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Pitfalls in the analysis of the physiological antioxidant glutathione (GSH) and its disulfide (GSSG) in biological samples: An elephant in the room

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Abstract

Glutathione (GSH) is the most abundant low-molecular-mass thiol within cells and one of the major antioxidant compounds in body fluids. Under pro-oxidant conditions, two GSH molecules donate one electron each and are converted into glutathione disulfide (GSSG). The GSH/GSSG molar ratio is considered a powerful index of oxidative stress and disease risk. Despite high interest in GSH/GSSG titration as measures of thiol redox balance, no broad agreement has yet been reached as to the best pre-analytical and analytical methods for the quantitation of these molecules in biological samples. Consequently, measured concentrations of GSH and GSSG and calculated GSH/GSSG molar ratios vary widely among laboratories. Here, we describe in detail the main analytical and pre-analytical problems related to the artificial oxidation of the sulfhydryl (SH) group of GSH that occur during sample manipulation. We underline how this aspect has been neglected for long time after its first description more than fifty years ago. Finally, selected reliable procedures and methods to measure GSH and GSSG in biological samples are discussed.

Keywords

Glutathione; Glutathione disulfide; Oxidative stress; Protocols

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1. Introduction

Glutathione (γ -glutamyl-L-cysteinylglycine, GSH) is an acidic molecule characterized by a cysteine residue and a γ -linked amino acid which confers protection against hydrolysis by cellular proteases. It is the non-protein cell compound with greatest abundance of sulfhydryl groups (-SH) and it is one of the most reactive cell functionalities. GSH 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 (chemical) and enzymatic neutralization of toxic reactive species and, in particular, for cellular defense towards oxidants [1]. The situation is somewhat different in extracellular matrices, e.g., human plasma, where a substantial amount of -SH is present as free cysteine, whereas GSH concentration is in the range of 2-20 μ M [2,3].

Under oxidative conditions, the sulfur atoms of two GSH molecules donate one electron each and convert into glutathione disulfide (GSSG), which can be reduced back to GSH by the action of GSSG reductase (GR). Therefore, a decrease in GSH and the ratio GSH/GSSG are interpreted as evidence of redox unbalance and have been associated to a variety of human diseases including diabetes, renal failure, malignancy, neurodegenerative diseases, and many others [4-6]. In clinical studies, GSH and GSSG are most often measured in whole blood or in isolated red blood cells (RBCs) based on the assumption that, although indirect, this minimally invasive type of analysis provides valuable information on the redox balance of less accessible tissue and organs and of the whole organism [7].

Despite high interest in GSH and GSSG titration as measures of redox balance, no broad agreement has yet been reached as to the most appropriate pre-analytical and analytical methods for the quantitation of these molecules in biological samples. Consequently, measured concentrations of GSH and GSSG vary considerably between laboratories [6,8]. Many examples are available of interference of such discordance with the interpretation and comparison of studies; this problem may also be the reason why many large clinical trials were unable to confirm the results of smaller scale preliminary investigations.

2. The use of *N*-ethylmaleimide to mask GSH: an old story

The most critical point in GSH and GSSG analysis is surely represented by the pre-analytical manipulation of the biological sample. Almost five decades ago, Srivastava and Beutler 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." [9]. The authors reached this conclusion by protecting GSH's artificial oxidation to GSSG by masking the -SH group with *N*-ethylmaleimide (NEM). This point was also addressed by Frank Tietze [10], who developed the GSH- recycling assay for the analysis of GSH and GSSG. The main advantage of the GSH-recycling assay [10] is the specificity, since it uses GR to reduce GSSG to GSH in the presence of NADPH. GSH occurring in the sample reacts with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) to form the mixed disulfide GS-TNB and the chromophore 5-thio-2-nitrobenzoic acid (TNB). GS-TNB is then reduced back to GSH by GR and NADPH (prevailing reaction) or by direct reaction with any GSH still present in the

