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Micro-method for the determination of glutathione in human blood

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Abstract

A new procedure is described for the visible-range spectrophotometric analysis of glutathione (GSH) in microvolumes of blood (as low as 0.5 μ L) collected by fingerstick. Samples are diluted 1 to 300 (v/v) in a stabilizing solution, followed by determination of hemoglobin concentration and by acid deproteination. GSH is then measured in the clear supernatant by colorimetry using DTNB, i.e., 5,5'-dithio-bis(2-nitrobenzoic acid), in aqueous solution at pH 7.8. The DTNB reagent is prepared and kept at pH 6.2 until just prior its addition, thus avoiding spontaneous decomposition of the reagent. The assay is rapid, easy to adapt to large-scale studies and it avoids artifactual oxidation of GSH, a common methodological shortcoming. The method is precise with 1.7 to 3.4% intra-day relative standard deviation (RSD) and 2.2 to 4.2% inter-day RSD, and accurate with -1.4% to 2.3% intra-day relative error (RE) and -2.8% to 1.6% inter-day RE. GSH is recovered by 97.5 to 100% at all tested concentrations. The new colorimetric micro-method was validated by a reliable previously reported HPLC method. The procedure is suitable for minimally invasive investigation of oxidative stress in peripheral blood.

Keywords

Blood; DTNB; Fingerstick; Glutathione; Oxidative stress

1. Introduction

Glutathione (γ -glutamyl-cysteinyl-glycine, GSH) is a non-proteinogenic tripeptide and the most abundant low-molecular-mass sulfhydryl groups (-SH) containing thiol. GSH is

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present in most tissues at concentrations ranging between 1 and 10 mM. Because of its ability to provide reducing equivalents, GSH is essential for cellular redox homeostasis [1]. The enzyme glutathione reductase normally maintains the ratio of GSH to its glutathione disulfide (GSSG) between 200:1 and 800:1 in most tissues including blood. Elevated oxidative stress, however, results in increased formation of GSSG and GSH conjugates with electrophiles, thus leading to decreased intracellular GSH levels [2]. GSH deficiency predisposes to oxidative damage which is thought to contribute to onset and progression of many disease states [3, 4]. Conversely, elevated GSH levels generally confer resistance to oxidative stress as observed in many types of cancer cells [5].

A variety of spectrophotometric and HPLC methods are available to measure GSH in blood [6, 7]. Red blood cells GSH reflects much better oxidative status compared to other less accessible tissues [8]. An important limitation of many presently existing methods is artifactual auto-oxidation of GSH to GSSG primarily during sample deproteination by strong acids. This practice can cause up to 30-40% oxidation of GSH to GSSG, is the source of analytical inaccuracy and widespread GSH and GSSG levels in blood [9].

Spectrophotometric analysis of GSH is commonly based on the colorimetric reaction of GSH with 5,5'-dithiobis(2-nitrobenzoic acid), i.e., DTNB, also known as Ellman's reagent [10]. DTNB reacts practically with all biological thiols, and DTNB-based colorimetric methods enable accurate measurement of GSH in most tissues, since GSH comprises >98% of all thiols in most tissues with the notable exception of the kidneys. In this reaction, DTNB is converted into 2-nitro-5-mercapto-benzoic acid (TNB) which possesses its absorption maximum at 412 nm. The DTNB-based method has the great advantage of being rapid, inexpensive, precise and accurate.

Here we propose a new HPLC-validated, micro-colorimetric DTNB-based method that allows for precise and accurate measurement of GSH in very small volumes of blood (down to 0.5 μ L). The method effectively avoids artefactual auto-oxidation of GSH during sample processing. The method is best suited for the analysis of GSH in human blood obtained by fingerstick.

