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Abstract

S-glutathionyated proteins (GSSP), i.e., protein mixed disulfides with glutathione (GSH), are considered a suitable biomarker of oxidative stress. In fact, they occur within cells at low level and their concentration increases markedly under pro-oxidant conditions. Plasma is something different since it is physiologically reach in *S*-thiolated proteins (RSSP), i.e., protein mixed disulfides with various types of low molecular mass thiols (LMM-SH). However, albumin, which is largely the most abundant plasma protein, possesses a cysteine residue at position 34 that is mostly reduced (about 60%) under physiological conditions, but easily involved in the formation of additional RSSP in the presence of oxidants.

The quantification of GSSP requires special attention to sample handling since their level can be overestimated as a result of artefactual oxidation of GSH. We have developed the present protocol to avoid this methodological problem. Samples should be treated as soon as possible after their collection with the alkylating agent *N*-ethylmaleimide that masks –SH groups and prevents their oxidation. The GSH released from mixed disulfides by reduction with dithiothreitol is then labeled with the fluorescent probe monobromobimane and quantified by HPLC.

The method can be applied to many different biological samples, comprising blood components, red blood cell plasma membrane, cultured cells, and solid organs from animal models. The HPLC separation conditions have been set in order to detect not only GSSP but also RSSP. This is particularly useful when analyzing plasma samples.

Keywords: *S*-glutathionylation, HPLC, whole blood, cells, protocols, quantification

Introduction

Protein thiol groups (P-SH) can be present in biological systems in different redox forms, one of which is represented by mixed disulfides with low molecular mass thiols (LMM-SH), also defined as *S*-thiolated proteins (RSSP). More than 95% of RSSP within cells is represented by protein mixed disulfides with glutathione (GSSP). The tripeptide glutathione (GSH) occurs at millimolar concentration within all mammalian cells (Hansen 2009; Giustarini 2017). The –SH group of the cysteine moiety confers to GSH a high reactivity towards a plethora of compounds. By reaction with oxidants, GSH can be converted to its disulfide forms, i.e., glutathione disulfide (GSSG) and *S*-glutathionylated proteins (GSSP). GSSP can form either by direct reaction with reactive oxygen species (ROS) or by reaction with secondary products of oxidative stress such as GSSG (Dalle-Donne 2008). The susceptibility of P-SH to form mixed disulfides with LMM-SH depends on both the solvent accessibility of the thiol within the three-dimensional structure of the protein and the redox potential of the Cys residue.

GSSP have been investigated essentially for two main reasons. First of all, they are considered a good biomarker of oxidative stress in addition to GSSG. Inside mammalian cells, GSH exists almost totally in the reduced form, but both GSSG and GSSP can increase during oxidative perturbations (Schafer 2001**).** Additionally, it is worth noting that, whereas GSSG is rapidly reduced by glutathione reductase, GSSP are less prone to enzymatic reduction (Giustarini 2019). Secondly, *S*-glutathionylation can regulate protein functions by allosteric modification of their conformation. Therefore, *S*-glutathionylation of sensitive proteins can lead to a change in the activity or function of the oxidized protein, thus suggesting a role in physiological signaling (Grek 2013). Moreover, since *S*-glutathionylation is a reversible process, it has been regarded as a protective mechanism against irreversible P-SH oxidation to sulfinic/sulfonic acids (Schafer 2001**)**.

Thus, only accessible P-SH with high thiol-disulfide oxidation potential are likely to undergo *S*-glutathionylation under physio-pathological conditions. For example, *S*-glutathionylated hemoglobin has attracted interest as a clinical biomarker of oxidative stress. Human hemoglobin has an accessible

Cys residue in position β93 (Garel 1982) that occurs almost totally in the reduced form in healthy people but that it is supposed to be *S*-glutathionylated under specific pathological conditions, such as diabetes mellitus and Friederich ataxia (Niwa 2007; Piemonte 2001). Albumin too possesses a free Cys in position 34 that can form mixed disulfides with LMM-SH (Sengupta 2001). In contrast to the intracellular environment, the concentration of GSH in plasma is low (~ 2 μM) and other LMM-SH are present, namely cysteine, cysteinylglycine, homocysteine, and γ-glutamylcysteine. Under physiological conditions about 60% -70% of Cys34 occurs as a free thiol, whereas the remaining Cys34 are involved in the formation of mixed disulfides with the physiological plasma LMM-SH (Sengupta 2001; Turell 2013). It is hypothesized that the percentage of *S*-thiolated albumin can increase in some diseases where oxidative stress has a role for the onset and/or progression of the disease (Candiano 2009).

In vitro experiments with whole blood, red blood cells (RBCs), platelets or cultured cells showed that GSSP increase rapidly under pro-oxidant conditions, i.e., after treatment with peroxides, diamide, disulfiram or menadione (Rossi 2001; Rossi 2006b; Giustarini 2015). Moreover, GSSP concentration is found to be age-dependent in several rat tissues (Giustarini 2009).

We must point out that this field of research is plagued by several pre-analytical artifacts, which usually lead to a large overestimation of GSSP. It is evident that, in order to better understand and define the physio-pathological role of GSSP, you need to pay attention to methodological procedure used to detect them. We have demonstrated that –SH groups can be artefactually oxidized during sample handling in the pre-analytical step, thus raising the levels of both GSSG and GSSP (Rossi 2002). This methodological problem should be particularly taken into consideration when the analysis of intracellular GSSP is carried out. In fact, the higher the real ratio GSH/GSSP, the higher the artificial increase in measured GSSP.

The protocol we describe and discuss here is commonly applied in our laboratory for GSSP quantification in cells and tissues. It has been developed in order to minimize all possible pre-

analytical problems related to artificial oxidation of the –SH group with consequent overestimation of GSSP.

