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Enzymatic glutaredoxin-dependent method to determine glutathione and protein *S*-glutathionylation using fluorescent eosin-glutathione

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ABSTRACT

Glutathione is an abundant low-molecular-weight thiol, up to 10 mM in mammalian cells, and exists in three major forms: reduced sulphydryl (GSH), glutathione disulfide (GSSG) or bound to Cys residues in proteins (PSSG). The ratio GSH/GSSG has been used as an indicator of the cells redox level but this parameter can also be estimated by the quantification of PSSG. In fact, PSSGs have the advantage of being more stable than GSSG.

Here we present a highly sensitive fluorescent-based method for detection of low concentrations of glutathione in complex samples such as cell lysates, tissues and plasma. The method is based on our previously described protocol to study Glutaredoxin (Grx) activity. The whole procedure was optimized to measure the fluorescence increase of the di-eosin-glutathione disulfide (Di-E-GSSG) reduced by Grx in the presence of Glutathione Reductase and NADPH, keeping GSH as the limiting factor to drive the reaction.

The methods to selectively measure PSSG are expensive and not widely accessible, therefore we optimized our glutaredoxin protocol to quantify this post-translational modification using common laboratory equipments.

Overall, our method has simplicity and rapidity combined with high sensitivity as its main advantages; therefore, it may be particularly suitable for large-scale clinical studies.

1. Introduction

Glutathione (GSH) is a tripeptide (γ-Glu-Cys-Gly) present at very high concentrations throughout living organisms (from 1 to 10 mM). However, the comprehension of its roles had greatly advanced only over the past decades [1–3]. From its first described role in heavy metals and xenobiotics detoxification, it is now known that GSH plays a key role in cell redox homeostasis, gene expression, cell proliferation and apoptosis mainly by binding to Cys residues in proteins, a process known as protein *S*-glutathionylation. However, when the oxidant concentrations increase over a certain threshold, protein *S*-glutathionylation becomes a protective mechanism to avoid over-oxidations of protein Cys residues [4–10].

GSH also acts when coupled to different enzymes like Glutathione Peroxidase and Glutaredoxin (Grx). In particular for Grx, GSH represents the reducing power for the enzyme and can directly regulate the activity of other important proteins like Ribonucleotide Reductase (RNR), that catalyzes the rate limiting step of the DNA synthesis [11]. It has been shown that the RNR activity with Grx is very much dependent on the concentrations of GSH since a disulfide is to be reduced [12]. Another important GSH function is in maintenance of the iron-sulfur cluster structure of the Grx2 mitochondrial isoform [13,14].

Within mammalian cells, glutathione exists mainly (> 98%) in the thiol-reduced form (GSH); in fact the oxidized form, GSSG, is toxic in the cytosol and it is either reduced by Glutathione Reductase (GR), in a reaction requiring NADPH as electron donor, or exported from cells [15,16].

Because GSH and PSSG are implicated in many essential cellular functions, a lot of effort has been spent to develop precise and accurate methods for their identification and quantification. However the technology available to assay PSSG is usually expensive and not widely accessible.

We have developed and characterized a new protocol to detect GSH, GSSG and PSSG in different cell lines, tissues and total GSH in plasma. The method is based on the Grx assay we previously described, exploiting its very high sensitivity [17,18].

Abbreviations: Grx1, human Glutaredoxin-1; GR, yeast glutathione reductase; NADPH, nicotinamide adenine dinucleotide phosphate; GSH, glutathione; DIA, diamide; BSA, bovine serum albumin; Di-E-GSSG, di-eosin-glutathione disulfide; E-GSH, eosin glutathione

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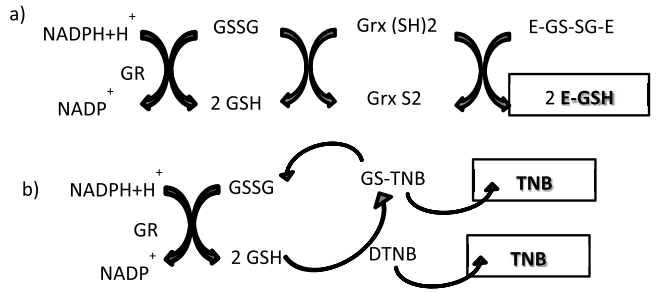

Scheme 1. Schematic representation of the reactions 1–3 a) and DTNB based assay b).