2. Experimental

2.1. Chemicals and materials

GSH (98%), DTNB (98%), *N*-ethylmaleimide (NEM, 98%), diethylene triamine pentaacetic acid (DTPA, 99%), ethylene glycol tetraacetic acid (EGTA, 98%), tripotassium ethylenediaminetetraacetic acid (K_3 EDTA, 99%), trichloroacetic acid (TCA, 99.5%), and all other reagents were obtained from Sigma-Aldrich, Milan, Italy. HPLC grade solvents were purchased from Mallinckrodt-Baker (Milan, Italy).

2.2. Study subjects and blood sampling

Twenty-three healthy subjects (14 females and 9 males, ranging from 27 to 35 years) participated in the study. At screening, they had normal physical examination and laboratory analyses including blood counts, lipid profile, comprehensive metabolic panel, uric acid, iron, urinalysis and urine electrolytes. Subjects were asked to avoid ingestion of *N*-

acetylcysteine (NAC), vitamins C and E, and non-steroidal anti-inflammatory drugs for at least 4 weeks prior to blood collection. Subjects with established diagnosis of diabetes, cancer, HIV, HCV, cardiovascular diseases, or inflammatory diseases were excluded from participation. All participants signed an informed consent prior to enrollment in the study.

Peripheral blood was obtained by phlebotomy of the antecubital vein and capillary blood by fingerstick using standard procedures. The venipuncture samples were collected into tubes containing either (K_3EDTA) for studies on stability or a mixture of K_3EDTA and NEM [11, 12]. Fingerstick samples were collected in a haemolyzing and stabilizing solution as described below.

2.3. Analysis of GSH in blood collected by fingerstick

Two microliters blood were diluted with 0.6 mL of a haemolyzing and stabilizing solution containing 300 μM of each of the following reagents: K_3EDTA , DTPA, and EGTA. Hemoglobin (Hb) concentration was measured in an aliquot of haemolyzed sample by spectrophotometry (500-700 nm, $\epsilon=13.8 \text{ mM}^{-1} \text{ cm}^{-1}$ at 541 nm) [13]. Four-hundred microliter of the diluted and haemolyzed sample were acidified by the addition of 25 μL 60% (w/v) TCA. GSH was measured in the clear supernatant by using a modification of a previously reported colorimetric DTNB-based method [14]. Specifically, after protein removal by centrifugation at $15,000\times g$ for 1 min a slight alkaline pH was restored adding 50 μL of 2 M TRIS to 400 μL of the acid supernatant. DTNB reagent was prepared as a 3 mM stock solution in 0.2 M Na^+/K^+ phosphate buffer, pH 6.2, and added to the cuvette at final concentration of a 10 μM . Reaction between GSH and DTNB was analyzed at 412 nm wavelength by a Jasco UV/Vis 530 spectrophotometer.

2.4. Analysis of GSH by HPLC

Two-hundred microliters blood pre-treated with NEM were acidified by the addition of an equal volume of 12% (w/v) TCA. After protein removal by centrifugation ($15,000\times g$, 1 min), the supernatant was injected into the HPLC apparatus. Chromatographic separation was performed on a Zorbax Eclipse XDB-C18 column (4.6 $\times$ 150 mm, 5 μm ; Agilent Technologies) as described elsewhere [11]. An additional aliquot of blood (10 μL) was haemolyzed by 1:200 (v/v) dilution to measure Hb concentration [13]. An Agilent 1100 instrument equipped with a diode detector was used for absorbance detection.

2.5. Validation of the method and stability analyses

Accuracy, precision and recovery of the method were determined on 3 separate days in blood that was haemolyzed by 1:3 (v/v) dilution with H_2O . Haemolysate was then cleared from low-molecular-mass thiols by gel filtration (Pharmacia PD10 columns, equilibrated with 50 mM phosphate buffer, pH 7.4). Eluted samples were further diluted in H_2O to obtain a 1:300 (v/v) final dilution. To the haemolysate GSH was spiked at 2.5, 5 and 10 μM . The precision of the method was expressed as the relative standard deviation (RSD). Accuracy was expressed as the relative error (RE) and calculated by the Formula: $[(\text{mean observed concentration} - \text{spiked concentration})/(\text{spiked concentration}) \times 100\%]$. The lower limit of quantification (LLOQ) was defined as the lowest concentration of added GSH that was measured with a signal-to-noise ratio >10 .