assay mix (with formation of GSSG; Scheme 1). The reaction proceeds in a cyclic way and the analysis of the steady-state production of TNB by spectrophotometry at 412 nm wavelength is performed. The method is generally employed to estimate the sample concentration of GSH plus GSSG, i.e., total GSH (tGSH), while addition of a -SH masking agent to the sample allows for accurate and precise estimation of GSSG even at the very low levels. The concentration of GSH can be then determined by subtracting GSSG from tGSH. Actually, the concentration of GSSG within control cells is more than two orders of magnitude lower than that of GSH, thus, usually, the measure of tGSH resembles that of GSH alone. The study carried out by Tietze confirmed that only the addition of NEM before sample acidification is capable of preventing large artifactual overestimation of GSSG. By application of its method to several biological tissues he also substantiated that GSH/GSSG ratio in blood (or RBCs) is around 400, and a similar value also occurs in most mammalian tissues [10].

However, since NEM is also a potent inhibitor of GR (which is required for the GSH-recycling assay), the procedure also calls for extraction of excess alkylating agent before proceeding with the GSSG titration. To obviate to the somewhat laborious extraction step, Griffith [11] modified the procedure by Tietze's method by introducing 2-vinylpyridine (2-VP) in order to mask GSH.

3. The devil is in the details

The method developed by Griffith [11] has become popular for two main reasons: first, 2-VP causes minimal inhibition of GR, thus allowing for the avoidance of the alkylating agent extraction step; and second, it facilitates the titration procedure by - quite a paradox - artificially raising the GSSG blood levels from ~5 μ M to ~100 μ M. In fact, the main disadvantage of 2-VP is that its reaction with GSH is very slow (about 500-fold slower than that of NEM) and it does not permeate cell membranes [12,13]. Because of this, if applied to intact cells or tissues, this method requires cell lysis which is usually carried out by acidification with consequent onset of large amounts of GSSG as an artifact. Indeed, in comparative analyses, where GSSG was determined after treatment of blood aliquots with either 2-VP or NEM, the levels of GSSG were at least 12 times higher in 2VP-treated samples with a corresponding decrease of the GSH/GSSG ratio [14]. A direct comparison between the two methods for analysis of GSH and GSSG in several mouse tissues has been performed also recently [15]. Results confirm that treatment with 2-VP does not entirely prevent GSH oxidation resulting in inaccurate data and significantly higher values for GSSG in all analyzed samples. In analogy to 2-VP, even the related compounds 1-methyl-2-vinylpyridinium trifluoromethane sulfonate and 1-methyl-4-vinylpyridinium trifluoromethane sulfonate successively proposed to mask GSH seem to give inaccurate results [15].

It should be pointed out that Griffith [11] originally validated this method in plasma where the artifactual oxidation of GSH is minimal, i.e., tolerable, because the GSH/GSSG ratio in plasma is much lower than in cells (~2 instead of ~400) [3], but this procedure has later been applied broadly to whole blood, red cells or other tissues in which GSH massively exceeds GSSG without proper modification.

4. The need to select a proper GSH alkylating agent

Notwithstanding the fact that the main feature of the sulfhydryl groups is that they oxidize easily to form disulfides, scarce attention is still devoted to this notion. Many different skilled scientists worldwide do handle biological samples containing high concentrations of GSH with minimal attention to the possible oxidation of the -SH group itself. In most of the available and widely used procedures now a days the only precaution used is the acidification of the sample. This behavior likely derives from the popular belief that the -SH group is much more stable than its ionized thiolate form S^- . Generally this is true, but in the presence of biological molecules with different characteristics and concentrations the situation deeply changes and many subtle errors can occur. In fact, it is now known that the -SH group indeed oxidizes during sample acidification, probably as the consequence of the release of reactive oxygen species from the biological matrices. Accurate analysis of GSSG requires even more analytical attention with respect to GSH. The main aspect to consider is that within cells GSH is 300- to 800-fold higher than GSSG. Therefore, even a minimal oxidation of GSH can induce a dramatic increase of GSSG. In Figure 1, the theoretical calculation of the impact of GSH's artificial oxidation on GSSG rise is shown, considered their actual ratio in blood (e.g., 500:1). If only 1% of GSH oxidizes, it would result in a ~150% bias of the measured GSSG concentration. We too ignored this aspect in the past and handled blood samples without any particular precaution [16-18]: as a matter of fact, we reported biased (high) levels of GSSG and a wrong ratio GSH/GSSG (about 10) in whole blood of healthy people.