Materials

Chemicals and reagents

Acetonitrile (HPLC-grade)	Sigma-Aldrich # 34851
Acivicin	Sigma-Aldrich # A2295
Boric acid	Sigma-Aldrich # B6768)
Bovine serum albumin	Sigma-Aldrich #A3294
Bradford reagent	Supelco #B6916
Brij® L23 solution	Sigma-Aldrich # B4184
Disodium hydrogen phosphate	Sigma-Aldrich, # 30412
Dithiothreitol	Millipore # 111474
Drabkin's reagent	Sigma-Aldrich, # D 5941
Glacial acetic acid (HPLC-grade)	EMD Millipore Chemicals # AX0074
l-Glutathione, reduced	Sigma-Aldrich # G4251
HPLC Zorbax Eclipse XDB-C18 column 4.6 × 150 mm, 5 μm	Agilent Technologies
Human hemoglobin	Sigma-Aldrich # H7379
Hydrochloric acid 37%	Sigma-Aldrich # 320331
Methanol (HPLC-grade)	Sigma-Aldrich #34860
Monobromobimane	Millipore # 596105
N-ethylmaleimide	Sigma-Aldrich # E1271
Phosphate buffer solution 1.0 M pH 7.4	Sigma-Aldrich # P3619
Potassium dihydrogen phosphate	Sigma-Aldrich, # 229806
l-Serine	Sigma-Aldrich # S4500

Sodium chloride	Sigma-Aldrich # S7653
Sodium hydroxide solution, 2 M	Fluka # 35254
Trichloroacetic acid	Sigma-Aldrich # T6399
Tripotassium EDTA	Fluka, # 03664
Tris base	Sigma-Aldrich # T1503
Water (HPLC-grade)	Sigma-Aldrich # 34877

Equipment

The chromatographic separations reported in this article were performed by using an HPLC Agilent 1100 series with fluorometric detector (Agilent Technologies). For each set of analyses, the column was first conditioned with 100% mobile phase B (HPLC grade acetonitrile), 1.2 ml/min for at least 5 min and the temperature equilibrated at 25 °C. Then, the mobile phase composition was changed to 94% phase A (sodium acetate 0.25% (v/v) pH 3.10) and 6% phase B. Run conditions: 0-5' 6% phase B, 5'-10' gradient until 10% phase B, 10'-10'50'' 10% phase B. After each injection, at the end of the run, the column was flushed with 100% phase B for 2 min and then the system was re-equilibrated to the initial isocratic conditions before the subsequent injection. The signals were recorded setting excitation at 390 nm and emission at 480 nm.

The determination of the protein content was performed by a UV-vis spectrophotometer (Jasco, V-550).

Small laboratory equipment

1. PD-10 desalting columns, bed volume 3.5 ml.
2. Semi-micro disposable cuvettes (Kartell, code 1938)
3. Microcentrifuge tubes, 1.5 ml (Eppendorf or equivalent)
4. Microcentrifuge (Mini Spin, Eppendorf)

5. Vortex 3 (IKA) with test tube inset

6. Homogenizers (Potter Elvehjem P7859 or IKA Ultraturrax with S10 N-8G dispersing element)

7. HPLC vials (Agilent Technologies)

8. pH meter

Stock and working solutions

A. 310 mM NEM: dissolve 388 mg *N*-ethylmaleimide in 10 ml water.

B. 100 mM NaCl/20 mM phosphate buffer pH 7.4: dissolve 584 mg NaCl in 2 ml 1.0 M phosphate buffer solution and 98 ml water.

C. 20 mM Tris base: prepare 2 M Tris base by dissolving 24.2 g Tris in 100 ml water and then dilute it 1:100 in water.

D. 100 mM NaCl/20 mM Tris base: dissolve 584 mg NaCl in 1 ml 2 M Tris base and 98 ml water.

E. 0.1 M phosphate buffer pH 6.5: dissolve 0.95 g KH_2PO4 and 0.427 g Na_2HPO_4 in 80 ml water. Adjust the pH to 6.5 and make the volume up to 100 ml.

F. 5 mM phosphate buffer pH 6.5/NEM: prepare 10 ml buffer by mixing 0.5 ml 0.1 M phosphate buffer pH 6.5, 0.065 ml 310 mM NEM and 9.43 ml water.

G. PBS/NEM: mix 9.73 ml of normal saline solution (normal saline solution; 9 g of NaCl per liter), 0.065 ml 310 mM NEM and 0.2 ml of 1 M phosphate buffer pH 7.4.

H. TCA solutions: all the TCA solutions are prepared by diluting 60% w/v trichloroacetic acid (60 g TCA brought to a final volume of 100 ml with water).

I. Tris-BSAN: prepare 50 mM Tris buffer in water with serine/boric acid/acivicin/NEM (pH 8.0). Dissolve 3.03 g of Tris in 430 ml of water and add 0.62 g of boric acid, 105 mg of serine, 2 mg of acivicin and 50 ml of 310 mM NEM. Adjust the pH to 8.0 with 1 M HCl; adjust the volume to 500 ml with water.

L. 0.5 M Tris pH 7.8 containing 1 mM K_3EDTA: dissolve 3g Tris in 30 ml water. Add 22 mg K_3EDTA, adjust the pH at 7.8 and make the volume up to 50 ml.

M. 10 mM DTT: dissolve 15.4 mg dithiothreitol in 10 ml water.

N. 40 mM mBrB: dissolve 10.8 mg monobromobimane in 1 ml methanol.

O. Mobile phase A (solution for HPLC): prepare 1 liter of 0.25% (vol/vol) acetic acid by diluting 2.5 ml of glacial acetic acid with HPLC-grade water. Add a few drops of 2 M NaOH to bring the pH to 3.1.

