Analysis of GSH and GSSG after derivatization with *N*-ethylmaleimide

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This protocol describes a procedure for determining glutathione (GSH) and glutathione disulfide (GSSG) concentrations in blood and other tissues. Artifactual oxidation to GSSG of 5–15% of the GSH found in a sample can occur during deproteination of biological samples with any of the commonly used acids, with consequent marked overestimation of GSSG. This can be prevented by derivatizing GSH with the alkylating agent *N*-ethylmaleimide (NEM) to form GS-NEM before acid deproteination, followed by back-extraction of excess NEM from the deproteinized samples with dichloromethane. GSSG concentration is then measured by spectrophotometry with the GSH recycling method, on the basis of conversion of GSSG to GSH by glutathione reductase and NADPH and reaction with 5,5′-dithiobis-(2-nitrobenzoic acid). GSH concentration is instead measured by either of two methods: by analysis of GS-NEM conjugates by HPLC in the same sample that is used to measure GSSG or, alternatively, by analysis of GSH by spectrophotometry (GSH recycling method) on one additional sample aliquot that has not been derivatized with NEM. The procedure can assay GSH and GSSG in blood and other tissues in 30 min or less.

INTRODUCTION

Of all the nonprotein compounds present in cells, glutathione (γ-glutamyl-L-cysteinylglycine, GSH) has the greatest abundance of sulfhydryl groups (–SH). It is present in tissues at concentrations ranging from 1 to 10 mM and, on account of its ability to donate reducing equivalents, it is essential for both direct and enzymatic neutralization of toxic reactive species and for the maintenance of the cellular redox balance[1]. In extracellular matrices (e.g., plasma), in which a substantial amount of –SH is present as free cysteine, GSH concentration is in the range of 2–20 μM (refs. 2,3).

Under oxidative conditions, two GSH molecules donate one electron each to GSSG, which can be reduced back to GSH by the action of GSSG reductase (GR). A low GSH and/or high GSSG concentration and a low ratio of the concentrations of these two molecules (GSH/GSSG ratio), are interpreted as evidence of redox unbalance and weakened reducing power[4]. A low GSH/GSSG ratio is a well-established manifestation of the aging process and of an array of human disease states including diabetes, renal failure, pneumonitis, malignancy, amyotrophic lateral sclerosis, Parkinson's disease, Alzheimer's disease and cataracts[5–8]. Recent research has gone further by trying to define the subcellular compartmentalization of GSH and GSSG and by opening new avenues for both basic research on oxidative stress and therapeutic targets[9,10].

Despite substantial interest in GSH and GSSG titration as measures of redox balance, no broad agreement has yet been reached as to the best protocol for sample preparation and analysis for the quantification of these molecules in biological samples. Consequently, numerous highly reputable research groups have observed and published rather different levels of GSH and GSSG, even in the blood of healthy individuals (used as controls)[7,11].

We reported in 2002 on the major artifacts that can occur during GSH and GSSG titration, with the main one being a 5–15% oxidation of GSH during sample deproteination with acids, which leads to marked overestimation of GSSG[11]. The use of blood acidification derives from the notion that pure preparations of thiols are more stable in acidic *in vitro* environments. In biological samples, however, other organic molecules can markedly influence the otherwise straightforward chemical interaction between thiols and acids. We found that all acids commonly used for this purpose, including the trichloroacetic, metaphosphoric, perchloric and sulfosalicylic acids, oxidize GSH, but to different exents[11]. Three decades before that, Srivastava and Beutler[12] had written: "Although had been believed that 10–20% of total glutathione was present in the oxidized form, it has now become apparent that the true level of GSSG in the erythrocytes is in the order of 0.25% of the GSH level." Around the same time, this point was further addressed by Tietze[13] who revisited the findings of Srivastava and Beutler[12] and confirmed that (i) only the addition of the thiol alkylating agent NEM before sample acidification is capable of preventing large artifactual overestimation of GSSG, (ii) the GSH/GSSG ratio in blood (or RBCs) is ~400 and (iii) a similar situation probably occurs in most mammalian tissues[13].

In 2006, *Nature Protocols* published a protocol for determining the GSH/GSSG ratio[14] that is similar to a previous one published in *Methods Enzymology*[15]. These two protocols add the alkylating agent simultaneously or after deproteination of the sample under acidic conditions, an approach that is followed by many research groups[7,16–18]. Here we provide a similar procedure, in which the main difference is that NEM is added immediately on sample collection, and auto-oxidation of GSH due to acidic deproteination is avoided.

Another important aspect that needs more agreement is the choice of the best alkylating agent for GSH conjugation. NEM has been substituted over the years with other thiol reactants. The main motivation for avoiding NEM has been that this derivatizing agent is a potent inhibitor of GR, and it interferes with the enzymatic assay of GSH and GSSG with GR; consequently, NEM must be removed from the sample before analysis[13] with a procedure that is considered quite complex and time-consuming. In 1980, Griffith[19] introduced the use of 2-vinylpyridine (2-VP)

as a derivatizing agent for the analysis of plasma GSH, the primary advantage being that 2-VP does not substantially inhibit GR and does not need to be removed from the samples before analysis. The two major limitations of 2-VP, i.e., poor permeability through cell membranes and slow reactivity with GSH and other –SH groups, are not crucial for testing plasma, and because it undergoes minimal (i.e., tolerable) artifactual oxidation of GSH on account of its much lower GSH/GSSG ratio than that in cells (~2 instead of ~400)[20]. Over time, however, 2-VP has been applied broadly not just to analyze plasma but also whole blood, cells and tissues[14,16]. In this setting, the use of acid to lyse cell membranes (to allow contact between 2-VP and cell GSH) is responsible for GSH oxidation, eliciting the onset of large amounts of GSSG artifacts.

As part of our method development process, we have compared different sample preparation methods. Those that add NEM either at or after the acid deproteination step and also those that use alkylating agents other than NEM (e.g., 2-VP) perform very differently from those described by Srivastava and Beutler[12] and those we used in our research (see **Supplementary Table 1** for a direct comparison that we did in the preparation of this protocol). The main difference pertains to the quantification of GSSG, which is orders of magnitude higher when NEM is not added before acidification; we believe that these differences result from uncontrolled auto-oxidation of GSH with a consequent artificial increase in GSSG levels. Avoidance of artifactual GSH auto-oxidation by pretreating the samples with NEM allows the detection of GSH/GSSG ratios 500 in blood and in other tissues (**Supplementary Table 1**)[8,12,13,20].

