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GEOMETRICAL AND APPLIED OPTICS

Hemoglobin-Catalyzed Fluorometric Method for the Determination of Glutathione[1]

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Abstract—A new spectrofluorometric method for the determination of glutathione based on the reaction catalyzed by hemoglobin was reported. The reaction product gave a highly fluorescent intensity with the excitation and emission wavelengths of 320.0 nm and 413.0 nm, respectively. The optimum experimental conditions were investigated. Results showed that low concentration glutathione enhanced the fluorescence intensity significantly. The line ranges were 1.0×10^{-6}–1.0×10^{-5} mol L^{-1} of glutathione and 6.0×10^{-10} mol L^{-1}–1.0×10^{-8} mol L^{-1}, respectively. The detection limit was calculated to be 1.1×10^{-11} mol L^{-1}. The recovery test by the standard addition method gave values in the range of 90.78%–102.20%. This method was used for the determination of glutathione in synthetic and real samples with satisfactory results.

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INTRODUCTION

Reduced glutathione (GSH) is formed by glutamic acid, half peptide methionine and glycine (Glu-cysteine-Cys). The GSH has many functions, such as implicating in the intracellular metabolism of medicine and hormone, combating free radicals effectively and antioxidizing stress, etc. [1]. It is a critical factor in protecting organisms against toxicity and diseases connected to oxidative stress. Its depletion is linked to a number of chronic diseases such as cancer, neurodegenerative and cardiovascular diseases [2]. Since the first discoverer found it in 1921, it has been investigated in various organisms [3].

The quantitative detection of GSH is very important for the research of physiological and pathological mechanism of life. Up to date, a range of analytical methods is available for the determination of GSH in clinical and biological samples, including spectrophotometry [4–6], high-performance liquid chromatography [7–14], electroanalytical [15–19], enzymatic [20–22], capillary electrophoresis [23, 24], spectrofluorimetric [25–27] and chemiluminescence-based methods [28].

Spectrofluorimetry is valuable in the field of biological and medical sciences because of its simplicity, sensitivity, relative selectivity and low cost. Many fluorescent reagents have been synthetized and used for the determination of GSH [29]. However, it is complicated to synthetize new compounds.

Enzymatic assays have considerable advantages over the traditional analytical methods because of their rapidity and high selectivity [30]. However, some enzymes are expensive and unstable. Hemoglobin (Hb), a necessary vehicle for oxygen carriage in the body, has the natural quaternary structure as enzymes. It contains four subunits of polypeptide, and each polypeptide chain contains a heme group that may be able to serve as the active center. What's more, it is cheaper than other enzymes. There have been some reports on the application of Hb as a mimic enzyme for HRP [31, 32]. Up to the present, there has been no report on the application of Hb to the determination of GSH.

In this study, based on the Hb-catalyzed reaction, it was also found that a small amount of GSH could strongly enhance the fluorescence intensity of the reaction product. However, a huge amount of GSH decreased the fluorescence intensity. There was a good linearity between the fluorescent intensity change and the amount of GSH both at low and high concentration. Thus, a sensitive spectrofluorometric method for the determination of GSH was established.

EXPERIMENTAL

Apparatus

Fluorometric detection was carried out on a 970 CRT Spectrofluorometer (Shanghai). The temperature was controlled by using a D2KW-4 thermostat water-bath (Beijing). The pH value was measured

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with a pH-3 digital pH meter (Shanghai). The 3D fluorescence spectra were obtained using Hitachi F-4500 spectrofluorophotometer (Japan).

Reagents

P-cresol (Aldrich) was 99% pure; a stock solution was prepared by mixing 0.15 mL of p-cresol with 99.85 mL of water, which was diluted to 6.0×10^{-4} mol L^{-1} during the experiments. Hydrogen peroxide solutions were prepared by appropriate dilution of a 30% solution with water and standardized by titration with $KMnO_4$. GSH and Hb were obtained from Sigma–Aldrich. A hemoglobin (bovine erythrocytes) solution was prepared by dissolving 0.6450 g of Hb in 100 mL of water and stored at 4°C. The GSH solution was prepared with water daily and diluted to 100 times for use. Double distilled water was used throughout and all other chemicals were of analytical grade.