Protocol

General aspects

The protocol presented here has been developed in order to measure the total GSSP content in cells and tissues. It is based on the quantification of GSH released by reduction of GSSP. The main steps required for the application of the metods are shown in Figure XX. A key step in GSSP analysis is the separation of proteins by the application of easy and time-saving methods. This step is usually carried out by acidification, followed by protein separation by centrifugation. However, we thoroughly demonstrated that the use of acids induces artificial oxidation of thiols to disulfides (Rossi 2002). As a consequence, a fair amount of artificial GSSP can be formed during this phase. Even if this artifact can be limited by addition of chelating agents (e.g., EDTA) or the use of TCA instead of perchloric acid or metaphosphoric acid, we reported that also under these conditions it cannot be avoided (Rossi 2002). Since the cytoplasmic concentration of GSH is usually two or three orders of magnitude greater than that of GSSP, a minimal percentage of oxidation leading to the production of GSSP causes a massive artificial alteration of their levels. In a few words, the measured concentrations of intracellular GSSP are not physiological but are mostly due to an artifact. We experienced this issue, in particular, when working with RBCs, probably due to the presence of iron and oxygen bound to hemoglobin, which during acidification formed reactive oxygen species (ROS) (Rossi 2002). However, a treatment with an agent that can quickly block all free thiols before any further processing can solve the problem. We proved that the alkylating agent *N*-ethylmaleimide (NEM) is perfect for this purpose. This molecule was shown to easily cross membranes and to rapidly bind all present -SH groups, thus preventing their oxidation during sample handling (Rossi, 2002).

Therefore, a common aspect of this protocol is the sample pre-treatment with NEM, which should be done as soon as possible after its collection. The excess of NEM can be easily removed by performing several washings of protein pellet under slight acidic conditions. At the end of this procedure, after elimination of any trace of soluble GSH, a purified protein pellet is obtained, ready to undergo the proper treatment for the release of protein-bound GSH. An exception is the detection of *S*-glutathionylated hemoglobin (HbSSG) in RBCs. For detecting HbSSG, we separate the protein by gel-filtration to remove excess NEM, GSH, and GSSG at once (Giustarini 2003). This procedure is preferred to the use of washed acid precipitated proteins as we noted that, after restoring neutral pH, the presence of a massive amount of denatured hemoglobin induces oxidation of the reducing agent used to detach GSH from GSSP, hampering a precise and accurate measurement of GSSP. Also GSSP from RBC plasma membrane can be measured because membrane separation is a preparative step performed before gel-filtration. For this purpose, membranes are washed three times with PBS to eliminate GSH and GSSG before further processing.

The homogenization buffer for GSSP analyses in solid tissues must be slightly alkaline in order to speed up alkylation of all -SH groups with NEM. However, the presence of γ-glutamyl transpeptidase (γGT) may present a challenge because it can not only decompose soluble GSH but also GSSP. We obviate this potential drawback by including the γGT inhibitors borate, serine, and acivicin into the homogenization buffer.

For the cleavage of the S-S bridge, a solution of dithiothreitol (DTT) at slight alkaline pH is used. GSH released from GSSP is then labelled with monobromobimane (mBrB) and quantified by HPLC. It is worth of note that this procedure can detect not only GSSP but also protein mixed disulfides with other LMM-SH. This is particularly useful when analyzing plasma samples, where several different LMM-SH are bound to albumin (Sengupta 2001). Conversely, in the intracellular milieu, GSSP represent almost 100% of mixed disulfides present under both physiological conditions and oxidative stress conditions.

We report here, in detail, the procedure to measure GSSP in animal solid organs but the same can be applied to human tissues. As for blood samples, the protocol for human samples is described but it can be applied to blood samples from virtually all animal species too. Nevertheless, since rat hemoglobin can form precipitates when handled at low pH (Brunori, 1982), a specific procedure is described accordingly.

Biological samples and preparative procedure

For the analyses in blood components collect ~3 ml of whole blood from the antecubital vein into K_3EDTA vacutainers and add immediately 300 µl of 310 mM NEM. Invert gently the tubes for 10 times and then apply the procedure described below for the specific kind of blood component.

Whole blood

Cytosolic whole blood GSSP: lyse 0.2 ml of whole blood by addition of three volumes of water. Centrifuge at 15,000×g for 10 min to separate membranes. Desalt the supernatant with PD10 columns equilibrated with 100 mM NaCl containing 20 mM Na^+/K^+ phosphate buffer pH 7.4. Eluted sample is ready for analysis or can be stored at -80°C for up to six months.

!WARNING: since rat hemoglobin crystalizes in hyposmotic buffers, for cell lysis and desalting buffer a 20 mM Tris base solution and a 20 mM Tris base solution containing 100 mM NaCl are used, respectively.

Cell membrane GSSP from whole blood: centrifuge cell lysates as above described at 15,000×g for 10 min to separate membranes. Wash membranes with 5 mM Na^+/K^+ phosphate buffer pH 6.5 containing 2 mM NEM. Repeat this step three times suspending each time the membrane pellet with a glass rod. Membrane samples are now ready for analysis or can be stored at -80°C for up to six months.

Red blood cells

Cytosolic RBC GSSP: purify RBCs by washing three times 400 μl of whole blood with 1 ml of PBS containing 2 mM of NEM. Then lyse 100 μl of packed RBCs by addition of three volumes of water (or 20 mM Tris base for rat RBCs). Centrifuge at 15,000×g for 10 min to separate membranes. Desalt the supernatant with PD10 columns equilibrated with 100 mM NaCl containing 20 mM Na^+/K^+ phosphate buffer pH 7.4 (or 20 mM Tris base for rat RBCs). Eluted sample is ready for analysis or can be stored at -80°C for up to six months.

Cell membrane GSSP: wash the membrane pellets obtained as above described with 5 mM Na^+/K^+ phosphate buffer pH 6.5 containing 2 mM NEM. Repeat this step three times suspending each time the membrane pellet with a glass rod. Membrane samples are now ready for analysis or can be stored at -80°C for up to six months.

Washed platelets

Centrifuge 1.5 ml of whole blood at 4,200×g for 40 s to separate platelet rich plasma (PRP), which is stratified in the upper part of the tube. Collect PRP carefully avoiding the buffy coat, which lies over RBCs. Wash three times the platelets with 1 ml PBS containing 2 mM NEM and, after the third washing, discard the supernatant and add 1 ml of 3% (w/v) TCA. Mix well and centrifuge for 2 min at 14,000×g. Repeat this step for three times resuspending with a glass rod. Finally, discard the supernatant. Samples are now ready for analysis or can be stored at -80°C for up six months.

