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ORIGINAL ARTICLE / ÖZGÜN MAKALE

Development of an Inhibition-Based Colorimetric Method For Glutathione Determination

GSH Tayini için İnhibisyon Temelli Kolorimetrik Bir Yöntemin Geliştirilmesi

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Abstract

Objectives: Glutathione (GSH, L-γ-glutamyl-L-cysteinyl-glycine), one of the major cellular antioxidants, is an important non-protein intracellular physiological antioxidant with sulphhydryl groups for detoxification of reactive oxygen species (ROS) in all living organisms. GSH deficiency has been shown to be associated with many human diseases, including cardiovascular, immune and ageing diseases, arthritis and diabetes. Therefore, the development of an accurate, reliable and sensitive method for the determination of GSH in biological fluids is essential for the understanding of GSH homeostasis in medicine and biochemical research

Material and Methods: In this study, a very inexpensive, practical, rapid, sensitive, and highly specific colorimetric method for the determination of glutathione (GSH) that can be detected by the naked eye was developed. This method is based on the inhibition of horseradish peroxidase (HRP) by GSH. As the concentration of glutathione increases, a pink coloured compound consisting of 4-chlorophenol, H2O2 and 4-aminoantipyrine (4-AAP) decomposes as a result of the reaction catalyzed by HRP, thus reducing the intensity of the colour.

Results: While the linear range of the developed method was found to be between 15.6-1000 mM, the intra- and inter-day repeatability % coefficient of variation values of the method were less than 15%. The effect of potential interfering substances on the developed method was tested, and no interference was found, except for cysteine. Cysteine increased GSH response by 10%. The developed method was used for the determination of GSH in commercial serum samples, and results were obtained between 91-106%.

Conclusion: In conclusion, this study has developed a very simple, inexpensive and unique colourimetric method for the determination of GSH.

Keywords: Glutathione, Horse radish peroxidase, 4-AAP, Inhibition based

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Öz

Amaç: Başlıca hücresel antioksidanlardan biri olan glutatyon (GSH, L-γ-glutamyl-L-cysteinyl-glycine), tüm canlı organizmalarda reaktif oksijen türlerinin (ROS) detoksifikasyonu için sülfhidril grupları içeren önemli bir protein olmayan hücre içi fizyolojik antioksidandır. GSH eksikliğinin kardiyovasküler, bağışıklık ve yaşlanma hastalıkları, artrit ve diyabet dahil olmak üzere birçok insan hastalığı ile ilişkili olduğu gösterilmiştir. Bu nedenle, biyolojik sıvılarda GSH tayini için doğru, güvenilir ve hassas bir yöntemin geliştirilmesi, tıpta ve biyokimyasal araştırmalarda GSH homeostazının anlaşılması için gereklidir.

Gereç ve Yöntem: Bu çalışmada, glutatyon (GSH) tayini için çıplak gözle tespit edilebilen çok ucuz, pratik, hızlı, hassas ve oldukça spesifik bir kolorimetrik yöntem geliştirilmiştir. Yöntem, horseradish peroksidazın (HRP) GSH tarafından inhibisyonuna dayanmaktadır. Glutatyon konsantrasyonu arttıkça, 4-klorofenol, H2O2 ve 4-aminoantipirinden (4-AAP) oluşan pembe renkli bir bileşik, HRP tarafından katalize edilen reaksiyon sonucunda ayrışır ve böylece rengin yoğunluğu azalır.

Bulgular: Geliştirilen yöntemin doğrusal aralığı 15,6-1000 mM arasında bulunurken, yöntemin gün içi ve günler arası tekrarlanabilirlik % varyasyon katsayısı değerleri %15'in altında bulunmuştur. Potansiyel interferans maddelerinin geliştirilen metot üzerindeki etkisi test edilmiş ve sistein dışında herhangi bir interferansa rastlanmamıştır. Sistein, GSH için yanıtı %10 oranında artırmıştır. Geliştirilen yöntem ticari serum örneklerinde GSH tayini için kullanılmış ve %91-106 arasında sonuçlar elde edilmiştir.