Procedures

To a 10.0 mL colorimetric cylinder, 1.00 mL of 4.0×10^{-6} mol L^{-1} H_2O_2, 1.00 mL of 6.0×10^{-4} mol L^{-1} p-cresol, 2.00 mL of the buffer solution (pH 9.50) containing 1.2 mol L^{-1} NH_3–NH_4Cl, a GSH standard solution, and 1.00 mL of 6.0×10^{-8} mol L^{-1} Hb were added. The solution was diluted with water up to the mark before shaking. After placing the colorimetric tube in a thermostat water bath (30°C) for 15 min, the relative fluorescence intensity of the system (blank (F_0) and sample (F)) were measured at an excitation wavelength of 320 nm and an emission wavelength of 413 nm. Then, the value of $\Delta F = F - F_0$ was calculated. Blank solutions contained all components except GSH. When samples were determined, GSH standard solution was substituted by the real sample solution prepared before.

Both synthetic and real samples were analyzed for GSH content by the proposed method. Ten tablets were weighed, and the average weight of a tablet was calculated before being ground into a fine powder. A portion of the powder, equivalent to the average weight of a tablet, was dissolved in water and filtered before using.

RESULTS AND DISCUSSION

Optimization of Experiment Conditions

The univariate method was applied to optimize the experiment variables by measuring the fluorescence intensity in establishing a method for assay of GSH. The pH dependence of ΔF was studied over the pH range 8.00–11.00 using a 1.2 mol L^{-1} NH_3–NH_4Cl buffer solution. Maximum signal was achieved in the pH values between 9.30 and 9.70 in NH_3–NH_4Cl buffer as shown in Fig. 1. The pH 9.50 was chosen for the method.

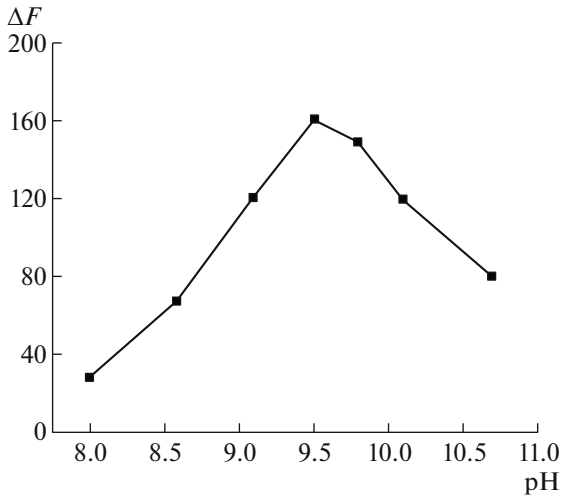

Fig. 1. Influence of the pH on ΔF. H_2O_2, 4.0×10^{-6} mol L^{-1}; p-cresol, 6.0×10^{-4} mol L^{-1}; NH_3–NH_4Cl 1.2 mol L^{-1}; Hb, 6.0×10^{-8} mol L^{-1}; GSH, 1.0×10^{-8} mol L^{-1}.

The buffer volume (1.2 mol L^{-1} NH_3–NH_4Cl) from 1.0 to 3.0 mL was investigated. When 2.0 mL of NH_3–NH_4Cl buffer was added, the fluorescence reached the maximum value and remained at a plateau. Therefore, 2.0 mL of 1.2 mol L^{-1} NH_3–NH_4Cl buffer was used in the procedure.

The reaction of p-cresol with hydrogen peroxide was chosen for the determination of GSH [33]. The influence of p-cresol concentration on ΔF was studied as shown in Fig. 2. No fluorescence was observed in the absence of p-cresol. The amount of p-cresol must be sufficient to react with the H_2O_2. On the other hand, an excessively large concentration of the reagent is detrimental, because it contributes to the blank fluorescence, which caused ΔF to decrease with increasing p-cresol. Thus, 6.0×10^{-4} mol L^{-1} p-cresol was used during the experiments.

The effect of H_2O_2 concentration was investigated in the range of 4.0×10^{-7}–6.0×10^{-6} mol L^{-1} and the value of ΔF increased with increasing concentration as illustrated in Fig. 3. When the H_2O_2 concentration was higher than 5.0×10^{-6} mol L^{-1}, ΔF began to decrease. Thus, 4.0×10^{-6} mol L^{-1} H_2O_2 was recommended for the test.

The influence of the Hb concentration on the ΔF was studied over the range of 6.0×10^{-9}–9.0×10^{-8} mol L^{-1} as shown in Fig. 4. No reaction was observed in the absence of Hb. The value of ΔF increased with the Hb concentration over a certain range, a too high concentration resulted in a decrease of the ΔF. Thus, 6.0×10^{-8} mol L^{-1} Hb was selected during the experiments.