The stability of GSH in the haemolyzed samples was studied by storing aliquots (3 mL) either at room temperature or at 4°C. Measurement of GSH was carried out at various times in the clear supernatant by reaction with DTNB after acidification with TCA as described above. The stability of GSH in blood supernatants was studied after immediate deproteinization of the haemolyzed samples by the addition of TCA at a final concentration of 3.5% (w/v). Aliquots of supernatants (2 mL) were stored either at room temperature or at 4°C.

2.6. Statistical analysis

Data were expressed as mean $\pm$ standard deviation. Agreement between the HPLC and spectrophotometric methods for GSH determination was analyzed with the Bland-Altman plot [15].

3. Results and discussion

3.1. Development of the procedure to measure GSH in microvolumes of blood

The original DTNB procedure for GSH is commonly performed on samples volumes of at least 0.1 mL. GSH measurement in blood by using the Ellman's reagent generally requires collection of blood by peripheral venipuncture. The modified procedure we describe here can be performed on as little as 0.5 μ L of blood and allows thus GSH measurement in blood collected by fingerstick. The development of the micro-colorimetric for GSH in blood reported here is possible because blood contains about 1.3 mM GSH, and because the TNB, the reaction product of GSH and DTNB, has a quite high molar absorptivity ϵ of 13640 M⁻¹ cm⁻¹. Thus, analysis of GSH in 1000-fold diluted blood is possible without loss of optical transition in cuvettes of 1-cm path length.

Another important issue when analyzing GSH in blood is the measurement of Hb in the same samples. Dilution of the samples with a haemolyzing and stabilizing solution allowed for easy analysis of both, GSH and Hb. Normalization for hemoglobin is necessary when expressing GSH concentration because the latter is significantly influenced by the hematocrit. In fact, >95% of blood GSH resides inside red blood cells, and blood GSH and Hb concentrations correlate strongly each other [11]. In addition, normalization for Hb also allows for the analysis of GSH in blood samples of unknown volume. Importantly, the haemolyzing and stabilizing solution used in our procedure protects GSH against artefactual oxidation of GSH during the subsequent acid deproteinization step. Thus, blood dilution with haemolyzing/stabilizing solution is expected to ensure high precision and accuracy of the assay (see below).

Our modification of the commonly used conditions of DTNB-based assays further improved the method by facilitating analysis of a large number of samples within one assay. In fact, DTNB is usually dissolved in aqueous buffers of pH around 8.0, because of its low solubility at acidic pH values. Slightly alkaline pH enables preparation of mM-DTNB solutions which ensure large excess with respect to the expected concentration of GSH in the sample [10]. Under these conditions, however, accuracy of the assay can be assured only by the parallel processing of blank samples that are used to monitor and correct for spontaneous decomposition of DTNB, which increases with increasing pH. We dispensed

with this step by preparing DTNB at pH 6.2 and using it at a final concentration of 10 μ M. Under these conditions spontaneous breakdown of DTNB was negligible.

Our investigations revealed that TNB also tended to oxidize, presumably as a consequence of its extremely reactive aromatic SH group (not shown). TNB oxidation was a function of the sample acidity and of the time elapsed after the addition of DTNB. This reaction may result in underestimation of GSH. In our hands, however, TNB oxidation was avoided by using the stabilizing solution (not shown). Continuous spectrophotometric recording of the reaction allows precise determination of the maximum value of absorbance, even when the absorbance tends to decrease over time due to TNB oxidation. Fig. 1 shows an example from the application of the proposed procedure in a 2 μ L blood sample collected by fingerstick from a healthy donor. The maximal absorbance of the sample at 412 nm was well above the method LLOQ (0.7 μ M), and the absorbance of the blank was negligible.