The fluorescent substrate, Di-E-GSSG, is cleaved by reduced Grx. This generates two molecules of E-GSH that have high fluorescence emission; the reaction is coupled to oxidation of the active site in Grx (reaction 1–3 and Scheme 1). Oxidized Grx is reduced by one molecule of GSH and this mixed disulfide is subsequently reduced by a second GSH molecule, releasing GSSG that is further reduced by GR in the presence of NADPH. GSH represents the limiting factor of the Grx cycle, therefore an increase in the fluorescence depends only on the GSH concentration present in the sample.

$$Grx(SH)_2 + \text{E-GS-SG-E} \rightarrow Grx\begin{matrix}\diagup \text{S-SG-E}\\ \diagdown \text{SH}\end{matrix} + \text{E-GSH} \rightarrow Grx\text{-}S_2 + \text{E-GSH} \quad (1)$$

$$Grx\text{-}S_2 + 2GSH \rightarrow Grx\text{-}(SH)_2 + GSSG \quad (2)$$

$$GSSG + NADPH + H^+ \xrightarrow{(GR)} 2\ GSH + NADP^+ \quad (3)$$

To check the applicability of the method, we prepared the samples following a very well established assay, based on the Tietze *et al.* recycling method, DTNB-assay [19,20]. Each sample was analyzed in parallel using both DTNB-assay and the proposed new fluorescent assay. Data indicate that in samples (cells or tissues lysates) with high GSH concentration our fluorescent method gave the same results as the DTNB-assay. Whereas the ability to directly detect GSH in plasma and PSSG in cells and tissues under physiological conditions was significantly better in this new method. Demonstrating a higher sensitivity.

From a practical point of view, the assay is easy to perform and it can be undertaken in 96-well plates using a standard fluorescent plate reader. For these reasons, we believe the proposed method could be an important and useful tool in the study of redox signaling and can have high applicability in clinical studies.

2. Materials and Methods

2.1. Reagents

Except when indicated, all general reagents were of molecular biology or higher grade and purchased from Sigma-Aldrich. L-cysteine-glutathione disulfide (CSSG) was from Cayman chemical. Di-E-GSSG was prepared as previously described [18]. Spectrapor dialysis membranes were purchased from Spectrum Laboratories and Amicon ultra centrifuge filter tubes with 14,000 MW cut off were from Merck Millipore.

2.2. Preparation of alkylated BSA

Enzymes can often bind irreversibly to tubing, resulting in a decrease in the effective enzyme concentration. This can be thwarted by including BSA in the assay buffer [21,22]. However the free Cys residue in BSA (Cys34) could interfere with the assay by binding GSH and therefore we prepared alkylated BSA to avoid any interferences in the Grx activity as follows:

BSA (0.3 mM) was incubated with Iodoacetamide (IAM - 1 mM) in 0.1 M potassium phosphate (KPE) buffer, pH 7.5, for 60 min at 37 °C. Alkylated-BSA was separated from the non-reacted IAM by dialysis against 0.1 M potassium phosphate buffer, pH 7.5, using dialysis membranes with 14,000 MW cut off. The concentration of alkylated-BSA was determined both by the method of Bradford [23], calibrated with BSA, and measuring the absorbance at 280–310 nm, using the molar absorption extinction coefficient of 44,000 $M^{-1}cm^{-1}$ in 0.1 M potassium phosphate buffer, pH 7.5.

