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The Analytical Performance of a Microplate Method for Total Sulfhydryl Measurement in Biological Samples

[Biyolojik Örneklerdeki Total Sülfidril Gruplarının Ölçümü İçin Bir Mikroplaka Yönteminin Analitik Performansı]

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ABSTRACT

Objective: Sulfhydryl groups of protein, glutathione and free cysteine are readily oxidized to disulfides by oxidants, and are important indicators of oxidative stress. Our aim was to apply the Ellman method for assay of sulfhydryl groups to microplate scale and to determine this method's analytical performance.

Method: Reaction products produced by methanolic 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) were measured at 412 nm using a multimode microplate reader that contains a monochromator.

Results: In standard curve, linearity was obtained in the range of 0.01-4.0 mM concentration of reduced glutathione concentration. Reproducibility of the assay within-run, within-day and between-days were detected as between 6.2%-7.5%. Recovery of glutathione was 91–113%. The method could also be used with tissue homogenates, after solubilization by sodium dodecyl sulfate.

Conclusions: The Ellman's reaction can be applied at the microplate scale with good analytical performance and is a quick and reliable method for use with numerous plasma and tissue samples.

Keywords: Oxidative stress, sulfhydryl groups, Ellman's reagent, microplate method

ÖZET

Amaç: Protein, glutatyon ve serbest sisteinde bulunan sülfidril grupları oksidanların etkisi ile kolayca disülfidlere okside olurlar ve bu yüzden de oksidatif stres çalışmalarında önemli göstergedirler. Protein değişikliklerinin ölçümü oksidatif stres çalışmalarında yaygın olarak kullanılsa da bu ölçümlerin analitik performansını gösteren veriler sınırlıdır. Bu çalışmadaki amacımız, sülfidril gruplarının ölçümünde kullanılan Ellman reaktifini mikroplaka ile küçük hacimli ölçüme olanak verecek şekilde uyarlamak ve bu ölçüm koşullarının analitik performansını saptamaktır.

Yöntem: Çalışmada sülfidril grupları ile reaksiyona giren metanolik 5,5'-ditiobis (2-nitrobenzoik asid) çözeltisi kullanıldı. Reaksiyon ürünleri, monokromatör içeren, çoklu ölçüm moduna sahip bir plaka okuyucu kullanılarak ölçüldü. Ölçüm 412 nm'de gerçekleştirildi.

Bulgular: İndirgenmiş glutatyon kullanılarak çizilen standart grafiklerinde 0.01-4.0 mM aralığında geniş bir doğrusallık, deney içi, gün içi ve günler arası tekrarlanabilirlik çalışmalarında ise %6.2-7.5 aralığında tekrarlanabilirlik saptandı. Çalışmada %91-113'lük bir geriye kazanımın olduğu gösterildi. Ayrıca sodyum dodesil sülfat ile çözünürleştirme işleminden sonra plazma örneklerinden farklı olarak berrak olmayan doku homojenatlarının çözünür hale getirilerek dokulardaki total sülfidril gruplarının ölçülebileceği gösterildi.

Tartışma: Çalışmamızda klasik Ellman reaksiyonu küçük hacimli koşullara uyarlandı ve yöntemin bu koşullarda iyi analitik performansa sahip olduğu gösterildi. Yöntem çok sayıdaki plazma ve doku örneğine sahip çalışmalar için hızlı ve güvenilir bir alternatif olarak değerlendirilmiştir.

Anahtar kelimeler: Oksidatif stres, sülfidril grupları, Ellman reaktifi, mikroplaka yöntemi

Introduction

Oxidative stress leads to oxidative damage by reactive oxygen species (ROS) and is related with pathological processes such as atherosclerosis, cancer, and neurodegenerative disorders [1]. Due to their capacity of being easily oxidized, sulfhydryl groups are vulnerable to oxidative stress. This impairs the sulfhydryl-disulfide balance which is a key player in redox-sensitive processes [2]. Sulfhydryl groups occur as non-protein compounds (glutathione and free cysteine) and, in protein such as thioredoxin, glutaredoxin and albumin, which is the chief protein constituent of blood plasma [1, 3]. The total sulfhydryl group (TSH) content of a biological sample is a valuable indicator of oxidative stress and of oxidative protein damage.

Quantification of protein thiols relies on use of thiol-reactive reagents, such as 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB, Ellman Reagent), p-chloromercuribenzoate (PCMB) and iodoacetamide [4]. DTNB is the most commonly used reagent for the measurement of total sulfhydryl groups spectrophotometrically due to its simplicity and the validity of obtained results [5]. Reaction between sulfhydryl groups and DTNB produces a mixed disulfide and a yellow colored thiolate, 5-thio-2-nitrobenzoic acid (TNB) with maximal absorbance at 412 nm [6].

