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# Immediate stabilization of human blood for delayed quantification of endogenous thiols and disulfides

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ABSTRACT

Endogenous thiols undergo rapid and reversible oxidation to disulfides when exposed to oxidants and are, therefore, suitable biomarkers of oxidative stress. However, accurate analysis of thiols in blood is frequently compromised by their artifactual oxidation during sample manipulation, which spuriously elevates the disulfide levels. Here, we describe a validated pre-analytical procedure that prevents both artifactual oxidation of thiols during sample manipulation and their oxidative decay for months in biosamples that are stored at −80 °C. Addition of *N*-ethylmaleimide to blood samples from healthy donors was used to stabilize whole blood, red blood cells, platelets and plasma disulfides, whereas addition of citrate buffer followed by dilution of plasma with $H_2O$ was used to stabilize plasma thiols. The concentrations of thiols and disulfides were stable in all biosamples for at least 6 months when analyzed by UV/Vis HPLC at regular intervals. Only 3 ml of blood were needed to perform the analyses of thiols and disulfides in the different blood fractions. This pre-analytical procedure is reliable for use in both animal and human prospective studies. Its ease of implementation makes the method suitable for application to multicenter studies where blood samples are collected by different sites and personnel and are shipped to specific specialized laboratories.



## 1. Introduction

The sulfhydryl group (-SH), unless masked within proteins, is the most reactive chemical functionality in cells with a unique ability to undergo very rapid and readily reversible oxidation to disulfides [1]. Sulfhydryl groups are abundant in proteins (P-SH) – primarily as cysteine (Cys) residues – and in low molecular mass (LMM) molecules—including free Cys and glutathione (GSH), with concentrations and redox states that vary substantially between the cellular and extracellular compartments. For example, in human red blood cells (RBCs) glutathione is by far the thiol of highest concentration, with ~99.8% (~2.5 mM) occurring in the reduced form (GSH); only ~0.1% of total glutathione is represented by glutathione disulfide (GSSG) [2,3] and just ~0.05% as *S*-glutathionylated hemoglobin (HbSSG) [4,5]. Conversely, in human plasma cysteine is by far the thiol of highest concentration, but ~90–95% of it circulates in disulfide form, as either cystine (CySS) or *S*-cysteinylated albumin (CySS-Alb) [6,7]. The concentrations and oxidation percentages of thiols vary as a function of physiological and pathological conditions and, consequently, these molecules have attracted growing interest as markers of oxidative activity and burden in a variety of disease states [8,9].

Thiols, however, are highly reactive not only *in vivo* but also *ex vivo*, and analysis of the thiol redox status in whole blood, blood cells and plasma calls for particular attention to sample handling, in order to avoid artifactual oxidation of the —SH group and error

*Abbreviations:* Alb, albumin; Cys, cysteine; CysGly, Cysteinylglycine; CyGlySSP, protein mixed disulfides with CysGly; CySS, cystine; CySS-Alb, *S*-cysteinylated albumin; CySSGly, cystinylglycine; CySSP, *S*-cysteinylated proteins; DTNB, 5′-dithiobis-(2-nitrobenzoic acid); DTT, dithiothreitol; γ-GluCys, γ-Glutamylcysteine; GSH, glutathione; GSSG, glutathione disulfide; GSSP, *S*-glutathionylated proteins; Hb, hemoglobin; HbSSG, *S*-glutathionylated hemoglobin; Hcys, homocysteine; HcySS, homocystine; HcySSP, *S*-homocysteinylated proteins; LMM-SH, low molecular mass thiols; LMM-SS, low molecular mass disulfides; mBrB, monobromobimane; MGP, membrane *S*-glutathionylated proteins; NEM, *N*-ethylmaleimide; Plt, platelets; PRP, platelet-rich plasma; P-SH, protein sulfhydryl group; RBCs, red blood cells; RSSP, *S*-thiolated proteins; —SH, sulfhydryl group; TCA, trichloroacetic acid.

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in the calculation of the thiol to disulfide ratios. We have previously demonstrated that thiols must be stabilized immediately after blood collection in order to prevent artificial oxidation. In that context we showed that blood treatment with *N*-ethylmaleimide (NEM), a membrane permeating agent that rapidly alkylates the −SH group, is effective in avoiding ex vivo artifactual oxidation [3,4,10,11].

As an extension of this observation, here, we describe a validated new pre-analytical procedure that allows stabilization of thiols and disulfides and preservation of their chemical characteristics and concentration for several months after sample collection. This procedure enables precise and accurate measurement of thiols and disulfides in whole blood, RBCs, platelets or plasma for at least 6 months after sample collection.

The protocol represents a technical advancement in this line of research since it can be easily applied to single-center or multicenter basic and clinical prospective studies that often involve extended delays between the times of sample collection and of laboratory analysis.

## 2. Materials and methods

Monobromobimane (mBrB) was purchased from Calbiochem, Milan, Italy. HPLC grade solvents were

purchased from Mallinckrodt-Baker (Milan, Italy). All other reagents were obtained from Sigma-Aldrich, Milan, Italy. Thiol and disulfide standards (GSH, GSSG, Cys, CySS) were at least 98% pure. NEM was prepared as 0.5 ml aliquots of a 310 mM solution in water and stored at −20 °C. Citrate buffer pH 4.3 was prepared by mixing 0.5 M sodium citrate with 0.5 M citric acid and stored at −20 °C.