White blood cells

Centrifuge 1 ml of whole blood at 4,200×g for 40 s to separate PRP, which is stratified in the upper part of the tube. Collect the buffy coat, which lies over RBCs. Add 1 ml of PBS containing 2 mM NEM and centrifuge 30 s at 10000×g. Discard the supernatant and add to the pellet 1.5 ml of an RBC lysing solution (0.1 M sodium chloride in 0.05 M Na^+/K^+ phosphate buffer pH 7.4. Allow to settle for 30 min, then centrifuge for 5 min at 10000×g and discard the supernatant. This step eliminates

RBCs collected along with the buffy coat. Add 1 ml of 3% (w/v) TCA, mix well and centrifuge for 2 min at 14,000×g. Repeat this step for three times resuspending with a glass rod each time. Discard the supernatant. Samples are now ready for analysis or can be stored at -80°C for up six months.

Plasma

Centrifuge 500 µl of whole blood at 10,000×g for 20 s. Add to 100 µl of the supernatant 1 ml of 3% (w/v) TCA. Centrifuge 2 min at 10,000×g. Discard the supernatant and wash three times with 1 ml of 3% (w/v) TCA resuspending each time the pellet with a glass rod. Finally, discard the supernatant. Samples are now ready for analysis or can be stored at -80°C for up six months.

Cell cultures

Remove the culture medium and wash cells twice (1 min each) at room temperature with PBS/NEM. Lyse the cells by treatment with 1 ml of 4% (w/v) TCA. Collect the cells by scraping. Mix well and centrifuge for 2 min at 14,000×g. Discard the supernatant and repeat this step for three times resuspending with a glass rod each time. Finally, discard the supernatant. Samples are ready for analysis or can be stored at -80°C for up to six months.

Solid tissues from animal models

Remove the organ(s)/tissue from the anesthetized animal and rapidly wash them with ice cold saline. Weight the tissues and homogenize them 1: 10 (w/vol) in Tris-BSAN buffer, wait for one minute and then acidify by 1:1 addition of 10% (w/v) TCA. Mix well and centrifuge for 2 min at 14,000×g. Repeat this step for three times resuspending with a glass rod. Finally, discard the supernatant. Samples are ready for analysis or can be stored at -80°C for up to six months.

RSSP reduction and derivatization for HPLC analysis

A. Plasma, washed platelets, white blood cells, cultured cells, solid tissues, whole blood, and RBC membranes

Timing: 30 min

1. Resuspend pellet with 0.4 ml of 0.075 M TRIS base containing 1 mM EDTA and 1 mM DTT.

2. Put the samples in a gyratory shaker for 20 min.

3. Deproteinize 0.1 ml sample by acidification with 40 μl 60% (w/v) TCA

4. Centrifuge 2 min at 10,000×g.

5. React 0.1 ml supernatant with 5 μl of 40 mM mBrB and 20 μl 2 M TRIS base for 10 min in the dark.

6. Add 5 μl 37% (v/v) HCl.

7. Centrifuge 2 min at 10,000×g.

Samples are ready for HPLC analysis.

B. Whole blood and RBC cytosolic fractions

Timing: 30 min

1. React 0.1 ml protein fraction separated by gel filtration or purified membranes with 5 μl of 10 mM DTT for 15 min.

2. Add 5 μl of 40 mM mBrB.

3. Place the sample in the dark for 15 min.

4. Deproteinize the sample by addition of 10 μl of 60% (w/v) TCA.

Samples are ready for HPLC analysis.

Quantification by HPLC

Timing: about 15 min per sample

Chromatographic separation is performed on an Agilent series 1100 HPLC device with a Zorbax Eclipse XDB-C18 column and fluorometric detector.

Determination of concentration is performed by running standard concentrations of GSH (or other LMM-SH for plasma analyses)

Normalization for protein content

The normalization for protein content is carried out for whole blood, RBCs, membrane, white blood cells, and cultured cells analyses.

The concentration of hemoglobin in RBCs is obtained by colorimetric determination at 540 nm by using the Drabkin's reagent. The reagent is prepared according to manufacturer's instructions by dissolving it with Brij® L23 solution. Human hemoglobin is used as a standard.

The protein concentration in RBC membranes, cultured cells, white blood cells, and whole blood is determined by colorimetric reaction with the Bradford reagent (Bradford 1979). Protein pellets are resuspended in 0.1 N NaOH and mixed in a rotatory shaker in order to facilitate protein dissolving. An aliquot of the sample (typically 10 μl) is reacted with the Bradford reagent and analyzed at 595 nm wavelength. Bovine serum albumin is used as a standard.

Platelets values are normalized for cell count and solid tissue values for tissue weight.

Biomedical applications

RSSP in RBCs

The protocol described in this manuscript for detection of GSSP in RBCs as biomarkers of oxidative stress was applied in our laboratory to human and rat samples. In Table 1 the reference values for both cytoplasmic and membrane GSSP are reported. It is evident that the basal levels of intracellular GSSP are usually very low, being about 2-3 orders of magnitude lower than cytoplasmic GSH concentration (Giustarini 2003; Khazim 2013). Membrane GSSP occur at low levels in healthy people too (Giustarini 2003). However, we have recently demonstrated that membrane GSSP

concentration can increase more than that of cytoplasmic GSSP in RBCs under slight and intermittent oxidative conditions, which can mimic the physiological ones. In Fig. 1 the *in vitro* treatment of RBCs with a cyclic low concentration of *tert*-buthylhydroperoxide (*t*-BOOH) slowly delivered with a particular device is reported. *t*-BOOH induced a stepwise increase of both HbSSG and membrane GSSPs; nevertheless, membrane GSSP appear to be less influenced by the absence of the peroxide. As a matter of fact, membrane GSSP decreased more slowly than HbSSG when t-BOOH infusion was stopped, suggesting that membrane GSSPs decreased slowly when pro-oxidant conditions are absent. It should also be noted that the oxidant-induced membrane GSSPs were more abundant than HbSSG at all the analyzed times (Giustarini 2019). Interestingly, as reported in Fig. 1, under slight intermittent oxidative stress GSSG appears to rise and decrease much faster than both membrane and cytosolic GSSP, thus suggesting that HbSSG and even more membrane GSSP are stable biomarkers of oxidative stress, which are likely less affected than GSSG by either temporary variation of oxidant stimulus or sample manipulation.