Our principles for sample preparation for the GSH/GSSG assay

First, blood acidification decreases the stability of GSH, as all commonly used acids lead to the oxidation of ~10% of a given GSH concentration[11,20,21]. Second, derivatization of GSH should be achieved as soon as possible after sample collection in order to prevent its artifactual oxidation to GSSG. Third, derivatization of GSH should be performed by NEM; blood collection in a tube that has been prefilled with NEM allows immediate mixing of the chelator with the biosamples before acidification, efficiently avoids GSH oxidation[11,20–22] and allows for storage of blood samples for months at −80 °C before analysis. NEM is particularly well suited for this kind of analysis, because it is extremely rapid at permeating cell membranes and at blocking the –SH groups[11,13]. Fourth, thiols in blood and other tissues maintain very high *ex vivo* metabolic activity. In particular, GR is present in blood with the activity of 0.4 kIU per liter (ref. 23), with the theoretical capacity to reduce 0.1 mmol of GSSG per liter of sample within few seconds. A GR inhibitor should be added to limit artifactual *ex vivo* reduction of GSSG. As GR activity depends on the presence of two cysteinyl –SH groups within its catalytic site, chemical agents that react with –SH (for example, NEM) are capable of blocking GR activity. Fifth, the physiological concentration of GSH is millimolar, whereas that of GSSG is micromolar (i.e., almost three orders of magnitude lower), and it is maintained within a relatively tight range. Therefore, a painstaking attention to technical details must be given in particular for GSSG analysis, because oxidation of even 1% of a given GSH concentration results in an increase in GSSG of one order of magnitude.

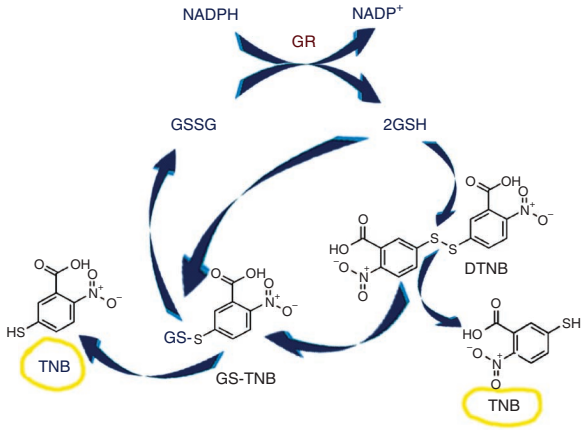


Figure 1 | Schematic representation of the mechanism of the GSH recycling method. The assay measures GSH and GSSG after its reduction to two molecules of GSH (2GSH) by GR in the presence of NADPH. GSH reacts with DTNB to form TNB and GS-TNB (glutathione adduct of TNB). GS-TNB is then reduced by GSH with the formation of GSSG and TNB or (more probably) by GR and NADPH with the generation of GSH and TNB. The formed TNB is measured at 412 nm. Yellow circles indicate the only compound that develops absorbance at 412 nm.

Development of the protocol

We have directly compared some of the most popular –SH masking agents, including 2-VP, iodoacetic acid, monobromobimane and NEM, and we have confirmed previous observations that the latter alkylating agent has the best masking characteristics[11] (**Supplementary Table 1**). We found that blood collection in tubes that are prefilled with both NEM and EDTA prevents sample manipulation–induced oxidation of GSH, as well as any GR-dependent reduction of GSSG; 25–30 mM NEM (final concentration) was optimal to secure both very rapid (within few seconds) alkylation of GSH and complete inhibition of GR activity[11].

The many analytical methods for GSH and GSSG fall under the broad categories of spectrophotometric and HPLC-based methods. The GSH recycling assay[13], which takes advantage of the specificity of GR to reduce GSSG to GSH in the presence of NADPH, features rapid execution and relatively low cost, and it is the most popular method. The reaction starts with the conjugation of GSH with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) to form the mixed disulfide GS-TNB and the chromophore 5-thio-2-nitrobenzoic acid (TNB); this is followed by back-reduction of GS-TNB to GSH by GR and NADPH (prevailing reaction) or by direct reaction of GS-TNB with any GSH still present in the assay mix (with formation of GSSG; **Fig. 1**). At this point, the conditions are present for recurrence of this reaction in a self-sustained closed cycle and for analysis of the steady-state production of TNB by spectrophotometry at a 412-nm wavelength. The method is generally used to estimate the sample concentration of GSH plus GSSG (i.e., total GSH (tGSH)), whereas the addition of an –SH masking agent to the sample allows for accurate and precise estimation of GSSG even at the very low levels that are typically encountered intracellularly. The concentration of GSH can then be determined by subtracting GSSG from tGSH.

Alternatively, GSH can be measured by endpoint colorimetric reaction with DTNB[24]. This method is also rapid and inexpensive,

but its main disadvantage is that it requires sample deproteination, which induces an artifactual oxidation of GSH that cannot be prevented by –SH masking.

The GSSG-endpoint assay[25] is another spectrophotometric method that measures the consumption of NADPH as a result of reduction of GSSG by GR; it is also relatively popular, but it lacks sufficient sensitivity to be useful for analytical work on blood or on other tissues.

Many HPLC methods are also available, with most protocols calling for long derivatization incubations, reduction of GSSG and/or derivatization of free amino groups of both GSH and GSSG[26]. In general, these methods are relatively complex and time consuming. In addition, considering that the –SH groups of GSH should be masked to prevent artifactual oxidation, this limits the concomitant use of other derivatizing agents that label these chemical groups. Initially, we addressed these drawbacks by modifying the HPLC method described by Reed *et al.*[27]. As its key feature, this method uses the iodoacetic acid to mask –SH groups and the Sanger reagent (DNFB) to label the amino groups of both GSH and GSSG[27]. Our modification of the method consisted of coupling the use of NEM and DNFB, which allowed for accurate and reproducible analysis of blood GSH and GSSG within the same run. However, a slow derivatization incubation step still was a major drawback, with more than 4 h being necessary to process and analyze each sample[21].

Here we describe a further improvement of the procedure for GSH and GSSG analysis that is relatively simple, rapid and free from the preanalytical pitfalls that very often bias these types of assays. In brief, the main step of this protocol consists of the immediate addition of NEM to the tissue sample to prevent any artifactual raise (or drop) in GSSG. GSSG can be analyzed by spectrophotometry with minor modifications of the GSH recycling assay[13], but we propose to use our recently developed HPLC method to analyze GSH as a GS-NEM conjugate[28]. If HPLC is not available, we recommend performing the spectrophotometric recycling assay for both GSSG and tGSH; this, however, requires collection and processing of a second blood aliquot without NEM. GSH is calculated as the difference between tGSH and GSSG. It should be noted that omitting NEM in this case is not problematic in that the fraction of GSH lost can be measured as GSSG by the same method.