Sonuç: Sonuç olarak, bu çalışmada GSH tayini için çok basit, ucuz ve benzersiz bir kolorimetrik yöntem geliştirilmiştir.

Anahtar Kelimeler: Glutatyon, Yabani turp peroksidazı, 4-AAP, İnhibisyon temelli

INRODUCTION

Glutathione (GSH) is a tripeptide consisting of the combination of glutamate, cysteine and glycine and containing sulfhydryl groups commonly found in animals. As an important metabolic substance in the body, it participates in the tricarboxylic acid cycle and glucose metabolism, can activate various enzymes to promote sugar, fat, and protein metabolism (1), and participates in many important biochemical reactions (2). Based on its antioxidant properties, it can remove free radicals and toxins, protect the sulfhydryl groups of important enzyme proteins from oxidation and inactivation, and ensure the normal operation of molecular physiological functions, such as proteins and enzymes (3). Abnormal GSH levels in the body are directly associated with specific diseases including cancer, human immunodeficiency virus (HIV), liver damage, and neurodegenerative diseases (3–5). Therefore, determination of GSH levels is very important for in vivo detection and biological diagnosis systems.

In the last 20 years, several methods have been proposed for GSH determination, such as methods based on high-performance liquid chromatography (HPLC) (6), UV-Vis spectrophotometry (6), fluorescence spectroscopy (7), and mass spectrometry (8). Despite acceptable sensitivity, some of these methods suffer from practical disadvantages, such as the need for expensive and high-tech instrumentation, time-consuming processes, and complex sample preparation processes. Therefore, it

is essential to develop a method for the rapid, inexpensive, and practical determination of glutathione.

Colorimetry is one of the most widely used methods to determine the concentration of a compound by measuring its colour or optical density (9) . In recent years, there has been an increasing interest in the colorimetry technique, which has advantages such as simplicity, low cost, and recognition even with the naked eye. This is because smartphones have become portable photometers with their advanced cameras and colour measuring software (10,11). In other words, when colorimetric methods are developed, the need for an extra device is reduced, unlike methods such as chromatographic and electrochemical methods.

Horseradish peroxidase (HRP) (E.C.1.11.1.7) is an enzyme that has been widely used and investigated for analytical purposes (12). It catalyses the reduction of hydrogen peroxide in the presence of a reducing compound. Methods involving peroxidases described in the literature are based on colorimetry, chemiluminescence, fluorescence and amperometric measurements (13,14). If the reducing agent is selected to produce a coloured product or to further react with a suitable chromogen to produce a coloured product, then hydrogen peroxide (or an oxidase substrate if HRP is combined with a hydrogen peroxide-producing oxidase) can be determined spectrophotometrically. A wide variety of reducing HRP substrates (such as phenol, aminophenols, indophenols, diamines and a number of other compounds) have been used in the spectrophotometric determination of H_2O_2. Trinder et al. (15) developed a method for the determination of H_2O_2 using HRP-catalysed oxidation of phenol in the presence of 4-aminoantipyrine. This method is the working principle of many oxidase-enzyme-based colorimetric kits. One of these is the glucose oxidase/peroxidase system, which was developed for glucose determination. In this reaction, glucose is catalyzed by glucose oxidase and converted to gluconic acid and H_2O_2. The resulting H_2O_2 reacts with 4-aminoantipyrine (4-AAP) and phenol to produce a pink-red-colored compound in the presence of HRP (16).

Some of the most important criteria for analytical method development are that the method should be simple, rapid, and inexpensive, without expensive equipment. GSH levels are directly related to specific diseases, including cancer, human immunodeficiency virus (HIV), liver damage, and neurodegenerative diseases. The inhibitory properties of the analyte to be determined also form the basis of some methods (17,18). Literature data indicate that Reduced glutathione is an HRP inhibitor (19). The aim of this study was to develop a fast, practical, inexpensive, and colorimetric method for the determination of glutathione, one of the most important cellular antioxidants, which can be detected even with the naked eye. Since HRP will be inhibited in the presence of glutathione, the intensity of the pink-red colour produced by the enzymatic reaction of 4-AAP, 4-chlorophenol and H_2O_2 with HRP will decrease. The decrease in absorbance constitutes the principle of determination of glutathione. No colourimetric method based on HRP inhibition for the determination of glutathione has been found in the literature. This is a unique aspect of this study.