We have applied the DTNB-based assay to the 96-well plate format. This permits to study with reduced amount of sample and reagents with satisfactory results in a short time period.

Method

DTNB, reduced glutathione (GSH), sodium dodecyl sulphate (SDS), high performance liquid chromatography (HPLC)-grade methanol and all other chemicals were purchased from Sigma-Aldrich (USA). Flat bottom polystyrene 96-well plates were supplied by Greiner (Germany). The multi-mode microplate reader Synergy HT, BioTek (USA) was used for absorbance measurements.

Experimental protocol was approved by the ethic comitte of the Medical School of Dokuz Eylül University. A male Wistar Albino rat (250-300 g) was sacrificed by exsanguination (heart puncture) under ether anesthesia. Its abdominal cavity was immediately opened, the liver was gently removed and washed with cold phosphate buffered saline (PBS; 10 mM, pH 7.4 containing 0.9% NaCl). Rat liver was homogenized in PBS (500 mg liver/5 mL buffer) and centrifuged at 10,000xg for 10 minutes and the supernatant was solubilized by mixing with an equal volume of SDS. The plasma was obtained from two healthy volunteers.

Two hundred microliters of Tris buffer (0.25 M Tris-HCl, pH 8.2 containing 20 mM EDTA) were pipetted into each well and 25 µL human blood plasma or solubilized supernatant was added. Ten microliters of DTNB reagent (4 mg/mL in methanol) was added to each well and incubated for 15 minutes at room temperature, then the absorbance (A_2) of the yellow-colored TNB at 412 nm was obtained against that of a similarly-treated blank (A1). The total sulfhydryl group content in samples corresponding to net absorbance (A_2-A_1) was calculated from standard curve constructed using reduced GSH (between 0 and 450 µM). The glutathione standards were assayed under the above conditions and the curve was constructed by linear regression analysis.

To determine the linearity and stability of the standard curve and precision of the method, several assay were performed by using GSH standards and plasma pool, respectively. For this purpose human plasma pool was obtained and divided into aliquots and stored at -85 °C. Plasma aliquots were thawed and GSH standards were prepared immediately before use. Six independent assays were performed by using plasma and GSH standards on four sequential days. Three of them were performed on three consequent days and the rest of them were on fourth day.

To determine the optimum incubation time, reaction was monitored for 50 minutes following the addition of DTNB reagent and the absorbance was measured for every 5 minutes. 15 minutes was determined to be the optimal.

For recovery studies, 20 µL of GSH solution (750, 1250 or 2500 µM) was added to 480 µL of plasma. Basal sulfhydryl levels were measured using plasma with distilled water in volume equal to that of GSH solution.

To determine the effect of bilirubin, hemoglobin and uric acid present in plasma a plasma pool was obtained. This contained 0.53 mg/dL of bilirubin, 0.006 g/mL of hemoglobin and 4.8 mg/dL of uric acid. Bilirubin stock solution was prepared with DMSO as solvent, hemoglobin was prepared in distilled water and uric acid was dissolved in hot water with a few drop of ammonium hydroxide solution (28%).

We also studied the effect of SDS concentrations between 1-8% on the absorbance. SDS concentration was increased with 1% intervals in a series of assay.

Results and Conclusion

GSH standard curve showed linearity between 0.01-4.0 mM. However we focused on the region 0-450 µM as it reflects the concentration in plasma samples and, solubilized and adequately diluted tissue supernatants. The correlation coefficients (R^2) for six standard curves were ranged between 0.9891-0.9989.

The slope (a) and intercept (b) values in the equation of GSH standard curves ($y=ax+b$) showed only minor differences indicating the stability of all assay conditions. When a constant absorbance ($y=0.2$) was replaced in all six GSH standard curve equation, there was only 3% differences between the lowest and highest concentration (x) values.

The sensitivity of an analytical method is determined from the slope of the GSH standard curve [7]. That deduced from standard curves was 0.0000111±0.000819 absorbance units per micromole sulfhydryl. These results were obtained with the same DTNB reagent and Tris buffer reflecting the stability of these reagents over 4 weeks of storage. They also show the cost-effective properties of the assay.

The limit of detection (LOD) and limit of quantification (LOQ) have been shown to be 1.06 and 3.52 µM, respectively [8]

Precision is the closeness of agreement between independent test results obtained under certain conditions [9]. The precision of our method was also calculated. Within-run (intra-assay) variations of six independent assays showed very similar variations that coefficient of variation (CV) values were profoundly restricted range between 6.2 and 6.7%. Within-day and between-day variation were 6.6 and 7.6% respectively, and represent satisfactory precision for an analytical method.

As shown in Table 1 our recovery results were within the acceptable range of 70-120% [10].