### 2.1. Blood collection for plasma thiol stabilization tests

Blood (3 ml) was collected from 4 healthy donors into vials containing EDTA and 0.3 ml citrate buffer, and immediately centrifuged at 10,000*g* for 30 s. A fraction of plasma (0.4 ml) was diluted with an equal volume of $H_2O$ and 40 μl citrate buffer and stored at −80 °C in 0.1 ml aliquots. The remaining plasma was stored undiluted at −80 °C in 0.1 ml aliquots. Two milliliters (2 ml) of blood were collected from the same donors into vials containing EDTA and immediately centrifuged at 10,000*g* for 30 s. Plasma was stored undiluted at −80 °C in 0.1 ml aliquots. Low-molecular mass thiols (LMM-SH) were measured in all the samples at the indicated times. Healthy donors granted informed consent to the research protocol.

### 2.2. Stability study

For the stability study, blood was obtained from 5 healthy donors (age 25–55 years), who gave oral informed consent at the time of enrollment. About 15 ml of peripheral venous blood were collected from the antecubital vein, in EDTA tubes, and then 12 ml were immediately treated with 1.2 ml of 310 mM NEM (NEM-blood).

#### 2.2.1. Analysis of GSH and GSSG in whole blood

One aliquot of NEM-blood (0.2 ml) was immediately deproteinized by addition of 0.15 ml 15% (w/v) trichloroacetic acid (TCA) and freshly analyzed; eleven aliquots of 0.2 ml blood were stored at −80 °C for delayed determination at the indicated times.

#### 2.2.2. Analysis of GSH, GSSG and GSSP in RBCs

Five ml of NEM-blood were centrifuged at 8000*g* for 30 s, deprived of plasma (used for plasma analysis of disulfides, see below) and washed twice with saline. RBC pellets were then divided into 24 aliquots of 0.075 ml each. One of these was immediately deproteinized by addition of 0.15 ml 15% (w/v) TCA and freshly analyzed for GSH and GSSG content. A second aliquot was used for analyses of *S*-glutathionylated proteins (GSSP) both in cytoplasm and in membranes after hemolysis by addition of 5 volumes of 5 mM phosphate buffer, pH 6.5, containing 1 mM NEM. The other 22 aliquots were stored at −80 °C for delayed determinations at the indicated times.

#### 2.2.3. Analysis of GSH, GSSG and GSSP in platelets

5 ml of blood-NEM were centrifuged at 4200*g* for 40 s to obtain platelet-rich plasma (PRP). PRP was then centrifuged at 8000*g* for 30 s to remove plasma, washed once with saline, resuspended in 1 ml of saline, counted for platelet number (by a Beckman coulter series Z) and divided into ten 0.1 ml-aliquots. One aliquot was treated with 0.01 ml of 60% (w/v) TCA and immediately used for determination of GSH, GSSG and GSSP content. The remaining aliquots were stored at −80 °C for delayed determinations at indicated times.

#### 2.2.4. Analysis of disulfides in plasma

Plasma separated as described above (see RBC paragraph), was divided into 12 aliquots of 0.1 ml each. One of these was freshly mixed with 0.1 ml of 12% (w/v) TCA and analyzed for disulfide content. The other aliquots were stored at −80 °C for delayed determinations at indicated times.

#### 2.2.5. Analysis of thiols in plasma

One ml and five hundreds microliters of blood were immediately treated with 0.15 ml citrate buffer solution, tilted 5 times, and centrifuged at 8000*g* for 30 s to obtain plasma. Six hundred microliters of plasma were diluted with 0.6 ml $H_2O$ and 0.06 ml citrate solution and divided into 12 aliquots of 0.1 ml each. P-SH and LMM-SH content was immediately determined in one aliquot. The other aliquots were stored at −80 °C for further determinations at indicated times.

To study the effect of storage on thiol and disulfide levels in samples that were not treated for stabilization, about 2 ml of blood were collected in EDTA tubes and handled as follows: (a) for analysis of whole blood, 2 aliquots of 0.2 ml blood were stored at −80 °C for further determinations at the indicated times; (b) for analysis of RBCs, 0.5 ml of blood were processed as described above for RBCs isolation and then 2 aliquots of 0.075 ml each were stored at −80 °C; (c) for analysis of platelets, 1 ml of blood was processed as described above for platelet isolation. Platelets were then resuspended in 0.2 ml of saline and divided into two 0.1 ml-aliquots.

### 2.3. Standard protocol to stabilize thiols/disulfides in whole blood and single blood components

3 ml of blood were collected from 5 healthy people (age 32–49 years) in EDTA Vacutainer tubes, tilted 3–4 times and then immediately treated to stabilize thiols and disulfides. Respective volumes and further details are described in Scheme 1.

### 2.4. Validation of the procedure

The accuracy, precision, and recovery of the above methods were tested in both RBCs and plasma. For this purpose, RBCs were hemolyzed with water (1:3, v/v), LMM-SH were removed by gel filtration (Pharmacia PD10 columns, equilibrated with 50 mM $Na^+/K^+$ phosphate buffer, pH 7.4), and then reconstituted to the initial hemoglobin (Hb) concentration by ultrafiltering excess solvent. Aliquots of the hemolyzed RBC preparation were then spiked with stock solutions of GSH, GSSG and HbSSG to the desired final concentrations, followed by addition of 30 mM NEM (final concentration). After 1-min incubation, the samples were analyzed for GSH, GSSG and HbSSG content. Sample preparation and analysis were performed on 3 separate days.