3.2. Validation of the method

Precision and accuracy of the method and GSH recovery were determined by adding three concentrations of GSH to aliquots of a haemolyzed blood sample that had been cleared from low-molecular-mass thiols by gel filtration. As shown in Table 1, intra- and inter-day RSD were consistently <5%; intra-day RE ranged between -1.4% and 2.3%, and inter-day RE between -2.8% and 1.6%. Recovery of GSH was consistently close to 100% irrespective of the amount of GSH that was added to the sample.

We also tested the stability of GSH in whole and haemolyzed blood and in the acidic supernatants. When kept at 4°C, whole blood GSH was stable for several days; GSH in haemolyzed blood was stable for at least 2 days. Conversely, GSH in acidic supernatants was quite unstable with GSH levels being dropped significantly after 3-6 h even when stored at 4°C (Fig. 2).

3.3. Application of the procedure to measure GSH in human blood

In a group of 23 healthy subjects, the GSH content in blood was determined by the present micro-method to be 8.54 ± 0.11 nmol/mg Hb, with a standard error of 0.024. Linear regression analysis of the GSH concentrations determined by parallel analysis of the same samples with the reference HPLC method showed a satisfactory correlation ($r = 0.9163$) between the two methods (data not shown). The close agreement between the two methods was further confirmed by the Bland-Altman method. Thus, bias was negligible and all data fell within the limits of agreement (Fig. 3). These data indicate that the main feature of the new micro-method is its excellent precision and accuracy for blood GSH.

A limitation of the new procedure is that GSH but not the molar ratios of GSH to its oxidized forms can be determined. HPLC-based protocols remain the reference method for the latter type of analysis [11].

The micro-method reported here for blood GSH can be utilized in clinical and basic research. For instance to assess oxidative stress which has been linked to onset and/or progression of many disease states and to monitor exposure to potentially hazardous xenobiotics with high reactivity against GSH.

4. Conclusions

The new HPLC-validated procedure for the determination of GSH in microvolumes of blood, that are collected by fingerstick in humans, sample volumes as low as 0.5 μ L are suitable for analysis. Being minimally invasive, the fingerstick method can be used to monitor frequently GSH concentrations while avoiding the discomfort and possible adverse events associated with phlebotomy. This method is likely to be equally suitable for GSH measurement in blood from small experimental animals by minimally invasive techniques which improve the chance of survival in long-term studies. The new procedure simplifies GSH analysis in blood by eliminating the need for the use of blanks but it also improves accuracy of the analysis because it avoids artifactual oxidation of GSH during sample manipulation. Moreover, this method, with minor modifications, can also be applied to the analysis of GSH in red blood cells.