2.3. Fluorescence measurement

The reduction of Di-E-GSSG and further release of E-GSH was measured by fluorescence detection in black 96-well microtiter plates, with a final volume of 100 µl, fluorescence emission was recorded at 545 nm, after excitation at 520 nm, using an Enspire 2300 Multilable Reader, from PerkinElmer. The standard curves for GSH, GSSG, CSSC, CSSG were made using 10 µl of each different standard concentration, 30 µl of 0.1 M phosphate buffer – EDTA 5 mM, pH 7.5, KPE, 50 µl of master mix (alkylated-BSA 0.1 mg/ml, NADPH 0.4 mg/ml, GR 0.1 µM, Grx1 1 µM (IMCO, Sweden) in KPE) and 10 µl of fluorescent substrate, Di-E-GSSG, 100 µM. Fluorescence was read at 545 nm after excitation at 520 nm for 30 min every 30 s.

2.4. DTNB measurement [19].

For the DTNB measurements 10 µl of standard or sample were mixed to 60 µl freshly prepared DTNB (0.3 mg/ml) and GR (1.67 U/ml) solutions together. After 30s incubation, for the conversion of GSSG to GSH via GR activity, 30 µl of β-NADPH 0.7 mg/ml were added to each well. Absorbance at 412 nm was recorded for 5 min every 20 s in a TECAN plate reader.

2.5. Cell culture

Cells (HEK293, PC3 and PANC-1) were cultivated in appropriate medium supplemented with 10% (v/v) of fetal bovine serum (FBS) and maintained at an atmosphere of 37 °C and a 5% (v/v) CO_2.

2.6. Sample preparation for total GSH and GSSG: cells and tissue

Sample preparation was performed as described previously [19] with the purpose to compare directly the results obtained by DTNB-assay versus the fluorescent-based method. Briefly, cells were scraped from 100 mm dish plates, washed twice in ice-cold PBS, lysed in 0.1% Triton-X and 0.6% sulfosalicylic acid (SSA) to precipitate the protein content, in KPE, and centrifuged for 10 min at 13,000 × *g* at 4 °C, in order to separate protein pellet and supernatant.

Snap-frozen or fresh heart, kidney, brain, spleen and liver samples were homogenized and sonicated in an ice-cold solution containing 5% phosphoric acid and 0.6% SSA mixture and clarified by centrifugation. (Animal housing, handling and experimentation were approved by the regional animal ethics committee of Northern Stockholm. We used tissues from male, 4 months old, C57BL/6 strain).

The acidified supernatants were divided in two and used directly for total GSH measurement or derivatized for GSSG assay, following the protocol proposed by Rahman et al. [19]. Importantly, to assay GSSG, the samples were fast mixed with vinylpyridine (4 µl of 1:10 diluted 2-vinylpyridine in KPE for 100 µl of supernatant) to form a stable complex with GSH, preventing it from participating in the enzymatic recycling reaction with GR. Standards of different concentration of GSSG were prepared and treated in parallel. All samples were vortexed 15 s and incubated for 2 h at room temperature in a fume hood. To inactivate the non-reacted vinylpyridine, 6 µl of triethanolamine (1:6 diluted in KPE)

were added to each sample, incubated for 10 min and divided in two aliquots for the DTNB-assay and fluorescent method analyses.

Standard curves were run in parallel to the samples in the appropriate range of GSH and GSSG for the sample GSH-content. The linear part of the kinetic curve was used to calculate the slope for each curve.

2.7. Sample preparation for total GSH: plasma

Human plasma was obtained from healthy blood donors at the Karolinska Hospital, Stockholm, Sweden. Plasma was aliquoted and stored at −20 °C until usage in further studies. Standard curves were run in parallel to the samples in the appropriate range of GSH. Briefly, 20 µl of plasma were used each well, and a plasma-background well with the master mix depleted of Grx was run for each different plasma sample in parallel to take in consideration every unspecific reduction of Di-E-GSSG due to unknown factors. The linear part of the kinetic curve (usually from 5 to 25 min) was used to calculate the slope for each curve.

2.8. Sample preparation for PSSG: cells and tissue

To avoid any GSH or GSSG contamination in the protein pellet and oxidation due to sample manipulation, a modified protocol of Bukowski and collaborators [24] was followed.