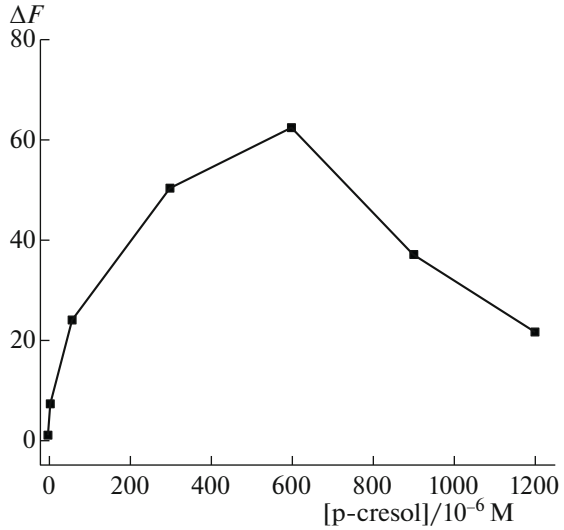

Fig. 2. Influence of the p-cresol on ΔF. H_2O_2, 4.0×10^{-6} mol L^{-1}; NH_3–NH_4Cl 1.2 mol L^{-1} (pH 9.50); Hb, 6.0×10^{-8} mol L^{-1}; GSH, 1.0×10^{-8} mol L^{-1}.

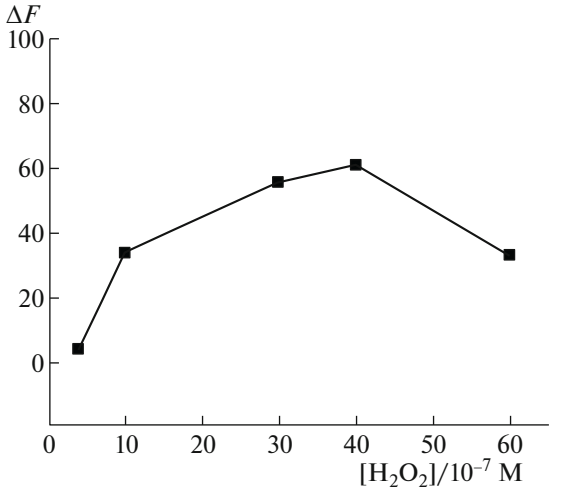

Fig. 3. Influence of the H_2O_2 on ΔF. p-cresol, 6.0×10^{-4} mol L^{-1}; NH_3–NH_4Cl 1.2 mol L^{-1} (pH 9.50); Hb, 6.0×10^{-8} mol L^{-1}; GSH, 1.0×10^{-8} mol L^{-1}.

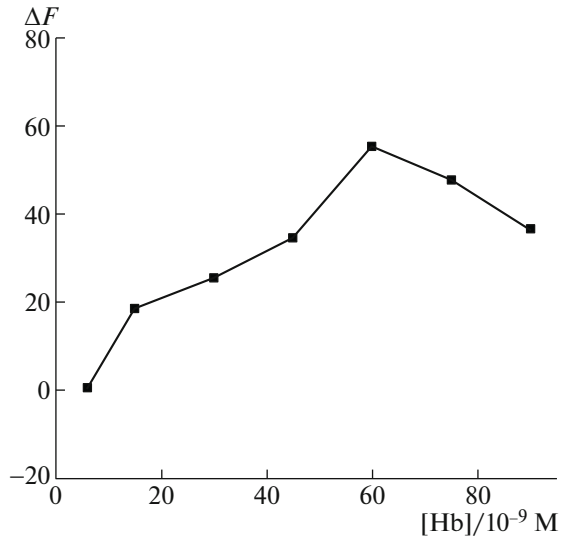

Fig. 4. Influence of Hb on ΔF. p-cresol: 6.0×10^{-4} mol L^{-1}; NH_3–NH_4Cl: 1.2 mol L^{-1} (pH 9.5); H_2O_2: 4.0×10^{-6} mol L^{-1}; GSH: 1.0×10^{-8} mol L^{-1}.