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References

1. Wu G, Fang YZ, Yang S, Lupton JR, Turner ND. Glutathione metabolism and its implications for health. *J Nutr.* 2004; 134:489–492. [PubMed: 14988435]
2. Schafer FQ, Buettner GR. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radic Biol Med.* 2001; 30:1191–1212. [PubMed: 11368918]
3. Giustarini D, Dalle-Donne I, Tsikas D, Rossi R. Oxidative stress and human diseases: Origin, link, measurement, mechanisms, and biomarkers. *Crit Rev Clin Lab Sci.* 2009; 46:241–281. [PubMed: 19958214]
4. Ballatori N, Krance SM, Notenboom S, Shi S, Tieu K, Hammond CL. Glutathione dysregulation and the etiology and progression of human diseases. *Biol Chem.* 2009; 390:191–214. [PubMed: 19166318]
5. Estrela JM, Ortega A, Obrador E. Glutathione in cancer biology and therapy. *Crit Rev Clin Lab Sci.* 2006; 43:143–181. [PubMed: 16517421]
6. Pastore A, Federici G, Bertini E, Piemonte F. Analysis of glutathione: implication in redox and detoxification. *Clin Chim Acta.* 2003; 333:19–39. [PubMed: 12809732]
7. Iwasaki Y, Saito Y, Nakano Y, Mochizuki K, Sakata O, Ito R, Saito K, Nakazawa H. Chromatographic and mass spectrometric analysis of glutathione in biological samples. *J Chromatogr B.* 2009; 877:3309–3317.
8. Veskoukis AS, Nikolaidis MG, Kyparos A, Kouretas D. Blood reflects tissue oxidative stress depending on biomarker and tissue studied. *Free Radic Biol Med.* 2009; 47:1371–1374. [PubMed: 19616614]
9. Rossi R, Milzani A, Dalle-Donne I, Giustarini D, Lusini L, Colombo R, Di Simplicio P. Blood glutathione disulfide: in vivo factor or in vitro artifact? *Clin Chem.* 2002; 48:742–753. [PubMed: 11978601]
10. Ellman GL. Tissue sulfhydryl groups. *Arch Biochem Biophys.* 1959; 82:70–77. [PubMed: 13650640]
11. Giustarini D, Dalle-Donne I, Milzani A, Rossi R. Detection of glutathione in whole blood after stabilization with N-ethylmaleimide. *Anal Biochem.* 2011; 415:81–83. [PubMed: 21545786]
12. Giustarini D, Dalle-Donne I, Milzani A, Fanti P, Rossi R. Analysis of GSH and GSSG after derivatization with N-ethylmaleimide. *Nature Protoc.* 2013; 8:1660–1669. [PubMed: 23928499]

13. Di Iorio EE. Preparation of derivatives of ferrous and ferric hemoglobin. *Methods Enzymol.* 1981; 76:57–72. [PubMed: 7329277]
14. Rossi R, Milzani A, Dalle-Donne I, Giannerini F, Giustarini D, Lusini L, Colombo R, Di Simplicio P. Different metabolizing ability of thiol reactants in human and rat blood: biochemical and pharmacological implications. *J Biol Chem.* 2001; 276:7004–7010. [PubMed: 11096069]
15. Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet.* 1986; 1:307–310. [PubMed: 2868172]

Highlights

Analysis of GSH in microvolumes of blood.

Use of a stabilizing solution that prevents oxidation.

Sample collection by fingerstick.

Fig. 1. Spectrophotometric analysis of the GSH content of a 2- μ L aliquot of human blood. Kinetic analysis of TNB appearance resulting from the reaction of DTNB reagent with either a 2 μ L whole blood sample (upper line) or acidified stabilizing solution (blank sample, lower line), as detailed in the Experimental section.

Fig. 2. Stability of GSH in blood. Three milliliter aliquots of whole blood (downward triangle), haemolyzed blood (1:300, v/v, dilution; white circle) and deproteinized supernatant of haemolyzed blood (dark circle) were stored at 4°C for 96 h, and GSH concentration was analyzed in aliquots at the indicated time-points by spectrophotometry. Blood was obtained from healthy donors by venipuncture. Each point represents the mean $\pm$ SD of four analyses.

Figure 3.

Bland-Altman plot of GSH as measured by the present DTNB-based spectrophotometric method of GSH in fingerstick capillary blood from 23 healthy subjects and by a reference HPLC method for GSH in NEM-spiked peripheral blood from the same subjects using a HPLC method, as described in Experimental section.

Table 1

Recovery, precision and accuracy of the method for GSH in haemolyzed human blood collected from healthy donors by fingerstick.

GSH added (μM)	2.5	5	10
Recovery (%)	98.3 ± 1.2	98.8 ± 0.8	99.3 ± 0.8
Precision (RSD, %)			
Intra-day	2.67 ± 0.36	3.04 ± 0.27	2.01 ± 0.27
Inter-day	3.84 ± 0.47	2.89 ± 0.18	2.75 ± 0.47
Accuracy (RE, %)			
Intra-day %	-1.35 ± 0.27	2.09 ± 0.13	2.34 ± 0.85
Inter-day %	1.63 ± 0.54	-1.27 ± 0.30	-2.84 ± 0.67