We offer particularly detailed guidelines for the analysis of GSSG, because its very low physiological concentration is difficult to measure accurately unless the procedure is carefully executed and monitored and the occurrence of interfering compounds is properly addressed.

This protocol is primarily intended for use with whole blood or isolated RBCs. It has not yet been validated for other corpuscular elements of blood. In our hands, the protocol yields poorly reproducible results when it is used for platelets and white cells because the GS-NEM conjugate translocates extracellularly—and is lost—with variable kinetics during the cell isolation step. We have also used the protocol to measure GSH and GSSG in solid tissues[8]. Finally, we recommend that the protocol be used with cultures of adherent or suspended cells with the caveat that export of the conjugates with different kinetics can occur in certain cell types. We have recently applied the procedure to measure GSH and GSSG in human gingival fibroblasts[29]. A specific protocol for each cell type is beyond the scope of this paper, but, in general, the addition of NEM to the culture medium (at a 2 mM final concentration) 10 min before cell collection (either by trypsinization or scraping), followed by double-washing of the cells with cold PBS solution containing 0.5 mM NEM is the most appropriate preanalytical procedure. The use of this protocol in plasma or other extracellular fluids is of less interest to us because GSH and GSSG levels are very low (both around 1–2 μM, i.e., 1,000 times lower than whole blood) and mostly determined by the release of GSH from cells within all tissues; we do not recommend the application of our method for this matrix, because there are other methods that are more appropriate that are also able to detect other thiols (e.g., cysteine), which occur at higher concentrations than GSH[3].

MATERIALS

REAGENTS

- Human blood samples **! CAUTION** Adhere to all relevant ethical regulations and guidelines for the collection and use of human blood.
  ! CAUTION To avoid potential contact with blood-borne pathogens, perform all work with appropriate personal protective equipment.
- Animal tissues **! CAUTION** Adhere to all relevant ethical regulations and guidelines for animal housing and care.
- Potassium dihydrogen phosphate (KH_2PO_4; Sigma-Aldrich, cat. no. 229806)
- Disodium hydrogen phosphate (Na_2HPO_4; Sigma-Aldrich, cat. no. 30412)
- Sodium chloride (Sigma-Aldrich, cat. no. S7653)
- NEM (Sigma-Aldrich, cat. no. E1271)
- Tripotassium EDTA (Fluka, cat. no. 03664)
- Trichloroacetic acid (TCA; Sigma-Aldrich, cat. no. T6399) **! CAUTION** TCA is corrosive; wear protective gloves when you are handling it.
- DTNB (Fluka, cat. no. 43760)
- β-NADPH (Sigma-Aldrich, cat. no. N-1630)
- Sodium bicarbonate (Sigma-Aldrich, cat. no. 56014)
- Tris base (Sigma-Aldrich, cat. no. T1503)
- L-Serine (Sigma-Aldrich, cat. no. S4500)
- Boric acid (Sigma-Aldrich, cat. no. B6768)
- Acivicin (Sigma-Aldrich, cat. no. A2295)
- Glutathione reductase (Sigma-Aldrich, cat. no. G3664-500UN)
- L-Glutathione, reduced (Sigma-Aldrich, cat. no. G4251)
- Glutathione, oxidized form (GSSG; Sigma-Aldrich, cat. no. G-6654)
- HPLC-grade glacial acetic acid (EMD Millipore Chemicals, cat. no. AX0074)
  ! CAUTION Acetic acid is corrosive; it should be handled in a fume hood.
- HPLC-grade water (CHROMASOLV; Sigma-Aldrich, cat. no. 34877)
- Sodium hydroxide solution, 2 M (Fluka, cat. no. 35254)
- HPLC-grade acetonitrile (CHROMASOLV; Sigma-Aldrich, cat. no. 34851)
  ! CAUTION Acetonitrile is harmful and highly flammable, and it should be handled in a fume hood.
- Dichloromethane (DCM; Sigma-Aldrich, cat. no. 676853) **! CAUTION** DCM is harmful and highly flammable, and it should be handled in a fume hood.
- Drabkin's reagent for the determination of hemoglobin (Sigma-Aldrich, cat. no. D5941)
- Brij L23 solution (Sigma-Aldrich, cat no. B4184)
- Hemoglobin, human (Sigma-Aldrich, cat no. H7379)
- Hydrochloric acid solution, 1 M (HCl; Fluka, cat. no. 318949) **! CAUTION** HCl is corrosive and volatile; wear protective gloves and handle it in a fume hood.

EQUIPMENT

- UV-vis spectrophotometer (Jasco, V-550)
- Semi-micro disposable cuvettes (Kartell, code 1938)
- Stirrer rod for cuvettes (Eurodyne, cat. no. CU/46610)

- Microcentrifuge tubes, 1.5 ml (Eppendorf or equivalent)
- Microcentrifuge (Mini Spin, Eppendorf)
- Vortex 3 (IKA) with test tube insert
- Homogenizers (Potter Elvehjem P7859 or IKA Ultraturrax with S10 N-8G dispersing element)
- HPLC Agilent 1100 series with UV-vis detector (Agilent Technologies)
- HPLC vials (Agilent Technologies)
- HPLC Zorbax Eclipse XDB-C18 column, 4.6 × 150 mm, 5 µm (Agilent Technologies)
- pH meter
- Refrigerator (4 °C)
- Freezer (−80 °C)
- Freezer (−20 °C)

REAGENT SETUP

▲ **CRITICAL** The procedures for sample collection differ on the basis of the instruments used for the analyses of GSH (HPLC for GSH or spectrophotometry for tGSH). This is specified for every kind of sample.

PB200 (phosphate buffer, 0.2 M, pH 7.4) Prepare 0.2 M KH_2PO_4 (27.2 g per liter) and 0.2 M Na_2HPO_4 (28.4 g per liter) solutions. These solutions are stable for at least 1 month at 4 °C. Add 40.5 ml of the Na_2HPO_4 solution to 9.5 ml of the KH_2PO_4 solution to obtain 50 ml of 0.2 M phosphate buffer (pH 7.4); check and adjust the pH as necessary with drops of either KH_2PO_4 or Na_2HPO_4 solution. This buffer is stable for at least 2 weeks at 4 °C.

PB50 (phosphate buffer, 0.05 M, pH 7.4) Dilute PB200 1:4 with water. This buffer is stable for at least 2 weeks at 4 °C.

PBS Prepare 0.154 M NaCl in water (normal saline solution; 9 g of NaCl per liter). Add 90 ml of normal saline solution to 10 ml of PB200. This solution is stable for at least 2 weeks at 4 °C.