The results of a clinical study, where the levels of HbSSG were measured in erythrocytes of healthy people (n = 21) and in maintenance hemodialysis (HD) patients (n = 33), are also reported in Table 1. We found that GSSP were 46% higher in HD patients than in age matched healthy controls. It is worth of note that a significant increase in cysteinylated hemoglobin (CySSHb) in HD patients was also observed (38.3 vs 11.5 pmol/mg Hb; $p< 0.001$] and that RBCs from HD patients contained twice as much CySSHb than HbSSG (Kazim 2013).

Rat hemoglobin possesses some extra-reactive –SH groups that make this protein exceptionally susceptible to *S*-glutathionylation under pro-oxidative conditions (Rossi 2001). As shown in Fig. 2, an *in vitro* treatment of RBCs with *t*-BOOH induced a dramatic increase in HbSSG in rat samples but not in human ones. This particular susceptibility was observed also in some mice strains (Giustarini 2006). *S*-glutathionylation of hemoglobin does not interfere with the allosteric capacity of hemoglobin to bind and transport oxygen. It can be thus interpreted as an extra antioxidant defense that RBCs of these animal species can exploit.

GSSP in platelets and white blood cells

Basal levels of GSSP in platelets from healthy humans measured with this protocol were 0.046 ± 007 nmol/10^9 plt (Table 1). Treatment of platelets with disulfiram (a drug used to prevent alcoholism that has a reactive disulfide bridge in his structure) induced a dose dependent increase in GSSP. When platelets were exposed to 1 mM disulfiram, irreversible formation of GSSP occurred, with all cytosolic GSH bound to proteins to form GSSP. Instead, at lower concentrations of the drug the recovery of both GSH and GSSP within one hour was observed. Interestingly, the concentration of GSSP was shown to greatly affect platelet aggregation indicating that P-SH have a key role for platelet activity. In fact, ADP-induced platelet aggregation was found to be inversely correlated with GSSP Figure 3 (Rossi 2006b). Actin can play a role in this process. Indeed, we found that, after treatment with disulfiram, actin is largely *S*-glutathionylated, probably because it has a solvent exposed and extremely reactive Cys residue at position 374 that is susceptible to *S*-glutathionylation (Dalle-Donne 2003).

Basal levels of GSSP in white blood cells were rather difficult to measure because, after treatment with NEM (which is necessary to avoid artifacts), it is not easy to separate them from other blood components. As a matter of fact, we have not been able so far to separate lymphocytes and polymorphonuclear cells using common techniques (separation based on distinct density differences) after treatment with NEM. Therefore, we can report only levels of GSSP on whole blood leukocytes.

RSSP in cultured cells and solid tissues

We recently applied the present protocol to measure GSSP levels in several cell lines (Giustarini 2015). Table 2 shows that GSSP levels in different cell lines fall in the range 14.0 ± 3.3 pmol/mg of protein for human prostate cancer-derived bicalutamide resistant cells (LNCap-Rbic) to 140 ± 26 pmol/mg of protein in human glioblastoma-derived cells (U87). In all the analyzed samples, the

treatment with the oxidant drug disulfiram induced a remarkable dose-dependent increase in GSSP even at very low concentrations (20 μM or even less).

Levels of GSSP in solid tissues from rats at different ages are reported in Table 3. GSSP were found to be significantly increased in some organs (lung, heart, spleen, brain) with ageing (Giustarini 2011), thus supporting a role of free radicals in this physio-pathological process (Viña 2007).

RSSP in plasma

Plasma content of RSSP is very different from the intracellular one both in terms of concentration and species of LMM-SH bound to proteins. In fact, plasma is quite poor in antioxidants and glutathione reductase is present only in traces, which probably derive from cellular disruption. As for thiols, plasma is characteristically rich in their disulfide forms and contains not only GSSP but also *S*-cysteinylated and *S*-homocysteinylated proteins. In addition, also protein mixed disulfides with CysGly and γ-GluCys are generally found (Turrell 2013).

Reference values in 22 healthy controls for different RSSP measured with the present protocol are reported in Table 4 (Fanti 2015). For some of these (CySSP, CyGlySSP, HcySSP), we observed a significant increase in maintenance HD patients. CySSP concentration resulted to be directly related to plasma neutrophil gelatinase-associated lipocalin (NGAL) in maintenance HD patients, suggesting functional coupling of thiol stress and acute-phase response in uremia. High circulating levels of NGAL are only in part the consequence of impaired renal elimination of the protein, as they also result from increased systemic production in response to chronic kidney disease-related inflammation and possibly to iron supplementation (Bolignano 2009, Chakraborty 2012).

Conclusion

S-glutathionilated proteins are investigated as powerful biomarkers of oxidative stress; nevertheless, their analysis is not easy, since their concentration can be overestimated as a result of the oxidation of thiols during the pre-analytical step. Here, we describe a protocol that avoids virtually all

methodological problems by protecting the –SH group from its artificial oxidation. By applying this procedure, we have been able to demonstrate that the basal levels of GSSP are very low in most biological samples, with the exception of the extracellular compartment. However, GSSP represent a sensitive biomarker of oxidative stress, since their concentration increase significantly also at low concentrations of oxidants (Rossi 2006, Giustarini 2019). The protocol can be applied to all biological samples and the quantification by HPLC with fluorometric detector allows the discrimination among the different LMM-SH involved in the RSSP formation. Obviously, this method is characterized by pros and cons. The main pro is that it is quantitative and can estimate with good precision and accuracy the total amount of GSSP in a biological sample. The main con is that it cannot discriminate among the various GSSP, thus it is impossible to identify every single protein undergoing *S*-glutathionylation.