Nevertheless, even when samples are treated with thiol-masking agents to prevent thiols artificial oxidation, in most cases unreliable compounds are used and/or they are not added at the proper time and the bias is still present. In fact, some different thiol-masking agents are routinely used to measure GSH and GSSG, such as alkylating agents (iodoacetic acid, NEM), 2-VP, 5-methyl methanethiosulfonate, monobromobimane [19]. In Figure 2, an example of the hematic levels of GSSG in healthy people measured considering different pre-analytical conditions (e.g., no pre-treatment, pre-treatment with NEM, NEM added together or after acid, 2-VP added together or after acid) reported in literature are shown. Apart a clinical study carried out in the late sixties, most data are available starting from the eighties. Interestingly, it can be noted that whereas NEM pre-treatment was a common feature in the first clinical studies, more recently this use was pretty abandoned. Additionally, in most cases samples are not pre-treated with a masking agent or the agent is added after acid addition, all conditions where measured basal levels result to be very high.

In 2002, we compared the effect of the use of NEM with that of other commonly used alkylating agents and reported that both iodoacetic acid and monobromobimane slowly react with -SH groups yielding too high values of GSSG. Additionally, only in samples treated with NEM before acid deproteinization we could obtain a good recovery of GSSG added to hemolysate [8]. These data together with those obtained for 2-VP [14,15] let us to select NEM as the most suitable alkylating agent to protect the oxidation of thiol groups, according to what suggested previously [Butler, Tietze]. NEM was found to be the best alkylating agent because, in addition to penetrate cell membranes and to rapidly block all available GSH within seconds, it is also a strong rapid irreversible inhibitor of GR [8]. The last effect

is important since it avoids any artificial reduction of GSSG to GSH during sample handling as the consequence of the shift to the right of the equilibrium (1):

Therefore, the reaction of NEM with –SH groups is so rapid and effective that any perturbation of the GSH-GSSG equilibrium is avoided.

The addition of NEM to the sample, as soon as possible after sample collection, and at the right concentration (e.g. about 40 mM in whole blood) was shown to be able to block any oxidation of thiols to disulfides and to represent the reference procedure to measure the intracellular GSH and GSSG concentration in a reliable way. The acidification step of samples for deproteinization must follow the reaction with NEM [8,10,14,20,34,35]. In fact, that addition of NEM together with acid or after acid deproteinization is not correct since it does not avoid artificial oxidation of GSH. In the first case, this is due to the fact that NEM does not react with GSH under acidic conditions. In the latter case, NEM treatment is not effective since GSH has been already oxidized to GSSG during acid addition [8]. The same is true for 2-VP, that being not cell membrane permeable, only works after cell disruption by acidification.

The acid-derived artifact is massive in blood or red cells, but it also occurs in solid tissues and cultured cells (Table 1). This notwithstanding, the problem of the artefactual oxidation of GSH is still neglected also by manufacturers that produce kits for GSH and GSSG assays. For example, the Glutathione Assay kit of Cayman (n. 703002) uses 2-VP in deproteinized samples as GSH masking agent. The same applies to the Glutathione Detection kit DetectX, Arbor Assays. As shown in Figure 3, by the use of these kits the values of GSSG (and of the ratio GSH/GSSG) resulted to be significantly different from those obtained by the reference method (sample pre-treatment with NEM) in some comparative experiments with several rat tissues and cultured cells (our laboratory, unpublished data). It is astonishing that notwithstanding the Tietze's method for glutathione titration is the most cited one in the literature (4111 hits on Scopus database) and most of the commercially available kits are still based on its procedure, his findings and warnings on GSSG values have been rapidly and are still consistently ignored.