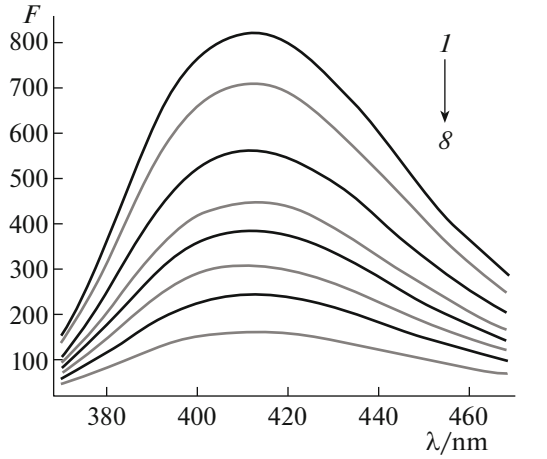

Fig. 5. The fluorescence spectra in the presence of different GSH concentration. GSH: 9.6×10^{-10}, 8.0×10^{-10}, 0, 1.0×10^{-5}, 2.0×10^{-5}, 4.0×10^{-5}, 6.0×10^{-5}, and 1.0×10^{-4} mol L^{-1} (from *1* to *8*). H_2O_2, 4.0×10^{-6} mol L^{-1}; p-cresol, 6.0×10^{-4} mol L^{-1}; NH_3–NH_4Cl (pH 9.5), 1.2 mol L^{-1}; Hb, 6.0×10^{-8} mol L^{-1}.

The effect of the temperature on ΔF was studied in the range of 20–55°C. Because a too high temperature would denature Hb and arouse the decomposition of H_2O_2, 30°C was chosen as the experimental temperature.

The influence of reaction time was investigated in the presence of different amounts of GSH. The relative *FI* was tested every 1.0 s over 40 min. Results showed that the reaction could reach its equilibrium after about 25 min. Therefore, the determination was begun after 30 min.

Fluorescence Spectral Characteristics

In this redox reaction between H_2O_2 and p-cresol catalyzed by Hb, it was found that different amounts of GSH had contrasting effects (activation or inhibition) on this reaction as shown in Fig. 5. Small amount of

