

Species	Tolerance ratio
K^+, Na^+, Cl^-, Ca^{2+}, Mg^{2+}, glucose, fructose, BSA	1000
Glycine, alanine, serine, threonine	500
Cystine, Cu^{2+}	100
Cysteine, Fe^{2+}	10
Fe^{3+}	1

The study carried out in this work was applied to determine glutathione in pharmaceuticals and vegetables. For analysis of reduced glutathione sodium for injection, accurate amount of powder were dissolved in double distilled water and then the solution was filtered into a 100 mL calibrated flask. Tomatoes and cucumbers were squeezed, respectively. The juice obtained was diluted quantitatively with double distilled water and the solution was centrifuged for 10 min. Working sulutions were made by appropriate dilution of the concentrated sample solution with buffer, so the final concentration was in the working range for further sample analysis. The results of determination are listed in Table-4.

TABLE-4
ANALYTICAL RESULTS OF SAMPLES

Samples	Added (10^{-5}mol L^{-1})	Found (10^{-5}mol L^{-1})	Recovery (%)
For injection	0	0.36	–
	0.36	0.73	102.7
	2.37	2.73	100
Cucumber	0	0.12	–
	0.36	0.46	94.4
	2.37	2.51	100.8
Tomatoes	0	0.10	–
	0.36	0.45	97.2
	2.37	2.46	99.6

Conclusion

A new spectrophotometric method for trace amount of glutathione determination was developed based on inhibitory effect of glutathione on hemoglobin-catalyzed reaction. This method can be used for the determination of glutathione in pharmaceuticals and vegetables with satisfactory results.

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