NEM310 Prepare 310 mM NEM in water (3.88 g of NEM per 100 ml); store 10-ml aliquots in disposable tubes at −20 °C. This solution is stable for at least 3 months at −20 °C. ▲ **CRITICAL** A slight warm-up of the solution may be necessary to completely dissolve the NEM.

Tripotassium EDTA Prepare tripotassium EDTA in water (75 mg of tripotassium EDTA per ml). This solution is stable for at least 2 weeks at 4 °C.

TCA60 Prepare 60% (wt/vol) TCA in water (60 g of TCA per 100 ml). This solution is stable for >12 months at room temperature (18–25 °C).

TCA15 Dilute TCA60 1:4 with water. This solution is stable for >12 months at room temperature.

TCA7.5 Dilute TCA60 1:8 with water. This solution is stable for >12 months at room temperature.

TCA7.5E Dilute TCA60 1:8 with water, and add 1 mg ml^{-1} tripotassium EDTA. This solution is stable for >12 months at room temperature.

$NaHCO_3$05 Prepare 0.5% (wt/vol) $NaHCO_3$ in water (50 mg of $NaHCO_3$ per 10 ml). This solution is stable for at least 1 month at 4 °C.

DTNB Prepare 20 mM DTNB in PB200 (15.8 mg of DTNB per 2 ml of PB200). ▲ **CRITICAL** DTNB is a light-sensitive solution; keep it wrapped or in the dark. It is stable for 1 month at 4 °C.

β-NADPH Prepare 4.8 mM NADPH in $NaHCO_3$05 (8 mg NADPH per 2 ml of $NaHCO_3$05). β-NADPH is stable for 1 month at −20 °C.

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 NEM310. Adjust the pH to 8.0 with 1 M HCl; adjust the volume to 500 ml with water. This solution is stable for 1 month at −20 °C.

GR Prepare a 20 IU ml^{-1} GR solution by diluting the commercial GR stock solution in PB50 (typically, 125 µl of commercial GR per 3 ml of PB50). This solution is stable for 1 week at 4 °C. ▲ **CRITICAL** Do not freeze the solution.

GSH, 20 mM Prepare 20 mM GSH in water (GSH 6.1 mg ml^{-1}). This solution is stable for at least 2 weeks at −20 °C. ▲ **CRITICAL** GSH tends to slowly oxidize; check its concentration every 2 weeks using the DTNB reaction endpoint method[20], as described in **Supplementary Figure 1**.

GSSG, 10 mM Prepare 10 mM GSSG in PB50 (24.5 mg of GSSG per 4 ml of PB50). GSSG is stable for >12 months at −20 °C.

GSSG, 10 µM Dilute 10 µl of 10 mM GSSG in 9.99 ml of water. This solution is stable for >12 months at −20 °C.

GSH standards (10–100 µM) Freshly prepare the 100 µM first GSH standard solution by diluting 5 µl of 20 mM GSH with 995 µl of water; prepare the other GSH standard solutions by sequential dilution of each standard with water. The reagent is stable for 1 d at 4 °C.

GS-NEM standards Prepare the 2 mM first GSH standard solution by diluting 100 µl of 20 mM GSH with 900 µl of PB50; prepare the other standard solutions by serial 1:1 dilutions of each standard with PB50; add NEM310 to each standard solution (6 µl of NEM310 per 500 µl of standard) and incubate it for 5 min. The reagent is stable for 3 months at −20 °C. Before use, add 494 µl of TCA15 to each standard.

Mobile phase A (solution for HPLC) Freshly 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.

Mobile phase B Mobile phase B is HPLC-grade acetonitrile. It can be stored at RT for 5 years.

Blood: GSSG spectrophotometry, GSH HPLC Collect human or animal blood in tubes prefilled with 23 µl of tripotassium EDTA and 100 µl of NEM310 per ml of blood (**Fig. 2**).

Blood: GSSG and tGSH spectrophotometry Collect one aliquot of human or animal blood in tubes prefilled with 23 µl of tripotassium EDTA per ml of blood (**Fig. 2**). Collect one additional aliquot of human or animal blood in tubes prefilled with 23 µl of tripotassium EDTA and 100 µl of NEM310 per ml of blood. Note that the protocol is also suitable for RBCs; see **Supplementary Methods**.

Solid tissue preparation Remove animal organs under anesthesia or after decapitation. ▲ **CRITICAL** Remove the organs and/or tissues of interest rapidly and in the same sequence. Wash them quickly in cold saline, and then homogenize them immediately in a known volume of Tris-BSAN homogenization buffer. ▲ **CRITICAL** The homogenization buffer must be slightly alkaline in order to speed up alkylation of –SH groups with NEM. However, tissues with high levels of γ-glutamyl transpeptidase (γGT) (e.g., kidney) present a challenge because γGT has high activity at the recommended homogenization pH, and because the procedure duration might be sufficient for this enzyme to metabolize a relevant amount of GSH and GSSG; we obviate this potential problem by including the γGT inhibitors borate, serine and acivicin into the homogenization buffer. ▲ **CRITICAL** We recommend the HPLC method for measuring GSH in tissues. If HPLC is unavailable, use the spectrophotometric GSH recycling assay, being careful to split the sample into two aliquots (for tGSH and GSSG, respectively) with comparable tissue characteristics (e.g., the same anatomic district within histologically complex organs such as kidney, intestine and brain).

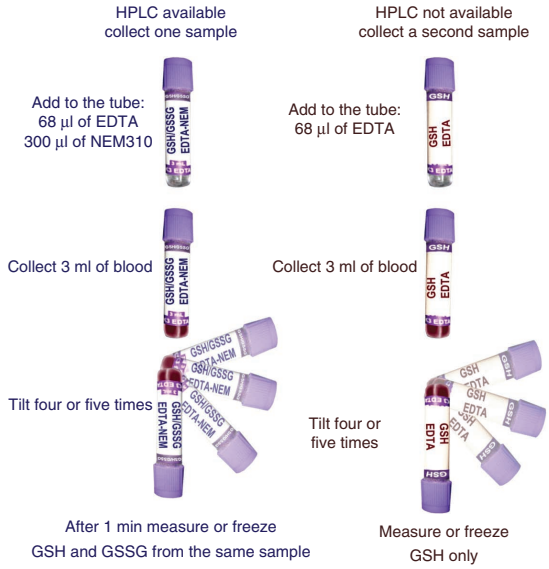


Figure 2 | Graphical representation of the preanalytical manipulation of blood samples for GSH and GSSG analyses. The easier procedure consists in detecting GSH by HPLC and GSSG by spectrophotometry. In this case, only one blood sample is collected, and a tube containing both tripotassium EDTA and NEM is used. If HPLC is not available, tGSH is measured by spectrophotometry. In this case, an additional blood sample needs to be collected into a tube containing just tripotassium EDTA. The figure refers to typical amounts of blood and reagents used for human samples.