Figure Legends

Figure 1. Cytoplasmic and membrane GSSP and GSSG in RBCs treated with *t*-BOOH. RBCs at a 10% hematocrit were treated by a slow flux of *t*-BOOH for 120 min (flux rate, 0.7 μmol/min) but with a cyclic 4-min time without exposure to the oxidant. At the indicated times, aliquots of sample were collected for HbSSG (triangle), membrane GSSP (circle) and GSSG (square) determination both from the treatment vessel and from a collection point not exposed to the oxidant (4 min after). Times of analysis from the treatment vessel: 0, 30, 60, 90, and 120 min. Times of analysis of samples not exposed to the oxidants: 34, 64, 94, 150 min. Data are the mean of four separate experiments (Giustarini, 2019).

Figure 2. Cytoplasmic HbSSG in human and rat RBCs treated with *t*-BOOH. RBCs were exposed to 1.5 mM *t*-BOOH (final concentration). At the indicated times, *S*-glutathionylated hemoglobin was measured in whole RBC lysates. Times of analysis: 0, 10, 20, 30, 40, 50, 60, 80, 100 and 120 min. Data represent the means ± SD of three separate experiments (Colombo 2010).

Figure 3. Correlation between GSSP concentration and the percentage of platelet aggregation. Human platelets were exposed to 0.1, 0.3, and 1 mM disulfiram. Platelet aggregation was initiated by the addition of ADP (10 μM, final concentration) and was monitored for 10 min by continuous recording of light transmission in a platelet aggregometer (Rossi 2006).

Compliance with Ethical Standards:

Conflicts of interest The authors declare that they have no conflict of interest.

Research involving Human Participants and/or Animals Data All animal handling procedures were carried out in accordance with the European Community guidelines for the use of laboratory animals. The experiments were authorized by the local ethical committee of the University of Siena.

The clinical study with hemodialized patients was approved by the Veteran Administration Research and Development Office Institutional Review Board and The University of Texas Health Science Center San Antonio. Written informed consent was obtained from every subject.

The clinical studies with healthy controls were conducted on volunteers after oral consent.

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Table 1. Reference values for GSSP measured in different blood components. Data are the mean ± SD.

Sample	**Red Blood Cells**		
	Cytoplasmic nmol/g Hb	**Membrane nmol/g protein**	**Ref**
Healthy humans (n=12)	9.90 ± 3.02	0.736 ± 0.159	Giustarini 2003
Healthy humans (n=21)	13 ± 0.3		Kazim 2013
HD patients (n=33)	20±0.5		Kazim 2013
Rat (n=10)	10.5 ± 2.57	0.682 ± 0.259	Giustarini 2003
Platelets			
Healthy humans (n=5)	0.046±007 nmol/10^9 plt		Giustarini 2015
White blood cells (cytoplasmic + membrane)			
Healthy humans (n=10)	37.7±12.4 nmol/10^6 cells		Unpublished results

Table 2. Levels of GSSP in several cell lines under basal conditions and after a 15-min treatment with the pro-oxidant drug disulfiram. Data (expressed as pmoles/mg protein) are the mean ± SD, n = 3.

Cell line	control	20 μM disulfiram
BAEC[a]	110±17	2378±67
HUVEC	24.3±1.2	476±54
Panc-1	87.1±10	1423±96
NT2D1	27.8±3.0	86.3±11.2
A549	50.1±7.5	96.9±5.5
RD	107±3	238±31
HEK	47.3±3.6	284±19
T98	330±41	379±22
HaCaT	20.6±1.4	85.1±6.3
U87	140±26	427±16
IMR-90	28.3±3.7	493±75
BRC-230	26.4±2.4	269±32
MCF-7	33.9±3.0	186±11
A 375	233±13	11050±715
LNCaP	20.7±7.1	45.9±1.1
LNCaP-Rbic	14.0±3.3	32.2±2.6

[a]**Abbreviations**: BAEC, bovine aortic endothelial cells; HUVEC, human umbilical vein endothelial cells; Panc-1, human pancreatic carcinoma-derived cells; NT2-D1, human pluripotent embryonal carcinoma-derived cells; A549, human lung carcinoma-derived cells; RD, human rhabdomyosarcoma-derived cells; HEK 293, human embryonic kidney-derived cells; T98G, human brain glioblastoma-derived cells; HaCaT, human spontaneously immortalized keratinocyte cells;

U87, human glioblastoma-derived cells; IMR90, human embryonic lung-derived fibroblasts; BRC-230, human breast cancer-derived cells; MCF-7, human breast cancer-derived cells; A375, human melanoma-derived cells; LNCaP, human prostate cancer-derived cells; LNCaP-Rbic, bicalutamide-resistant cells derived from LNCaP.