Table 1. Recovery of GSH added to plasma. Each value represents the mean of five assays.

Basal conc. (µM)	Added GSH (µM)	Measured conc. (µM)	Recovered GSH (µM)	% Recovery
260	30	294	34	113
	50	310	50	100
	100	351	91	91

The assay variability was the same as mentioned for intra-assay variation.

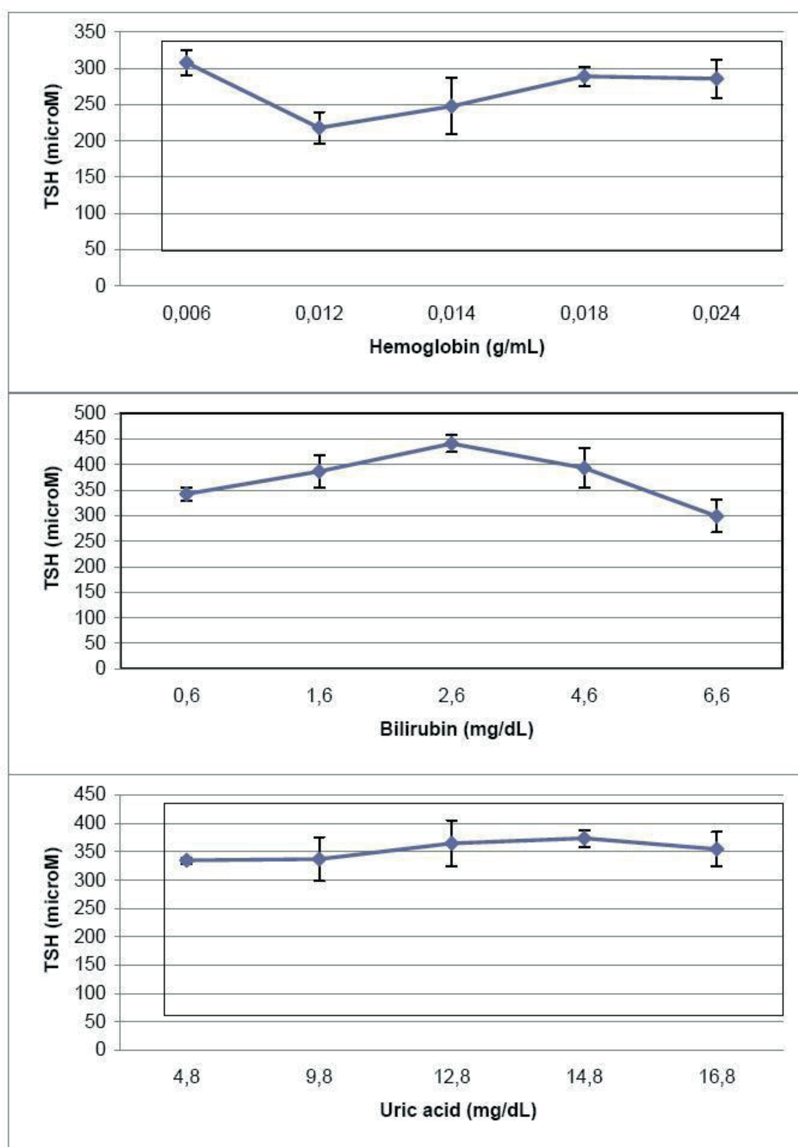

Figure 1. Effect of hemoglobin, bilirubin and uric acid on total sulfhydryl measurement. Each point on graphs represents the mean of five assays. The variability was the same as mentioned for intra-assay variation.

As shown in Fig. 1, hemoglobin and bilirubin had a significant concentration-dependent effect on total sulfhydryl levels, while uric acid showed relatively low interference.

SDS in concentration of 1-8% had no effect on the absorbance of sulfhydryl measurement. This is important in the case of samples having high absorbance and needs to be diluted. The dilution can be made by distilled water that reduces the SDS concentration in the solubilized sample. As a result, an absorbance is obtained within linear range of the standard curve and avoids foam formation caused by higher SDS concentration.

In conclusion, the method we used is a simple, reliable, cheap and can be used with different biological materials. It allows the analysis of numerous samples with good linearity, repeatibility and recovery. It can also be define as a quick method due to short incubation time.

Abbreviations used: TSH, total sulfhydryl groups; ROS, reactive oxygen species; PCMB, p-chloromercuribenzoate; DTNB, 5,5'-dithiobis (2-nitrobenzoic acid); GSH, glutathione; SDS, sodium dodecyl sulphate; HPLC, high performance liquid chromatography; EDTA, ethylenediaminetetra acetic acid; TNB, thiolate anion; LQD, limits of quantification; LOD, limit of detection; DMSO, dimethyl sulfoxide.

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