Tissue: GSSG by spectrophotometric recycling assay and GSH by HPLC. Organs (typically a fraction of them, e.g., 0.5 g) are homogenized 1/10 (wt/vol) in Tris-BSAN buffer on ice.

Tissue: GSSG and tGSH by spectrophotometric recycling assay Organs must be split into two parts with comparable tissue characteristics (e.g., the same anatomic district within histologically complex organs such as kidney, intestine and brain). One part (typically 0.5 g) is homogenized 1/10 (wt/vol) in Tris-BSAN buffer on ice for GSSG analysis. The other part (typically 0.5 g) is 1/10 (wt/vol) homogenized in TCA7.5E on ice for GSH analysis.

EQUIPMENT SETUP

HPLC analyses of GSH Chromatographic separations are performed on an Agilent series 1100 HPLC device or equivalent equipped with a Zorbax Eclipse XDB-C18 column (4.6 × 150 mm, 5 µm, Agilent Technologies) and a UV-vis diode array detector. At the start of a series of experiments, prepare a fresh set of mobile phases A and B as described in the reagents section.

Condition the column with 100% mobile phase B at 1.25 ml min^{-1} for at least 5 min and equilibrate the column temperature at 25 °C. Change the mobile phase composition to 94% phase A and 6% phase B with a flow rate of 1.25 ml min^{-1}. Run one or more blanks followed by standards and unknowns under isocratic conditions, and record the signals at a 265-nm wavelength with 400 nm as a reference. After each injection, at the end of the run, flush the column with 100% phase B for 2 min and then re-equilibrate the system to the initial isocratic conditions before the next injection. Under these conditions, the GS-NEM conjugate elutes as two well-separated symmetrical peaks (owing to the formation of two diastereoisomers[30]) of equal area at a retention time (r.t.) of 4.29 and 4.95 min. Two peaks are detected because the diastereomers have different physical and chemical properties and, consequently, they are resolved under achiral chromatographic conditions. Either of the two peaks can be used for calculations: we prefer using the peak with a 4.29-min r.t. because it is more likely to be free of interferences in all analyzed tissues.

Application of GSH recycling method, blank check Set the spectrophotometer as follows: 412-nm wavelength, 1-min continuous readings and 1-s data pitch. Perform one or more blank analyses to assess reagent quality and to be sure that the method works well. Add the following reagents in the specified order and in rapid sequence into a cuvette: 0.925 ml of PB200, 5 µl of DTNB, 20 µl of TCA7.5E, 20 µl of NADPH and 20 µl of GR; mix the reagents with a cuvette stirrer rod and read the absorbance for 1 min at 412 nm. At the end of the reading, immediately add 10 µl of 10 µM GSSG to the same solution and read the absorbance for one additional minute. ▲ **CRITICAL** Read the whole tracing, and not just the initial and final values. The rise of absorbance must be linear, with a slope for the blank preferably <0.005 absorbance units (A.U.) per minute. Slope after standard addition should be ≥ 0.025 A.U. min^{-1}. Continue with the analyses only if all these requirements are met. Otherwise, address the possibility of a contaminated reagent, as this is the most common problem. ▲ **CRITICAL** The measurements are generally performed at room temperature; as the reagents' temperature may influence the readings, start the assay only when all the reagents are equilibrated to room temperature.

PROCEDURE

Preparation of biological samples

1| The sample-processing protocol (options A–D) differs according to the instrumentation for GSH analysis (i.e., HPLC or spectrophotometer). In general, the HPLC procedure should be the first option because it allows for the measurement of GSH and GSSG starting from a single sample. Nevertheless, the GSH recycling method by spectrophotometry represents a suitable alternative in all cases.

(A) Whole blood: GSSG by spectrophotometry and GSH by HPLC ● TIMING 2 min

(i) Collect blood in tubes containing both NEM and tripotassium EDTA (Reagent Setup).

(ii) Mix the blood by slowly tilting the filled test tube for 1 min.

■ **PAUSE POINT** Samples can be stored at −80 °C. In our hands, compared with results from fresh samples, storage of samples for 3 months at −80 °C did not affect analytical results.

(iii) Proceed with HPLC analysis of GSH and spectrophotometric analysis of GSSG.

(B) Whole blood: spectrophotometric detection of GSSG and tGSH ● TIMING 1 min

(i) Collect blood into two test tubes: one containing both NEM and tripotassium EDTA, and the other containing only tripotassium EDTA (Reagent Setup).

(ii) Mix the blood by slowly tilting the two test tubes for 1 min.

■ **PAUSE POINT** The samples can be stored at −80 °C. In our hands, compared with results from fresh samples, storage of samples for 3 months at −80 °C did not affect analytical results.

(iii) Proceed with spectrophotometric analysis of tGSH (from the sample in tripotassium EDTA) and GSSG (from the sample in NEM/tripotassium EDTA).

(C) Solid tissues: GSSG by spectrophotometry, GSH by HPLC ● TIMING 8–10 min

(i) Remove organs or tissues and homogenize them in Tris-BSAN buffer (Reagent Setup).

(ii) Incubate the homogenized samples for 2 min at room temperature.

(iii) Acidify and mix one sample aliquot with TCA60 (typically 100 µl of TCA60 for 1 ml of homogenate).

(iv) Centrifuge the mixture at 14,000*g* for 2 min at room temperature.

■ **PAUSE POINT** The supernatants can be stored at −80 °C. In our hands, compared with results from fresh samples, storage of samples for 3 months at −80 °C did not affect analytical results.

(v) Proceed with HPLC analysis of GSH and spectrophotometric analysis of GSSG.

(D) Solid tissues: spectrophotometric detection of GSSG and tGSH ● TIMING 15–20 min

(i) Remove organs or tissues and homogenize them in Tris-BSAN buffer or TCA7.5E (Reagent Setup).

(ii) If the samples are homogenized in TCA7.5E, incubate them for 2 min at room temperature.

(iii) If the samples are homogenized in Tris-BSAN buffer, acidify and mix one sample aliquot with TCA60 (typically 100 µl of TCA60 for 1 ml of homogenate).

(iv) Centrifuge all samples at 14,000*g* for 2 min at room temperature.
■ **PAUSE POINT** The supernatants can be stored at −80 °C. In our hands, compared with results from fresh samples, storage of samples for 3 months at −80 °C did not affect analytical results.
(v) Proceed with spectrophotometric analysis of tGSH (from sample homogenized in TCA) and of GSSG (from sample homogenized in Tris-BSAN).