5. Suggested methods to measure GSH and GSSG

The methods should be selected depending upon the sample type and the analyte concentration and, therefore, they must be different for GSH and GSSG.

Given the high concentration of GSH in the intracellular compartment, its measurement is simpler and regarding the choice of the method, the feature of having a low detection and quantitation limit is of scarce importance. Much more important aspects to consider are robustness, simplicity, feasibility and, last but not least, the costs of the method.

Conversely, for GSSG, considered that its basal levels are very low, the choice of a method is mainly determined by a low detection limit and high sensitivity. It is to underline, here,

that the majority of errors we encountered working in this field mainly derive from pre-analytical factors rather than from the analytical method itself. Therefore, once avoided oxidation of GSH during sample handling, it is a quite easy task to measure both GSH and GSSG, and several methods are available for this.

As we recently reported, an easy method to measure GSH within cells is to detect the conjugate GS-NEM by UV/vis-HPLC [34]. Alternatively, GSH can be measured in samples not pre-treated with NEM as total GSH (tGSH, i.e., GSH+ GSSG) by the GSH-recycling method [35]. Under basal conditions this value can be reasonably considered equivalent to the GSH concentration since GSSG levels are very low (about 400- to 500-fold lower). Nevertheless, to study the modulation of this concentration under particular physiological or pathological conditions, the subtraction of GSSG is needed. As evidenced in our recent study [35] and as previously reported by others [36], the use of the GSH-recycling method is not easy and requires some particular advices. Thus, the method is not an end-point analysis but it depends upon the K_M value of GR. Further, the assay is affected by pH, salt concentration, temperature, kind of buffer, and contaminants that may be present in the cuvette.

Major differences between cellular and extracellular compartments exist both in terms of the concentrations of thiols and disulfides and their relative redox state. Specifically, thiol concentration is very low and similar to that of disulfides and other thiols occur either as precursors or metabolites of GSH [37]. Therefore, the most suitable way to measure the redox thiol homeostasis here is labeling of thiols with fluorescent molecules, such as monobromobimane and benzofurazans [for a comprehensive description see 38].

GSSG too can be measured by different methods after removal of excess NEM by extraction. It can be assayed by spectrophotometry by using the GSH-recycling method or alternatively by HPLC [39, 40]. Apart from the GSH-recycling method, GSSG is generally reduced to GSH by thiol exchange type reagents (like dithiothreitol) or by phosphines and then the thiol group is labeled as mentioned above.

Some mass spectrometry-based methods have also been developed for GSH and GSSG measurement. Thus far, no GC-MS methods have been reported for GSSG. Measurement of GSH by GC-MS is possible after a two-step derivatization (Scheme 2). Thus, GC-MS analysis of GSH succeeded after its derivatization to the *N,S*-carboxyethyl dimethyl esters [41,42]. Yet, these derivatives require electron ionization (EI) which lacks sensitivity. GC-MS analysis of GSH can be improved by acylating the three amino groups of the GSH dimethyl esters with pentafluoropropionic anhydride (PFPA) and by using electron-capture negative-ion chemical ionization (ECNICI) (Scheme 2). However, LC-MS/MS methods are clearly the most preferred approaches to measure simultaneously GSH and GSSG. Given the indispensability of NEM or other alkylating agents such as iodoacetamide (IAA) for the accurate analysis of GSH, the majority of recently reported and validated LC-MS-based methods measure the GSH-NEM or GSH-IAA thioethers in parallel with native GSSG (Scheme 3). The main reason for using NEM, IAA or 2-VP is not the improvement of the LC and MS/MS properties of GSH, but to avoid artificial oxidation of GSH to GSSG, even if, as stated before, they are not equally effective to this purpose. Some LC-MS methods