MATERIALS AND METHODS

Chemicals and instruments

HRP (1200 U/L) was obtained from Biolabo (France), and 4-AAP, 4-chlorophenol, H2O2, potassium phosphate dibasic, glutathione, and other chemicals were obtained from Sigma Aldrich (USA). The solutions were prepared using ultrapure water. Spectrophotometric measurements were carried out with Multiscan FC Thermo Scientific device.

An Ethics Committee Approval Certificate is not required for studies to be conducted on commercially sold human cadavers, cadaver parts and other biological materials» (ETHICS COMMITTEE APPROVAL DOCUMENT INFORMATION NOTE 2020, n.d.). We declare that our study, the information of which is included above, is among the studies that do not require ethics committee permission due to the use of commercially purchased

Chemicals Required in the Study

Working buffer: 100 mM pH:7 Phosphate Buffer containing 5 mM 4-chlorophenol and 0.25 mM 4-AAP

Substrate: 0.5 mM H2O2 was prepared using water.

HRP: Prepared in 1200 U/L working buffer.

Glutathione: A 20 mM stock solution was prepared in artificial serum.

Preparation of artificial serum: 111 mM NaCl, 2.9 mM $NaHCO_3$, 2.2 mM K_2HPO_4, 0.8 mM $MgCl_2$, 2.5 mM urea, 5 mM KCl are mixed and the pH is adjusted to 7.4 (20).

Principle of measurement

The working principle of this method is illustrated in Figure 1. Accordingly, in the absence of GSH, the enzymatic reaction will take place with high efficiency and the pink-red colour of quinoneimine formed as a result of the reaction will be apparent. In the presence of GSH, HRP is partially inhibited (depending on the concentration), and therefore, the intensity of the color decreases. A decrease in the intensity of the color causes a decrease in absorbance. GSH can be determined from the graph drawn between the decrease in absorbance and GSH concentration.

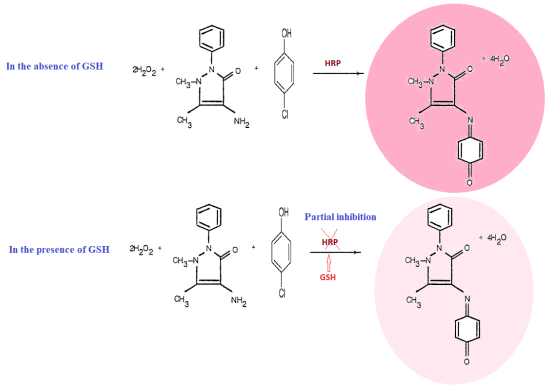

Figure 1. Working principle of the inhibition-based colorimetric glutathione determination method

Test protocol

100 mL HRP solution + 50 mL GSH + 50 mL (0,5 mM) H_2O_2 is added to an ependorf tube and left to incubate at room temperature and in the dark. The reaction was complete within 1 min. The absorbance of the formed color was measured using a spectrophotometer at a wavelength of 504 nm.

Determination of linear range

In order to determine the linear range of determination, standards are prepared by serial dilution from 4 mM GSH. A calibration graph was drawn with 16 points between 0-4 mM, and the range of linearity was determined as the linear range of determination.

Determination of intra-day and inter-day repeatability

The repeatability experiment was performed in 6 replicates at 3 levels, low (0.0625 mM), medium (0.25 mM) and high (1 mM) GSH concentrations, both within and between days, and the mean (x), standard deviation (S.D.±) and % coefficient of variation (% CV) values were calculated.

Determination of substrate specificity and interference effect

In order to determine the substrate specificity of the developed colorimetric GSH method, cysteine, alanine, glycine, glutamic acid, serine, tryptophan, and histidine substrates were used as 0.5 mM instead of GSH. The GSH response was accepted as 100% and compared with the GSH responses of other substrates. The data are plotted as a column graph.