Analytical procedures

2| There are three analytical procedures. One is for GSSG analysis, and the other two can be applied for GSH measurement. For GSH analysis the procedure used depends on instrument availability (HPLC or spectrophotometer). If the spectrophotometric procedure is used, tGSH is measured instead of GSH alone; therefore, GSH is then calculated by subtraction of GSSG from tGSH.

▲ **CRITICAL STEP** Precision and caution in avoiding reagent contamination are crucial to the success of this procedure.
▲ **CRITICAL STEP** Conduct a blank analysis and reagent quality check before proceeding with the assay (Equipment Setup).
▲ **CRITICAL STEP** Details about calculations refer to experimental conditions for blood. To calculate the concentration of GSSG in tissues, refer to the different dilution conditions as described in option C or D and in Reagent Setup. Express GSSG values as μmol per gram of tissue.

(A) GSSG analysis ● TIMING 10–15 min

▲ **CRITICAL STEP** For whole blood, start from Step 2A(i); for all other tissues start with Step 2A(ii).
(i) (Whole blood only; for all other tissues start with Step 2A(ii)) Add to each NEM-treated whole-blood sample an equal volume of TCA15; shake the sample vigorously and centrifuge it at 14,000*g* for 2 min at room temperature.
(ii) Extract the supernatant of each acid-deproteinized sample with three volumes of DCM (typically 750 μl of DCM for 250 μl of supernatant, in a 1.5-ml microcentrifuge tube).
▲ **CRITICAL STEP** DCM is used to extract the excess of NEM; we use DCM instead of ethyl ether or other organics[13] because, with centrifugation, the DCM forms a layer below the aqueous sample, thus facilitating collection of the top layer that contains the sample.
(iii) Shake the mixture moderately on a vortex shaker at 800 r.p.m. for 5 min at room temperature.
(iv) Centrifuge the mixture for 30 s at 14,000*g* and collect the supernatant sample.
(v) Fill a cuvette with the following reagents in the specified order: 0.925 ml of PB200, 5 μl of DTNB, 20 μl of sample and 20 μl of NADPH; mix the contents. Finally, in rapid sequence, add 20 μl of GR, mix the cuvette with a stirrer rod, and then record the absorbance at 412 nm for 1 min.
(vi) After the initial 1-min reading, quickly add 10 μl of 10 μM GSSG to the cuvette, mix it with the stirrer rod and record the absorbance at the same wavelength for 1 additional minute. The tracing must be linear and similar to that in **Figure 3**.
? TROUBLESHOOTING
(vii) Calculate the concentration for blood of GSSG as follows:
Calculate S = slope sample − slope blank; Calculate St = (slope sample + GSSG) − slope sample;
GSSGb (concentration of GSSG in blood) = $S \times [GSSGc]/St \times 49.5$ (sample dilution factor in cuvette) × 2 (dilution due to acidification), where [GSSGc] is the final concentration of standard GSSG in the cuvette (0.1 μM at the indicated conditions). For example: slope sample = 0.0151 $\Delta_{A.U.}$ min^{-1}; slope blank = 0.0048 $\Delta_{A.U.}$ min^{-1}; slope sample + GSSG = 0.0398 $\Delta_{A.U.}$ min^{-1}. Therefore, S = 0.0103, St = 0.0247 and GSSGb = 0.0103 × 0.1/0.0247 × 49.5 × 2= 4.12 μM.
(viii) Measure hemoglobin concentration in blood samples (those with anticoagulants and NEM) according to the manufacturer's instructions (for the Drabkin's reagent).
▲ **CRITICAL STEP** Blood glutathione occurs mainly in cells, whereas only traces can be found in plasma, and thus normalization either to RBC number or to hemoglobin concentration is mandatory. We suggest normalization to hemoglobin. Express GSSG values as nmol per mg of hemoglobin.

(B) GSH analysis by HPLC ● TIMING 15–20 min for each sample

▲ **CRITICAL STEP** For whole blood, start from Step 2B(i); for all other tissues start with Step 2B(ii).
(i) (Whole blood only; for all other tissues start with Step 2B(ii)) Add to each NEM-treated whole-blood sample an equal volume of TCA15; shake each sample vigorously and centrifuge it at 14,000*g* for 2 min at room temperature.
(ii) Load 5 μl of standard samples containing 0.1, 0.2, 0.5, 0.75 and 1.0 mM GS-NEM onto the HPLC column, and then process them as detailed above (Equipment Setup).
? TROUBLESHOOTING
(iii) After the standard samples, load 5 μl of unknown sample supernatants, under the same conditions as above. The chromatographic tracing should be very similar to those shown in **Figure 4**.
(iv) Measure hemoglobin concentration in blood samples (those with anticoagulant and NEM) according to the manufacturer's instructions for the Drabkin's reagent.

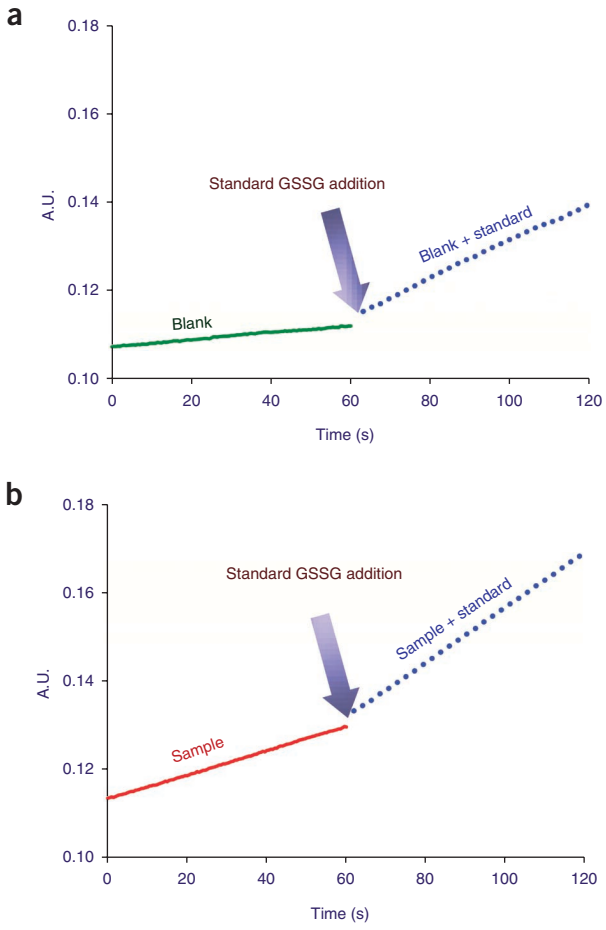


Figure 3 | Representative tracings obtained by applying the GSH recycling method for GSSG analysis in blood. (**a**) A blank analysis is carried out by adding into the cuvette all the reagents without the sample (substituted by TCA) and, after 1 min, 10 µl of 10 µM GSSG. Slope blank = 0.0048 $\Delta_{A.U.}$ min^{-1}; slope blank + GSSG standard = 0.0297 $\Delta_{A.U.}$ min^{-1}. (**b**) The measurement of GSSG in blood is performed by adding into the cuvette all the reagents and the acidified supernatant after NEM extraction. After 1 min, 10 µl of 10 µM GSSG is added and the reaction is registered for 1 additional minute. Slope sample = 0.015 $\Delta_{A.U.}$ min^{-1}; slope sample + GSSG standard = 0.0398 $\Delta_{A.U.}$ min^{-1}. Tracings must be linear.