have been reported, where NEM has been selected as alkylating agent, however, again some discrepancies regarding the use of NEM are evident. For example, Steghens et al. [24] developed a new method based on a LC-MS/MS in positive electrospray ionization mode after chromatographic separation on a specific column. As mentioned above, in this procedure the use of NEM was used to avoid the oxidation of GSH. However, NEM was added to sample together with the deproteinizing acid (sulfosalicylic acid). Under these acidic conditions (pH =2–2.3) NEM cannot react with thiols. More recently, a new LC-MS/MS method was developed, where compounds were separated by liquid chromatography using a Hypercarb column, particularly suitable for small polar compounds [43]. Also in this work, whole blood was mixed with a precipitating solution containing both NEM and sulfosalicylic acid. Even more recently, a new method based on molecular speciated isotope dilution mass spectrometry (SIDMS) has been developed. SIDMS entails spiking a known mass of sample with proper amounts of isotopically enriched analogues of the targets analytes GSH and GSSG. Then samples are analyzed by tandem mass spectrometry (NEM was used as masking agent and added to collecting tubes) and the analytes are quantified using the stable-isotope labelled analogs. Oxidation of GSH to GSSG was corrected by using mathematical relationships in double-spiking SIDMS [44]. These LC-MS/MS methods may represent a good alternative to spectrophotometric and HPLC with UV absorbance detection assays but a good agreement with these techniques still lacks. The measured values for GSH and GSSG and the used experimental conditions in these manuscripts are summarized in Table 2.

It is worth mentioning that LC-MS/MS methods that use NEM in combination with stable-isotope labelled analogs of GSH, GSSG and NEM, provide very low GSSG contents in human whole blood. For instance, Moore et al. reported $900 \pm 140 \mu\text{M}$ for GSH, $1.17 \pm 0.43 \mu\text{M}$ for GSSG and 880 ± 370 for the GSH/GSSG ratio in whole blood of 59 healthy adults [43]. In whole blood of 9 subjects, Fahrenholz et al. reported $1.46 \pm 0.07 \mu\text{mol/g}$ for GSH and $2.4 \pm 0.4 \text{ nmol/g}$ for GSSG, yielding a mean GSH/GSSG ratio of 608. In RBCs of 9 healthy children, this group reported $1.69 \pm 0.83 \mu\text{mol/g}$ for GSH and $6.1 \pm 3.8 \text{ nmol/g}$ GSSG, corresponding to a mean GSH/GSSG ratio of 277 [44]. These data indicate considerable discrepancies even between highly sophisticated LC-MS/MS methods and point out to additional not yet fully recognized pre-analytical and/or analytical problems. It should be also reminded that NEM was used at different experimental. In some cases NEM was added to the deproteinizing solution [24,43]. This procedure should be less effective in protecting GSH from its oxidation since NEM alkylation needs a neutral pH. This notwithstanding, the measured GSSG levels are very low, even if Steghens et al. found a concentration seven-fold higher in RBC than in whole blood; these points would be worth to be further studied to understand the reason for this unexpected result. Conversely, blood samples in the work of Fahrenholz et al. [44] were treated with NEM after sample collection and stored until analyses. These different pre-analytical treatments may be responsible for the observed discrepancies. Additionally, Baldwin and Kiick reported that NEM adducts of some thiols including *N*-acetylcysteine may undergo retro and exchange reactions at physiological pH and temperature [45]. Using the lipophilic and strong reductant thiol *N*-acetylcysteine ethyl ester (NACET) [46], we obtained by GC-MS strong indication for alternative reaction products of thiols with NEM (Fig. 4). Thus, our results indicate that the reaction of NACET

with NEM is a substitution but not a Michael-like addition reaction. Further investigations, preferably by LC-MS/MS, are warranted to investigate the reaction of NEM, IAA and 2-VP with thiols and characterize their reaction products.