After removed the acidified supernatants (section 2.6), the pellets were resuspended in 500 µl of 6 M urea buffer (0.1 M phosphate and 10 mM *N*-ethylmaleimide (NEM), to alkylate free thiols, pH 7.4) and incubated at room temperature for 30 min; 10 µl of each sample were aliquoted for protein quantification.

Proteins were precipitated with acetonitrile and centrifuged at 15,000 × *g* for 5 min. The supernatants were discarded, whereas pellets were rinsed three times with acetonitrile to remove excess of NEM. Pellets were then resuspended in 500 µl of 6 M urea/phosphate buffer (0.2 M, pH 7.4) with sonication and then precipitated again with 5% SSA solution and centrifuged three times to remove any residual GSH and GSSG. The final pellet was resuspended in 500 µl of 6 M urea/$NaBH_4$ (25 mM, pH 9.0) to release the GSH from protein and 10 µl/sample were collected for protein concentration measurement. SSA 5% was added and centrifuged for 5 min at 15,000 × *g*. The final supernatants were divided in two aliquots and processed following DTNB-assay or fluorescent-based method. For PSSG a standard curve of GSH was used as a reference.

2.9. Statistics

Data are presented as the mean ± SD and $n \geq 3$. Statistical significance was determined by paired Student's *t*-test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Standard curves

As previously described Di-E-GSSG is a bulky molecule that is a very poor substrate of GR but a very good substrate of Grx and can be used in presence of GR, GSH and NADPH to asses Grx activity [17].

To use this method as a GSH-content assay, we optimized the conditions to measure GSH concentration using Grx system keeping GSH as the limiting factor.

To check if this approach could be used in further analysis we first tested a broad range of GSH dilutions and the slope of the linear part of the curve "fluorescence vs time" shows a linear regression trend with increasing of GSH concentration. The results of the lower part of the curve (Fig. 1a) demonstrated that using this method, it is possible to detect GSH concentration as low as 0.1 µM. Moreover, we wanted to check if GSSG and mixed glutathione disulfide could be detected using

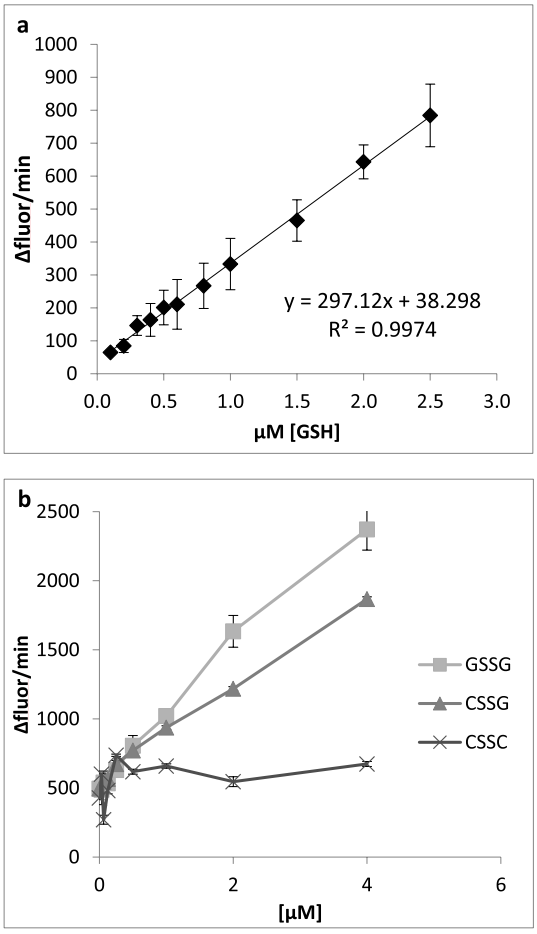

Fig. 1. Standard curves for GSH, GSSG, CSSC and CSSG. An aliquot of 50 µl of master mix of alkylated-BSA 0.1 mg/ml, NADPH 0.4 mg/ml, GR 0.1 µM, Grx1 1 µM were added to 10 µl of different standard solutions at different concentrations. After addition of the fluorescent substrate, Di-E-GSSG 10 µM, fluorescence at 545 nm was recorded after excitation at 520 nm. Slope of the linear part of the curve (fluorescence, time) were plotted against the GSH or disulfide concentration (n for "a" > 8; n for "b" = 4 result are average ± STD).

this method. Fig. 1b shows that only in the presence of disulfide with glutathione there is an increase in fluorescence over time, proportional to the increase in concentration, while CSSC does not lead to any increase in fluorescence.