In the interference effect experiment, the same substrates were added separately at the same concentration as that of the sample containing 0.5 mM GSH. The GSH response alone was accepted as 100%, compared with the responses to other substrates + GSH, and graphed.

Recovery

In the recovery experiment, commercially purchased serum samples were spiked with standard additives at 3 levels: low (0.0625 mM), medium (0.25 mM), and high (1 mM) GSH concentrations, and the percentage recovery was calculated.

RESULT AND DISCUSSION

Findings related to the working principle of the developed method

Colorimetric methods allow quantitative measurements to be performed using the relationship between the concentration and absorbance of a solution. To perform these measurements, a spectrum scan was first performed, and the wavelength with the maximum absorbance was selected for quantitative measurements. For this purpose, a spectrum scan was performed between wavelengths of 400-680 nm, including wavelengths in the visible region, and the results are shown in Figure 2. When examining the spectrum scan and the results of the solution obtained from the reaction in the absence of GSH and in the presence of 1 mM GSH (Figure 2), the wavelength at which the maximum absorbance was observed was 504 nm. Absorbance measurements were performed at this wavelength for further optimization and characterization. In addition, the principle of the method developed in this study is to determine GSH by the decrease in intensity of the pink colour formed by HRP activity and the inhibition of HRP by GSH. A careful analysis of Figure 2 shows that the decrease in the red peak is due to the presence of GSH. This confirmed the principles of this study. Optimization of the pH, temperature, buffer type, and concentration of the studied HRP-catalyzed method has been extensively studied (15,16). Therefore, in this study, the measurement conditions were established based on the literature information without repeated optimizations.

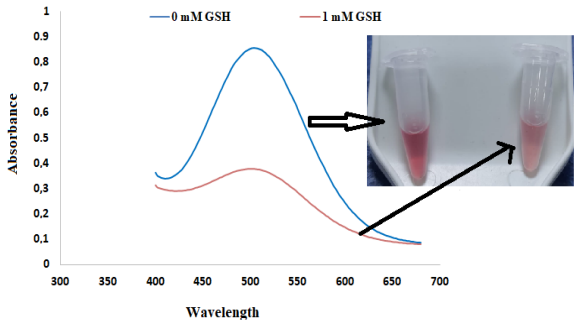

Figure 2. Colourimetric responses obtained in the absence and presence of 1 mM GSH

Results on the linear range of the developed method

The linear range of the developed method is one of the most important factors determining its limitations and performance. The developed method also aims to analyze serum. Therefore, calibrators were obtained by adding a standard to the artificial serum. Samples containing 4 mM GSH were serially diluted, and 16 calibration points between 0.245 m 4 mM were subjected to the test procedure and measured by spectrophotometry. The absorbance was plotted against concentration, and the results are shown in Figures 3 and 4. Figure 3 shows an image of the solutions formed as a result of the working method on the plate, the spectral scan of these solutions, and the GSH concentration-absorbance graph drawn using the absorbances at 504 nm. As shown in Figure 3, no decrease in colour intensity was observed between 0-7.8 mM GSH concentrations. Above 1 mM, the colour disappeared almost completely. The range in which the method showed linearity between concentration and absorbance was found to be between 15.6 mM-1 mM. The equation of the graph plotted in this range was y=0.0006x + 0.0301, and the R^2 value of the graph was 0.9945. The R^2 value of the graph is 0.9945, which is not only acceptable, but also close to perfect. Serum levels of GSH are in mM levels(21), and the linear determination range of the method we developed in this study includes serum GSH levels.