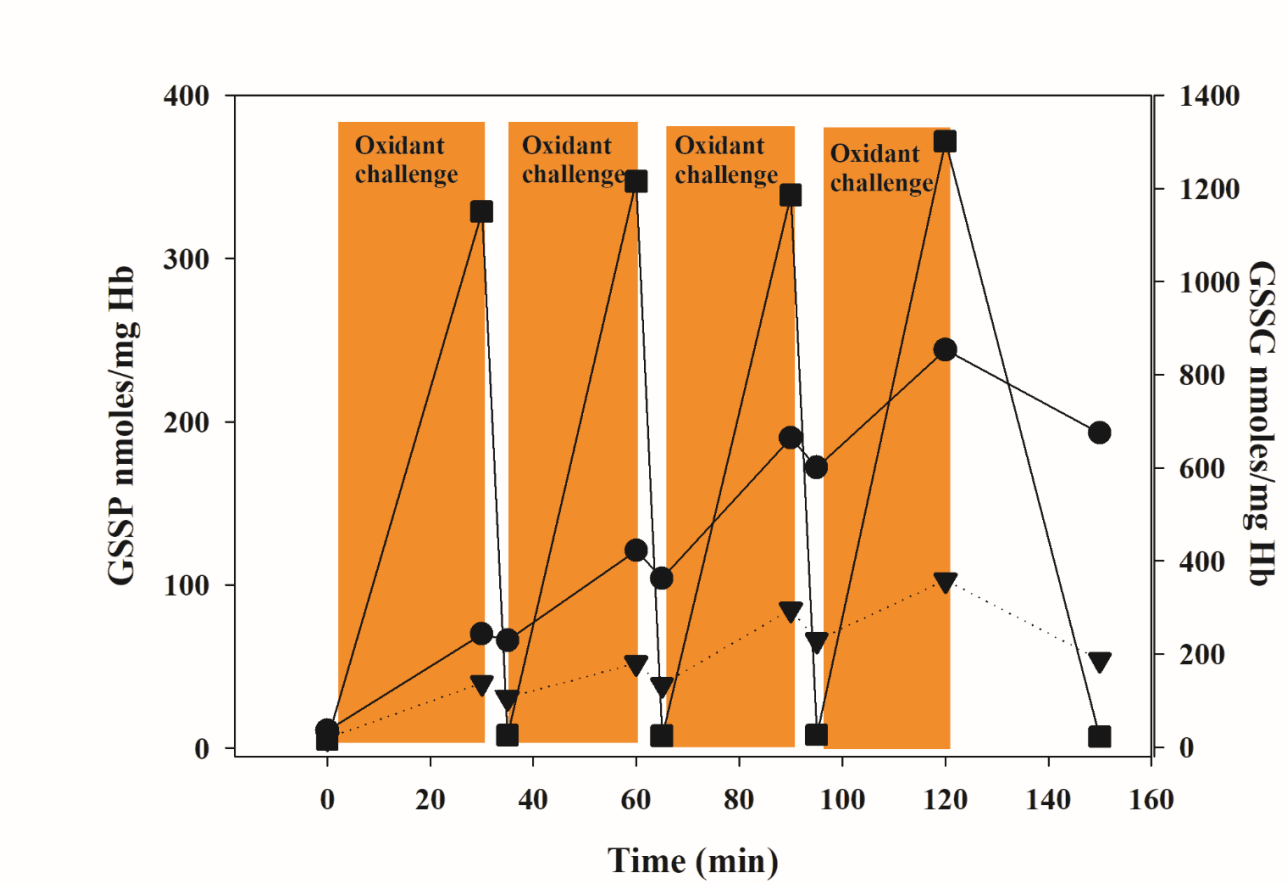
Table 3. Reference values for GSSP in solid rat tissues at different ages. Data are expressed as μM and are the mean ± SD, n = 4 for each study-group. *p < 0.05 vs 3-month-old rats; **p < 0.01 vs 3-month-old rats; #p < 0.05 vs 9-month-old rats; ##p < 0.01 vs 9-month-old rats.

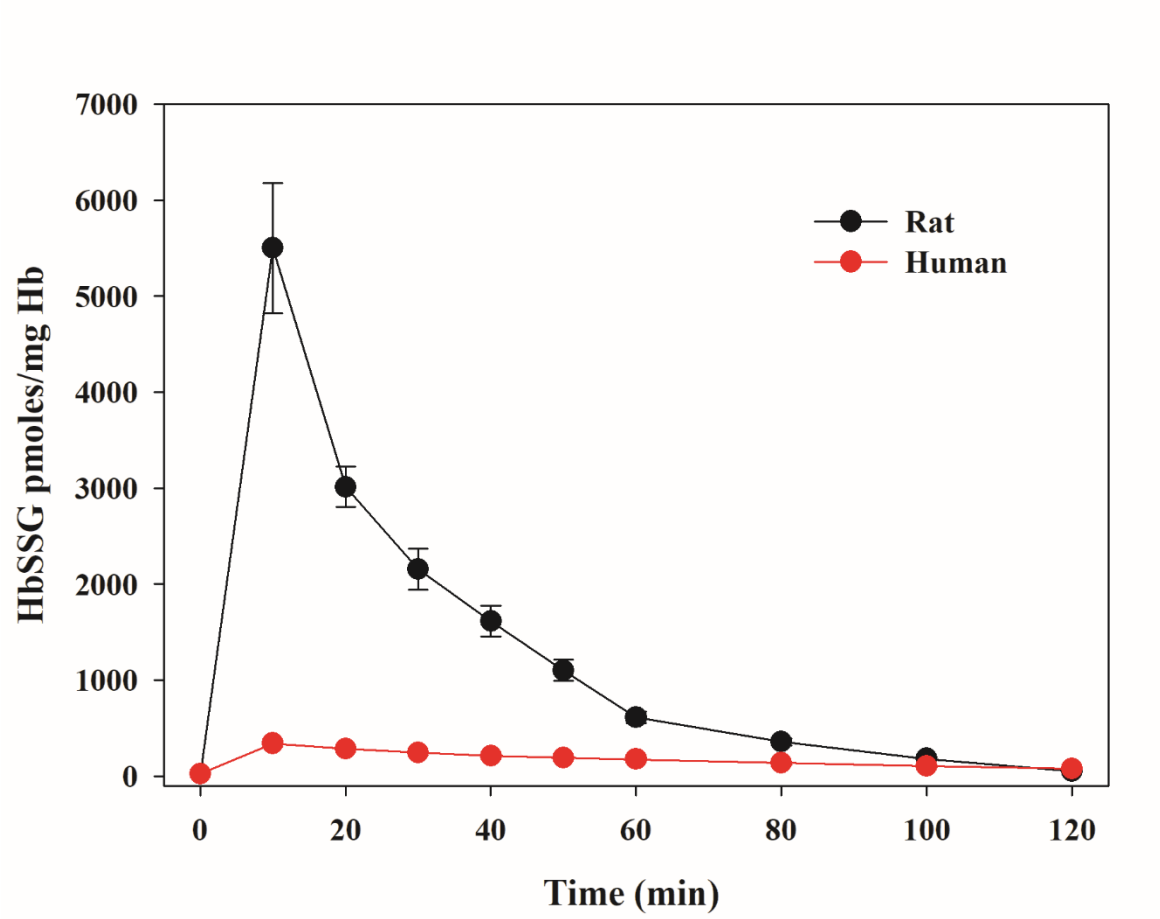
Tissue	3 months	9 months	20 months
Liver	4.71 ± 0.85	4.81 ± 0.64	5.99 ± 0.64
Kidney	2.25 ± 0.32	1.85 ± 0.12	1.85 ± 0.64
Lung	2.18 ± 0.33	1.95 ± 0.25	2.90 ± 0.64*,#
Heart	1.12 ± 0.08	1.20 ± 0.12	1.82 ± 0.64**, #
Spleen	6.84 ± 0.56	5.40 ± 0.93	9.20 ± 0.64**, ##
Testis	1.55 ± 0.21	1.59 ± 0.05	0.95 ± 0.64
Brain	6.04 ± 0.44	6.20 ± 0.80	8.40 ± 0.64*,#