▲ **CRITICAL STEP** When you are analyzing blood samples, it is essential to normalize the readings to hemoglobin concentration, because GSH occurs mainly in erythrocytes, with only traces present in plasma; normalization to the RBC number is less desirable, because cell hemoglobin content is variable depending on the physiology and/or disease state. Express GSH values as nmol per milligram of hemoglobin.

▲ **CRITICAL STEP** For other tissues, express GSH as µmol per gram of tissue.

(C) tGSH analysis by spectrophotometry ● TIMING 5 min

▲ **CRITICAL STEP** For whole blood, start from Step 2C(i); for all other tissues start with Step 2C(ii).

(i) (Whole blood only; for all other tissues start with Step 2C(ii)) Add to each whole-blood sample (without NEM) an equal volume of TCA15; shake it vigorously and centrifuge the mixture at 14,000*g* for 2 min at room temperature.

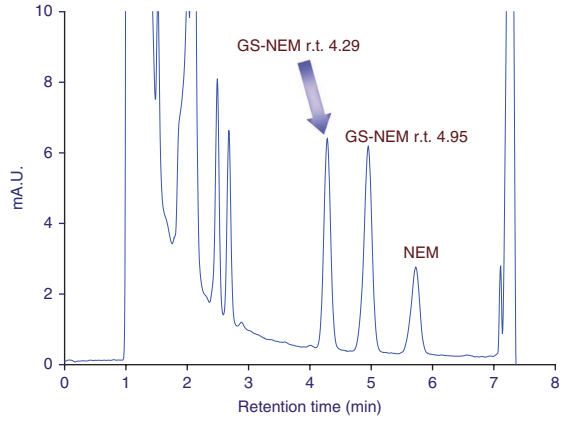
(ii) Dilute the samples to a 1:100 ratio with water.

(iii) Fill a cuvette with the following reagents in the specified order: 0.945 ml of PB200, 5 µl of DTNB, 10 µl of sample and 20 µl of NADPH, and mix the contents. Finally, in rapid sequence, add 20 µl of GR, mix it with a stirrer rod, and then record the absorbance at 412 nm for 1 min.

(iv) Use the same procedure to create a calibration curve, substituting the unknown sample with 10, 25, 50, 75 and 100 µM GSH standards.

(v) Measure hemoglobin concentration in blood samples (those with anticoagulants only) according to the manufacturer's instructions for the Drabkin's reagent.

▲ **CRITICAL STEP** This procedure measures tGSH (GSH + GSSG). To obtain the concentration of GSH, subtract the measured levels of GSSG (multiplied by 2, because in the recycling method one molecule of GSSG is reduced to two molecules of GSH) from the levels of tGSH.



▲ **CRITICAL STEP** Blood glutathione occurs mainly in cells, whereas only traces can be found in plasma, and thus normalization to hemoglobin concentration is important. RBC numbers can also be used to normalize GSH and GSSG, but this is less desirable. Express GSH values as nmol per milligram of hemoglobin.

▲ **CRITICAL STEP** For the other tissues, express GSH as µmol per gram of tissue.

Figure 4 | Representative chromatogram of the GS-NEM conjugate in blood samples. The GS-NEM conjugate was analyzed by reversed-phase HPLC with UV detection at 265 nm in supernatants obtained from NEM-treated blood after acidification. GS-NEM conjugate separation results in two peaks with the same area at 4.29 min and 4.95 min r.t. These two peaks form as a consequence of the generation of diastereomers, which are separable under achiral chromatographic conditions. The unreacted NEM gives a peak at an r.t. of 5.8 min. mA.U., milliabsorbance units.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**; more general issues are described below.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reasons	Solution
2A(vi)	Blank absorbance reading is too high	One or more reagents is/are contaminated	Prepare new reagents
	Slope is shallow	Buffer temperature is too low	Allow the buffer to equilibrate to room temperature
		Deteriorated reagents	Check NADPH and/or GR activity (**Supplementary Methods**)
	Sample absorbance slope is too steep, i.e., >0.1 A.U. min^{-1}	Sample GSSG concentration is too high	Dilute the sample with water
	GSSG addition does not adequately increase the slope	GR activity is insufficient	Increase the amount of GR (e.g., use 25 µl instead of 20) and run both the blank and GSSG addition samples again
	Slope steepness decreases during recording	Some NEM is still present in the sample	Perform one additional extraction with DCM
2B(ii)	HPLC peaks are broadened or unresolved, both in samples and standards	Unstable pressure	Check the instrument pressure
		Column is old	Replace the column

General advice about sample processing

NEM must be added as soon as possible to the biological samples after collection. This alkylating agent should be present in the test tubes at the time of blood collection, thus assuring immediate mixing with the biological samples. The conditions are not as exacting for solid tissues, as the earliest NEM can be added is during homogenization and, inevitably, the time elapsed between tissue collection and exposure to NEM can introduce bias. Under these circumstances, the best possible approach is to quickly collect the tissues, homogenize the samples as soon as possible in the presence of NEM and ensure that the timing of this preanalytical procedure is consistent from sample to sample.

General advice regarding the spectrophotometric GSH recycling assay

The GSH recycling assay is not an endpoint method, because it is based on the GR property of cyclically reducing both the GSSG that is originally present in the sample and that formed by reaction of GSH with DTNB. Since it is based on an enzymatic reaction, the assay is affected by pH, temperature, kind of buffer, and contaminants that may be present in the cuvette. In particular, the reaction can be slowed down by the inhibition of GR activity by both the acid used for protein precipitation and by the presence of NEM, as well as by interaction of NEM with other thiols present in the sample (e.g., GSH and TNB). The protocol calls for the use of DCM to extract excess NEM from the samples. Extraction with DCM is easy and rapid to perform, but, as for all extraction processes, some traces of the compound can remain in the sample. DCM extracts part of TCA too. To correct for possible assay variability caused by trace NEM and by TCA, we recommend performing a two-step analysis that includes adding a known quantity of GSSG to the cuvette after the sample reaction has achieved steady state (i.e., after 1 min). We carry out this step for every sample in which we measure GSSG, as it allows for a more precise calculation of the analyte concentration. Conversely, measurement of tGSH does not require the addition of standard GSH to each sample for calculations, because of the absence of NEM from the reaction mix and because of the analyte's generally high concentration in the sample. In this case, only one curve with five standards diluted in buffer is usually performed.