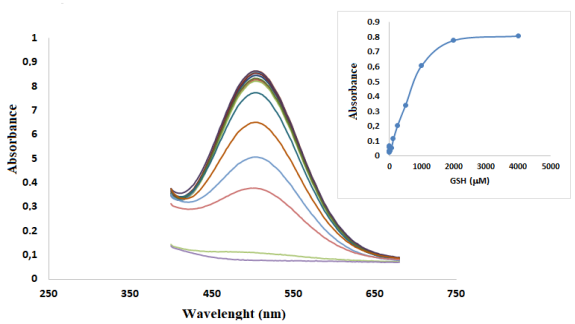

Figure 3. The resulting colours obtained in the presence of 0-4 mM GSH and their spectrum scan between 400-680 nm wavelength

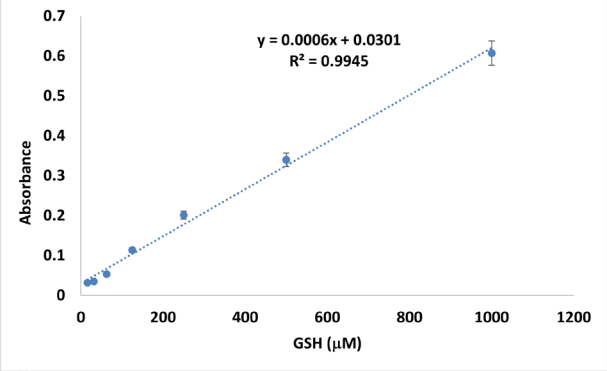

Figure 4. Standard graph for GSH concentrations between 15.6-1000 mM

Selectivity and interference effect of the developed method

To determine the selectivity and interference effect of the colorimetric method developed for GSH determination, cysteine, alanine, glutamic acid, serine, tryptophan, glycine, and histidine were tested at the same concentration (0.5 mM) as GSH. Although these compounds were added alone in the selectivity assay, they were added together with GSH (0.5 mM) in the interference effect assay. The response obtained in the presence of GSH (0.5 mM) was considered 100% and the response obtained from other compounds was plotted as a ratio to the GSH response. The results are shown in Figure 5. According to this, only cysteine gave a response of 13% among the compounds tested, whereas no response was obtained

with the other compounds. In the interference effect experiment, only cysteine increased the response of the method by 11%, whereas the other compounds had no significant effect on the GSH response. These results show that there was no interference effect on the developed method, except for cysteine.

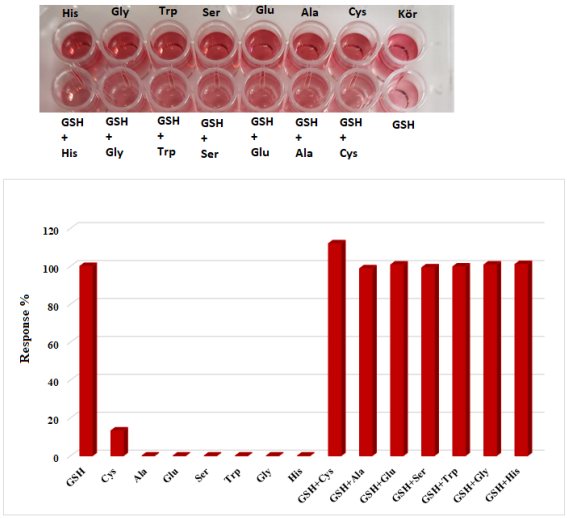

Figure 5. Findings on selectivity and interference effects of the developed method. Cys: Cysteine; Ala: Alanine; Glu: Glutamic acid; Ser: Serine; Trp: Tryptophan; Gly: Glycine; His: Histidine

Accuracy and precision test of the developed method

Intraday and inter-day repeatability and % recovery tests were performed at three levels: low (62.5 mM), medium (250 mM), and high (1000 mM). The repeatability study showed the precision of the method, and the recovery study showed the accuracy of the method. The results are presented in Tables 1 and 2. The repeatability of the method was lower at low concentrations and higher at medium and high concentrations. However, in all cases the repeatability of the method is below the acceptable limit of 15%. This shows that the results of the method are of acceptable precision. The retrieval results were between 91.6-106.9%. These values indicate that the accuracy of the method was close to 100%. The % recovery experiment was performed by standard addition to serum samples obtained commercially from Bio-Rad. The standard was plotted for both the artificial serum and real serum samples (Figure 6). Although the linearity of the graphs plotted with both matrices is good, the slopes of the graphs show a reduction of approximately 33% in the real serum matrix (0.006 vs. 0.004). This suggests that the blank used when studying serum samples should be a real serum sample.