6. Conclusions, possible consequences and perspectives

Measurement of GSH and GSSG, in particular for the calculation of the GSH/GSSG ratio which is considered an excellent index of the oxidative status of a tissue, is plagued by many severe artifacts. Results obtained so far using inappropriate GSH and GSSG methodologies are void of any value. To our knowledge only a few relevant clinical studies have been carried out so far using robust and artifact-free methods. In 1968, a significant difference in the hematic GSH/GSSG ratio in people affected by G6PDH deficiency was observed. The content of GSSG in the RBCs deficient in G6PDH was about 3 times that in normal RBCs [47]. More recently the GSH/GSSG ratio has been measured in blood of premature newborns. Results indicate that both GSSG and the GSSG/GSH values of premature infants with idiopathic respiratory distress syndrome (IRDS) markedly exceeded the levels of control newborns of the same age and maturity. The authors also found a decrease in GSH concentration in premature infants with IRDS [40]. An increased GSSG/GSH ratio was also seen in blood of patients younger than 3-months old with simultaneous active retinopathy of prematurity [48]. In our recent work aiming to study the glutathione redox state in end-stage renal disease patients we observed a doubling of GSSG levels in RBCs of these patients as compared with age-matched healthy control subjects [49]. In a few words, at present, the question “do people affected by a certain disease have abnormalities in the GSH/GSSG levels?” is still unanswered. However, this can be taken as an opportunity: the correct assessment of the GSH/GSSG ratio in blood (as well as in tissues and cultured cells) is still an unexplored field. Furthermore, how and to what extent the GSH/GSSG ratio can be influenced by physiological and pathological conditions, is still largely an unanswered question.

In previous work, we have pointed out to the pre-analytical and analytical factors that may occur in many approaches that are widely used for the measurement of GSH and GSSG in biological fluids, especially including blood. Only the reliable measurement of biological GSH and GSSG can provide dependable values for the GSH/GSSG ratio in health and disease, in experimental and clinical studies, in the area of oxidative stress. We have recently reported detailed protocols for the artefact-free and accurate measurement of GSH and GSSG in blood and other biological samples [35]. In consideration of the current wide use of questionable procedures, frequently including the use of commercially available, yet non-binding kits, by many researchers in the area of oxidative stress, our focus in the present article was on the emphasis of potential but avoidable pitfalls in the analysis of GSH and GSSG in biological samples, notably whole blood and RBCs. Non-consideration of these long-known and well-recognized pitfalls will inevitably lead to artificial oxidation of GSH to GSSG thus generating invaluable biased GSH/GSSG ratios, falsely cheating altered oxidative stress. GSH is considered to be involved and to play crucial roles in ageing, disease and last but not least in pharmacotherapy including intoxication, for instance by paracetamol (acetaminophen). The issue of GSH and GSSG measurement in blood resembles the “Elephant in the room”, a metaphorical for an obvious truth that is ignored, as

an elephant in a room would be impossible to overlook (https://en.m.wikipedia.org/wiki/Elephant_in_the_room).

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Abbreviations

ECNICI	electron-capture negative-ion chemical ionization
EI	electron ionization
GSH	glutathione
tGSH	total GSH
GR	GSSG reductase
GSSG	glutathione disulfide
IAA	iodoacetamide
NEM	N-ethylmaleimide

PFPA	pentafluoropropionic anhydride
2-VP	2-vinylpyridine
SIDMS	speciated isotope dilution mass spectrometry

Highlights

- GSH artificially oxidizes during sample handling producing GSSG
- Most of GSH to GSSG ratios reported in the literature are erroneous
- Immediate –SH alkylation with *N*-ethylmaleimide is mandatory

Figure 1. Effect of GSH oxidation on GSSG levels

The theoretical impact of GSH oxidation on the increase in GSSG is represented. The values refer to a ratio GSH/GSSG of ~500, typical of whole blood (or RBCs).

Figure 2. GSSG levels measured in whole blood of healthy people

GSSG values were obtained from selected papers published in the time period 1968-2015.

Data are discriminated according to the pre-analytical conditions: NEM pre-treatment before acid (circle), acid without any pre-treatment (triangle up), NEM added together or after acid addition (triangle down), 2-VP added together or after acid addition (diamond). Data are from [6,8,20-31]

Figure 3. GSH/GSSG values in different biological samples analyzed by different methods
Tissues and cultured cells were processed as previously described [33,35] or according to the Glutathione Assay kit of Cayman (commercial kit 1) and Glutathione Detection kit DetectX, Arbor Assays (commercial kit 2) for GSH and GSSG analysis.