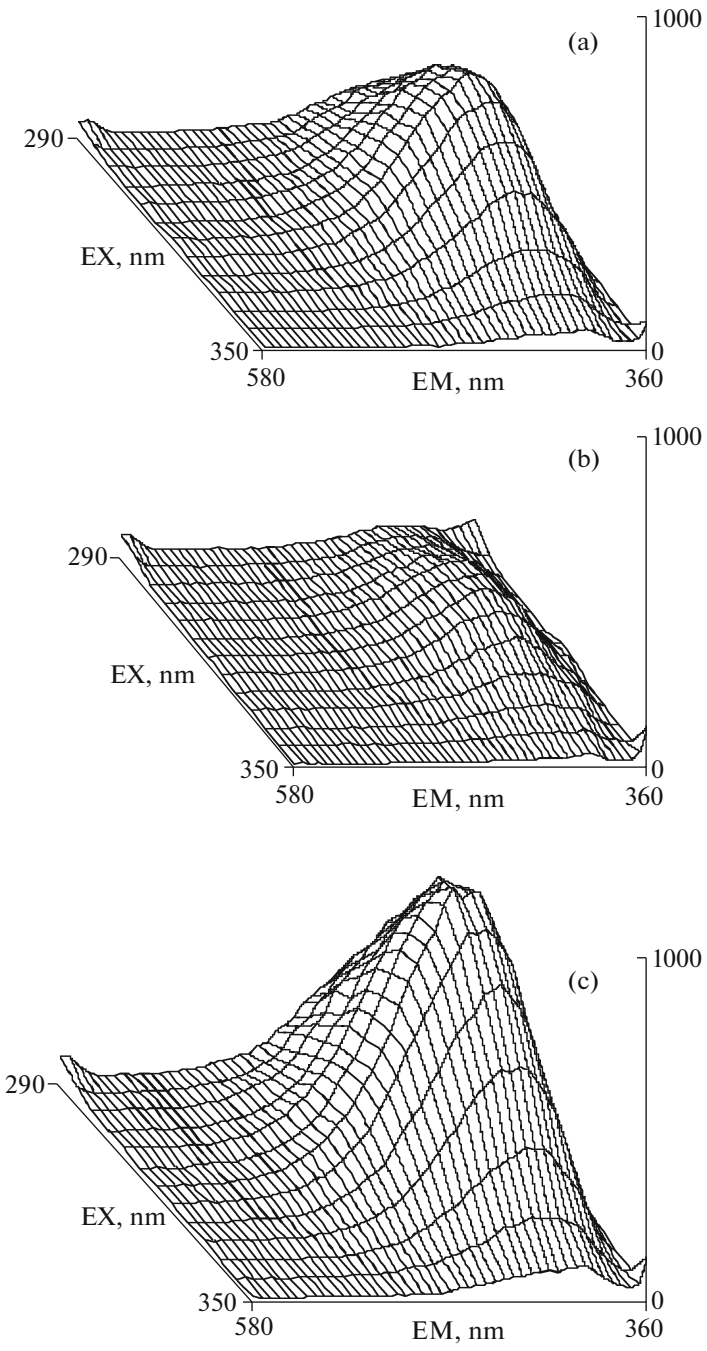

Fig. 6. The 3D fluorescence spectra of the reaction product. H_2O_2, 4.0×10^{-6} mol L^{-1}; p-cresol, 6.0×10^{-4} mol L^{-1}; NH_3–NH_4Cl (pH 9.5), 1.2 mol L^{-1}; Hb, 6.0×10^{-8} mol L^{-1}. (a) in the absence of GSH, (b) the presence of 2.0×10^{-6} mol L^{-1} GSH, (c) in the presence of 1.0×10^{-8} mol L^{-1} GSH.

GSH will enhance the fluorescence intensity as shown in Fig. 5 (curves *1* and *2*), and large amount of GSH will inhibit the reaction as shown in Fig. 5 (curves *4*–*8*). The 3D fluorescence spectra of the products were shown in Fig. 6. It was noted that both in the presence of GSH and the absence of GSH, the 3D fluorescence spectral shapes of the products were similar. The only and remarkable difference between these three was the size of peaks. The addition of GSH, no matter how little or how much, did not result in any new fluorescent products. GSH simply enhanced the ΔF of the product linearly at very low concentration.

Table 1. Influence of the order of reagent addition on the system

No.	System	*FI*
1	H_2O_2 + GSH + p-cresol + buffer solution + Hb	511.5
2	H_2O_2 + p-cresol + GSH + buffer solution + Hb	559.6
3	H_2O_2 + p-cresol + buffer solution + GSH + Hb	494.2
4	H_2O_2 + p-cresol + buffer solution + Hb	498.6
5	p-Cresol + buffer solution + GSH + Hb	26.6
6	H_2O_2 + buffer solution + GSH + Hb	25.2
7	H_2O_2 + p-cresol + buffer solution + GSH	26.0

Study of the Orders of Reagents Addition

The influence of orders of reagent addition on the fluorescence was studied. From Table 1, it was found that the highest fluorescence intensity appeared when GSH was added later than H_2O_2 and p-cresol. So the addition order (H_2O_2 → p-cresol → GSH → the buffer → Hb) was chosen.

In this reaction, p-cresol was oxidized by H_2O_2 to produce the fluorescence product. Hb catalyzed the reaction between p-cresol and H_2O_2. A small amount of GSH would enhance this reaction. The results in Table 1 also showed that this reaction did not occur if one of three substances (H_2O_2, p-cresol, and Hb) was absent.

Interference

The influence of common inorganic ions and amino acids on the measurement of 1.0×10^{-8} mol L^{-1} GSH was studied. If the relative ΔF changed by >5%, the added substance was considered to have caused interference. The results were given in Table 2.

Table 2. Influence of foreign species on the measurement of GSH

Foreign species	Tolerance ratio[a] [Foreign species]/GSH
K^+; Na^+; Ca^{2+}; Mg^{2+}	250
Al^{3+}; Mn^{2+}	50
L-Valine; L-leucine; L-threonine; L-cystine; L-serine; glycine	10

[a] The tolerance ratio is mole ratio.

Analytical Characteristics

Two linear calibration graphs were obtained in GSH concentration (C) ranges 1.0×10^{-6}–1.0×10^{-5}[a] mol L^{-1} and 6.0×10^{-10}–1.0×10^{-8} mol L^{-1}[b], respectively. R is the correlation coefficient. The linear responses can be fitted to equations, as follows:

$$Y^{[a]} = 164.27 + 27.16(C_a/10^{-6}) \ (R^{[a]} = 0.9967),$$

$$Y^{[b]} = 84.17 + 1.37(C_b/10^{-10}) \ (R^{[b]} = 0.9991).$$

The detection limit, calculated according to the three S_d/k criteria, in which k is the slope over the range of linearity used (6.0×10^{-10}–1.0×10^{-8} mol L^{-1}) and S_d is the standard deviation ($n = 11$) of the signal from the blank, is found to be 1.1×10^{-11} mol L^{-1}. The reproducibility of the system was tested by measurements on GSH 1.0×10^{-9} M ($n = 11$). The relative standard deviation is 1.8%.