Table 1. Findings related to intra-day and inter-day repeatability of the developed method (n=6)

GSH (mM)	Intraday repeatability CV%	Inter-day repeatability CV%
62.5	11.75	14.55
250	6.83	7.41
1000	3.63	4.45

Table 2. Results on the recovery of the developed method in commercial serum sample (n=3)

Added GSH (mM)	Found (mM)	Recovery %
62.5	57.25±6.81	91.6
250	267.25±21.55	106.9
1000	924.75±36.52	92.47

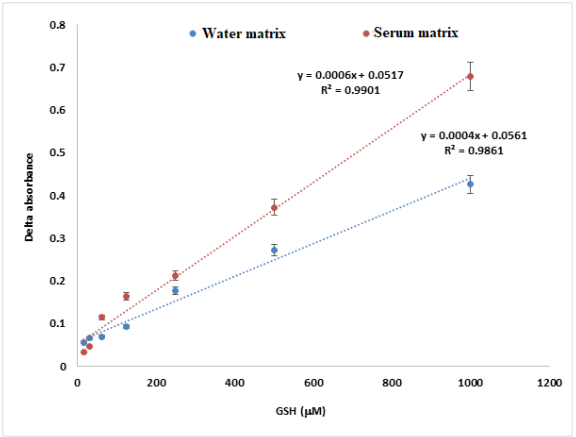

Figure 6. Standard plots obtained by standard addition to both artificial serum and real serum samples

CONCLUSION

In summary, this study demonstrated a colorimetric method for the determination of GSH using the mechanism of HRP inhibition by GSH. Since HRP will be inhibited in the presence of glutathione, the intensity of the pink-red colour produced by the enzymatic reaction of 4-AAP, 4-chlorophenol and H_2O_2 with HRP will decrease. The resulting decrease in absorbance constitutes the principle of glutathione determination. The developed method does not require any complex pretreatment process, does not require expensive equipment, substrate, and enzyme systems, allows rapid determination, is inexpensive, and very practical. Only a spectrophotometer or even smart phones with colour measuring software is sufficient for the method to work. The method showed linearity between concentration and absorbance within the range of serum GSH concentrations. Cysteine exhibited 13% response, whereas the other compounds showed no response. In the interference tests, only cysteine elicited an 11% increase in method response, indicating minimal interference, except for cysteine. The repeatability of the method was lower at low concentrations but improved at medium and high concentrations, remaining below the acceptable 15% limit in all cases, indicating acceptable precision. The recovery results fall between 91.6-106.9%, suggesting a method accuracy close to 100%. % recovery experiments using standard addition to commercially obtained serum samples from Biorad show satisfactory linearity in both artificial and real serum matrices. However, the slopes of the graphs for real serum were reduced by approximately 33%, indicating the necessity of using real serum as a blank in serum sample studies. Table 3 shows a comparison of GSH determination methods. Accordingly, when compared with the literature, it can be said that the determination method developed in this study has a wide determination range and an ideal sensitivity. At the same time, the time required for the method developed in this study was very short, that is, 1 min. Although the sensitivity of the developed method is ideal, the limit of determination can be reduced by using nanoparticles.

The limitations of the study are that it was not compared with a reference method, and it was not integrated into a mobile phone. With the software installed on the mobile phone, it is possible to easily measure glutathione through color measurement.

Table 3. Comparison of GSH determination methods

Method	Prob	Linear range	Reference
Colorimetric	Naphthalimide-capped AuNPs	0.025-2.28 mM	(22)
Colorimetric	Carbon nanodots	0-7 mM	(23)
Colorimetric	Cu-S nanoparticles	0.5-10 mM	(24)
Fluorescence	N-Doped Carbon Dots	0.2-1000 mM	(25)
Colorimetric	Cobalt oxyhydroxide nanosheets	0.1-300 mM	(26)
Colorimetric & Fluorescence	Red-emmiting N-doped CDs	12.5-800 mM	(27)
Colorimetric	TEMPO/ $Cu(acac)_2$/TMB	1-100 mM	(28)
Colorimetric	4-AAP, 4-Klorofenol ve H_2O_2	15.6-1000 mM	This study

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