Figure 4. GC-MS spectrum of a reaction product of NEM with *N*-acetylcysteine ethyl ester (NACET) in the electron-capture negative ion chemical ionization (ECNICI) mode Equimolar concentrations (1 mM) in 1 mL phosphate buffer, pH 7.4, were incubated at room temperature for 1 h. Thereafter, reaction products were extracted from 100 μ L of the mixture with 1 mL toluene and analyzed by GC-MS in the ECNICI mode. The mass spectrum of the major peak with the retention time of 7.4 min was obtained by scanning between the range m/z 30 to m/z 450. The inset shows the reaction of NEM with NACET. This mass spectrum supports the formation of an unsaturated NACET-NEM thioethers (substitution reaction, A) rather than a saturated NACET-NEM thioethers (Michael-like addition reaction, B).

Scheme 1. Schematic diagram of the GSH-recycling reaction

GSH reacts with DTNB to form TNB and GS-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. The assay measures total glutathione, i.e., the sum of GSH + GSSG.

Scheme 2. Schematic diagram of the two-step derivatization of GSH for GC-MS analysis

The carboxylic groups of GSH are converted to their methyl esters by using 2 M HCl in methanol (MeOH). Subsequently, the GSH dimethyl esters are converted to their dimethyl ester-*N,S*-ethoxycarbonyl derivatives by using ethyl chloroformate (EtOCOCI) for electron ionization, or to their dimethyl esters pentafluoropropionyl (PFP) derivatives with pentafluoropropionic anhydride (PFPA) for electron-capture negative-ion chemical ionization.

Scheme 3. Schematic diagram of the one-step derivatization of GSH for LC-MS/MS analysis

The sulfhydryl group of GSH is converted by *N*-ethylmaleimide (NEM), by iodoacetamide (IAA) or by 2-vinylpyridine (2-VP) for electrospray ionization in the positive mode. Although not definitely demonstrated, the reaction of NEM with GSH is generally considered a Michael-like addition reaction which forms the GSH-NEM thioether under saturation of the C=C bond of NEM.

Table 1

Reported values for GSSG in biological samples pre-treated (+ NEM) or not pre-treated (- NEM) with *N*-ethylmaleimide (NEM).

Sample	GSSG (μM) + NEM	GSSG (μM) - NEM	Reference
Human blood ^a	22.2±7	561±235 (2400%) ^b	[35]
Rat RBCs ^a	18.6±0.10	502±88 (2700%)	[32]
Rat liver	43.3±2.9	253±32 (484%)	[32]
Rat kidney	13.4±3.4	119±5 (788%)	[32]
Rat lung	17.2±0.8	119±12 (592%)	[32]
Rat heart	11.3±2.6	92±15 (714%)	[32]
Rat spleen	12.2±2.6	53.3±12.6 (338%)	[32]
Rat testis	15.0±1.0	87.8±10.6 (485%)	[32]
Rat brain	18.1±1.4	54.3±3.1 (200%)	[32]
BAEC ^{a,c}	218±38	525±23 (140%)	[32]

^aValues are expressed as pmoles/mg of protein

^bData in brackets indicate the percentage of increase of GSSG

^cAbbreviations: BAEC, bovine aortic endothelial cells

Table 2
Reported values for GSH and GSSG in human blood from healthy people

Data are expressed as nmol/mg Hb for GSH and pmol/mg Hb for GSSG. A value of 150 mg/ml Hb in whole blood was considered for normalization among different studies.

GSH	GSSG	NEM addition	Method	Reference
8.40±0.56	-	in collection tubes	HPLC	[34]
8.46±1.75	13.2±4.2	in collection tubes	HPLC/GSH recycling	[39]
9.5±2.1	15.2±3.3	in hemolyzed blood	GSH recycling	[40]
8.73 ±0.79	4.13	with acid	HPLC-ESI-MS	[24]
6.00±0.93	7.8±2.9	with acid	LC-MS/MS	[43]
5.63±0.28	20.3±12.7	in collection tubes	LC-MS/MS	[44]