Application

In order to evaluate the selectivity of the developed methods, the effect of the presence of foreign species was investigated. We prepared solutions which contained 1.0×10^{-6} mol L^{-1} or 1.0×10^{-9} mol L^{-1} GSH and increased concentrations of the potential interferences up to 5.0×10^{-5} mol L^{-1} or 5.0×10^{-9} mol L^{-1},

Table 3. Determination results of the synthetic samples

GSH, mol L^{-1}	Main interference	Found,[a] mol L^{-1}	Recovery	R.S.D. (%) ($n = 5$)[b]
1.0×10^{-9}	Glycine; L-leucine; L-serine[c]	9.90×10^{-10}	99.30%	1.71
1.0×10^{-9}	Glycine; L-leucine; L-serine; L-valine; L-threonine; L-cystine[c]	1.02×10^{-9}	102.10%	1.05
1.0×10^{-6}	Glycine; L-leucine; L-serine[d]	1.02×10^{-6}	102.30%	1.03
1.0×10^{-6}	Glycine; L-leucine; L-serine; L-valine; L-threonine; L-cystine[d]	1.00×10^{-6}	100.10%	0.92

[a] The found amounts were measured by standard curve.
[b] R.S.D. is relative standard deviation for five measurements of samples.
[c] The concentration of the foreign substances are 1.0×10^{-8} mol L^{-1}, respectively.
[d] The concentration of the foreign substances are 1.0×10^{-5} mol L^{-1}, respectively.

Table 4. Study of the recovery test by the standard addition method

Samples obtained, mol L^{-1}	Added, mol L^{-1}	Found, mol L^{-1}	Recovery	RSS ($n = 5$)
3.25×10^{-9}	2.5×10^{-9}	5.58×10^{-9}	93.33%–102.20%	3.70%
4.88×10^{-9}		7.26×10^{-9}		
6.00×10^{-9}		8.43×10^{-9}		
8.13×10^{-9}		10.76×10^{-9}		
9.25×10^{-9}		11.80×10^{-9}		
2.50×10^{-6}	2.5×10^{-6}	4.77×10^{-6}	90.78%–95.40%	1.20%
3.25×10^{-6}		5.56×10^{-6}		
6.25×10^{-6}		8.58×10^{-6}		
7.50×10^{-6}		9.87×10^{-6}		

Table 5. Determination results of GSH in real samples

Sample	Nominal value	The proposed method[(c)]	The reference method[(c)]	Difference
GSH tables, mg/table	100	125.0 [(a)]	120.9	3.3%
GSH tables, mg/table	100	123.4 [(b)]	120.9	2.0%

(a) High concentration linear calibration.
(b) Low concentration linear calibration.
(c) Average of five determinations.

respectively. The tolerance of each foreign species was taken as the largest concentration, yielding an error of <5% in the analytical signal of GSH. The L-valine, L-leucine, L-threonine, L-cystine, L-serine and glycine were tolerated in large amounts.

Firstly, the application of this method was assayed by determining the concentration of GSH in synthetic samples. According to the tolerances of foreign substances, three synthetic samples and six synthetic samples were constructed by adding co-existing components in the standard solution. The samples were determined with satisfactory results as shown in Table 3. The values of recovery for the proposed method were obtained in the range of 99.30%–102.3% as shown in Table 4. Both the accuracy and precision of were acceptable.

Secondly, to illustrate the practicability of the proposed method, the amount of GSH in tablet samples was determined following the procedure described in Section Procedures. The samples were also tested by ultraviolet spectroscopy (UV) [34]. As listed in Table 5, no marked difference was found in the results from both methods, and the results were reproducible and reliable. So, the hemoglobin-catalyzed fluorimetric method has been successfully developed for the determination of traces of GSH for the first time.

CONCLUSIONS

In this work, a spectrofluorometric method for the determination of GSH based on its activation on the hemoglobin-catalyzed reaction was proposed for the first time. Compared with the traditional method, the proposed method has advantages. For example, the proposed method has higher sensitivity, lower detection limit, low costs, simplicity, precision, and reproducibility. This method appears to be fit for determining the concentration of GSH both in synthetic samples and real samples with satisfactory results.

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