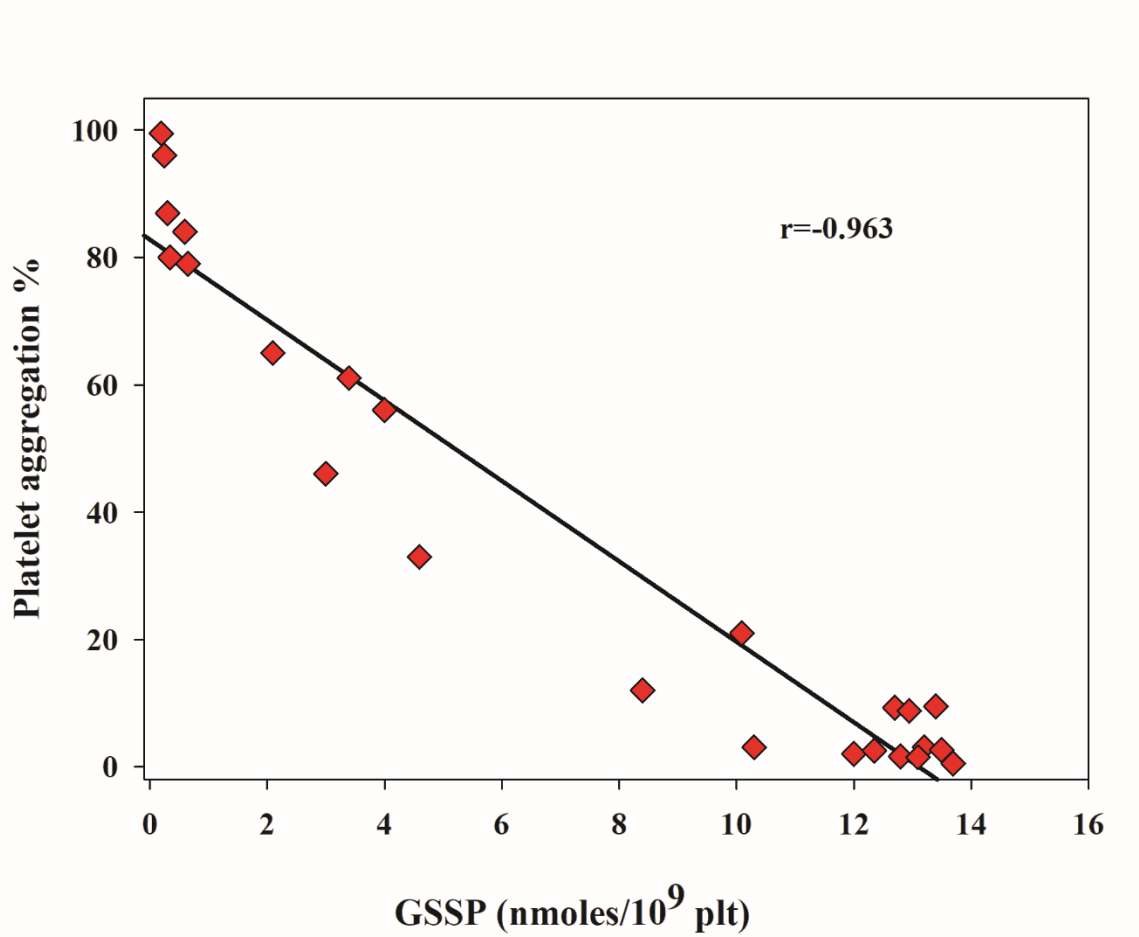
Table 4. Reference values for RSSP in plasma of healthy people and in maintenance hemodialysis (MHD) patients. Data are the median and are expressed as μM. **p < 0.001 vs healthy humans.

Sample	CySSP[a]	CyGlySSP	HcySSP	γ-GluCySSP	GSSP
Healthy humans (n = 24)	163 (150-195)	15.0 (13.3-18.1)	8.23 (6.45-9.96)	1.55 (1.31-1.71)	3.11 (2.66-3.38)
MHD (n = 71)	216** (182-254)	21.0** (16.5-25.5)	18.5** (14.9-23.4)	1.51 (1.22-1.78)	3.01 2.07-3.91)

[a]**Abbreviations**: CySSP, protein mixed disulfides with cysteine, CyGlySSP, protein mixed disulfides with cysteinylglycine; HcySSP, protein mixed disulfide with homocysteine; γ-GluCySSP, protein mixed disulfides with γ-glutamylcysteine; GSSP, protein mixed disulfides with GSH.


Oxidant challenge
Oxidant challenge
Oxidant challenge
Oxidant challenge
GSSP nmoles/mg Hb
GSSG nmoles/mg Hb
Time (min)
0
100
200
300
400
0
200
400
600
800
1000
1200
1400
0
20
40
60
80
100
120
140
160


7000
6000
5000
4000
3000
2000
1000
0
HbSSG pmoles/mg Hb
0
20
40
60
80
100
120
Time (min)
Rat
Human


Platelet aggregation %
100
80
60
40
20
0
r=-0.963
0
2
4
6
8
10
12
14
16
GSSP (nmoles/10^9 plt)