The GSH recycling assay is specific with minimal or absent interference from other thiols and disulfides; the lower limit of detection is 0.1 µM in most tissues.

General advice regarding GSH measurement by HPLC

On the one hand, HPLC analysis of GS-NEM conjugate is very simple, as it just requires loading TCA deproteinized samples onto the HPLC, but on the other hand, it is subject to all the pitfalls and complications that can occur when operating an HPLC apparatus. Tracings are generally easily analyzed, with the main possible pitfall being the occurrence of unspecific peaks

that, depending on the type of sample, can co-elute with one of the two symmetrical GS-NEM peaks of interest. This problem does not occur with blood samples, but it can come up when analyzing solid tissues. For example, in rat lung, we found interference with the 4.95-min (r.t.) GS-NEM peak (**Supplementary Fig. 2**). However, we have always been able to circumvent this possible problem by instead analyzing the GS-NEM peak with an r.t. of 4.29 min, which is always free from interference.

GS-NEM has a low extinction coefficient, but the concentration of GSH is very high in all tissues; therefore, the method has a sufficient sensitivity. The GS-NEM conjugate is stable and not substantially affected by leaving the samples at room temperature for 3 d before analysis (**Supplementary Fig. 3**).

● **TIMING**

Step 1A, whole blood, GSSG by spectrophotometry and GSH by HPLC: 2 min
Step 1B, whole blood, spectrophotometric detection of GSSG and tGSH: 1 min
Step 1C, solid tissues, GSSG by spectrophotometry, GSH by HPLC: 8–10 min
Step 1D, solid tissues, spectrophotometric detection of GSSG and tGSH: 15–20 min
Step 2A, GSSG analysis: 10–15 min
Step 2B, GSH analysis by HPLC: 15–20 min for each sample
Step 2C, tGSH analysis by spectrophotometry: 5 min per sample

ANTICIPATED RESULTS

Table 2 shows the GSH and GSSG concentrations measured in blood and solid tissues. In some cases[31,32], GSH and GSSG were measured in RBCs. All measurements have in common the sample pretreatment with NEM (i.e., before acidification), but different analytical methods have been used to detect GSH and GSSG. Specifically, values reported in **bold** were obtained by applying the procedures described here (i.e., measurement of GS-NEM conjugate by HPLC and/or measurement by GSH

TABLE 2 | Concentrations of GSH and GSSG in several tissues from humans and laboratory animals.

Tissue	GSH	GSSG	GSH/GSSG	Reference
Human blood	**8,342 ± 769 nmol per gram Hb**	**23.3 ± 5.06 nmol per gram Hb**	**358**	**Rossi *et al.*[11]**
Human blood	~2,500 μmol per ml RBC[a]	3.6 ± 1.4 μmol per ml RBC	~690	Srivastava and Beutler[12,b]
Human blood	1,378 ± 96 μM	3.21 ± 0.87 μM	429	Giustarini *et al.*[21,b]
Human blood	**8,620 ± 460 nmol per gram Hb**	**—**	**—**	**Giustarini *et al.*[28]**
Human RBCs	**8,460 ± 1,750 nmol per gram Hb**	**13.2 ± 4.0 nmol per gram Hb**	**693**	**Khazim *et al.*[31]**
Human RBCs (hemodialysis)	**8,720 ± 2,150 nmol per gram Hb**	**26.4 ± 8.7 nmol per gram Hb**	**363**	**Khazim *et al.*[31]**
Human blood	~7,800 nmol per gram Hb[a]	~14 nmol per gram Hb[a]	~550	Papp *et al.*[36,b]
Rat blood	**8,155 ± 918 nmol per gram Hb**	**36.7 ± 12.7 nmol per gram Hb**	**222**	**Rossi *et al.*[11]**
Mouse blood				
C57BL/6J	11,200 ± 237 nmol per gram Hb	20.3 ± 12.1 nmol per gram Hb	560	Rossi *et al.*[32,c]
Mouse blood				
DBA/2	6,960 ± 109 nmol per gram Hb	10.5 ± 4.01 nmol per gram Hb	696	Rossi *et al.*[32,c]
Rat liver	8,221 ± 528 nmol g^{-1}	43.3 ± 2.9 nmol g^{-1}	190	Giustarini *et al.*[8,c]
Rat kidney	2,221 ± 302 nmol g^{-1}	13.4 ± 3.42 nmol g^{-1}	166	Giustarini *et al.*[8,c]
Rat heart	2,314 ± 182 nmol g^{-1}	17.2 ± 0.8 nmol g^{-1}	134	Giustarini *et al.*[8,c]
Rat lungs	1,835 ± 244 nmol g^{-1}	11.3 ± 2.6 nmol g^{-1}	162	Giustarini *et al.*[8,c]
Rat spleen	2,648 ± 55 nmol g^{-1}	12.2 ± 2.6 nmol g^{-1}	217	Giustarini *et al.*[8,c]
Rat testis	4,354 ± 131 nmol g^{-1}	15.0 ± 1.0 nmol g^{-1}	290	Giustarini *et al.*[8,c]
Rat brain	1,801 ± 59 nmol g^{-1}	18.1 ± 1.4 nmol g^{-1}	100	Giustarini *et al.*[8,c]

[a]Graphically deduced. [b]Detection carried out by GSH recycling method with some modifications. [c]Detection carried out by HPLC with fluorescence detection.

recycling method). In the other cases, detection was performed either with the classical GSH recycling method (with minor modifications with respect to our protocol) or with HPLC, but by using fluorescence labeling of the free GSH.

The protocol described here has been validated for all these applications[8,28,32]. **Supplementary Table 2** shows recovery of GSH and GSSG after addition of the thiols to whole blood and measurement using the present protocol.

GSSG concentration in solid tissues determined using this protocol ranged from 10 to 50 μM in all solid tissues that we have tested, whereas 95% of the subjects we analyzed presented with blood GSSG levels from 2 to 5 μM, or 13–33 nmol per gram Hb. GSH/GSSG ratios ranged from 100 to 700. These ratios are higher than those reported by other research groups[8,33–35].

By applying this protocol, we have recently found that hemodialysis patients have increased GSSG levels in RBCs and a decreased GSH/GSSG ratio with respect to controls[31].

Note: Supplementary information is available in the online version of the paper.

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