3.2. Comparison between DTNB-assay and fluorescent-method in cells

The DTNB-recycle method is very commonly used to measure glutathione concentration.

We first compared the fluorescent and the DTNB-method [19] on the total GSH and GSSG content in different cell lines. Results were in the same range and did not show any statistical difference between the methods (Fig. 2a).

The total GSH measurements were compared to those obtained elsewhere with established methods in complex samples (DTNB, HPLC and biotinylated–GSH). The results were in the same range and, therefore, comparable [25–28]. However, when the PSSG content from different cell lines were measured, the best results were obtained with the fluorescent assay (Fig. 2b).

3.3. Comparison between DTNB-method and fluorescent-method in different organs and conditions

The analysis of total thiol-disulphide state of glutathione is

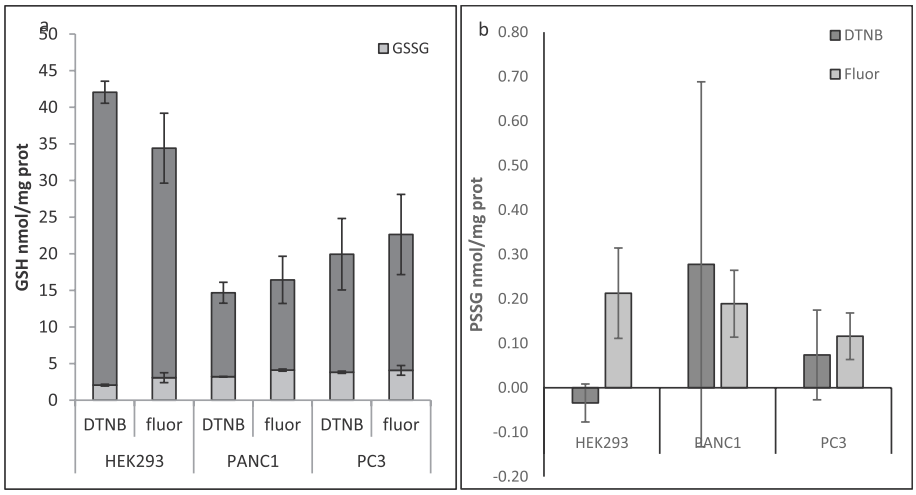

Fig. 2. Total GSH measurement and PSSG. Total GSH and GSSG concentration and PSSG were measured in different cell lines. Each sample was aliquoted in two and analyzed in parallel with the DTNB-assay and the fluorescent assay we present. The values obtained were normalized for the total protein content. No significant differences were observed comparing the two methods for both GSH and GSSG measurements. However the DTNB assay was not sensitive enough to detect the GSH released from a protein using 20 µl of sample, see material and methods 2.8. (n = 2 mean ± STD).

challenging not only for the low concentration of specific forms (PSSG) or samples (plasma) but also for the risk of artefact formation during sample collection, storage and manipulation. For this reason we compared the measurements of fresh samples and snap frozen tissues stored in −80 °C for a month.

The glutathione content in tissues is usually higher than the limit detectable by the DTNB-method. Therefore, we employed the classic DTNB-method to measure total GSH and GSSG in different samples and compared the results with the fluorescent-method.

Mice tissues were homogenized and the obtained supernatants (see Materials and Methods) were analyzed. There were no differences between the concentration values for total GSH and GSSG comparing the two methods (Fig. 3a and b). Moreover, the values obtained for total GSH are according to the data already reported in the literature.

In Fig. 3c, it is interesting to notice that the levels of PSSG in brain samples are quite high compared to that of other organs analyzed.

Fig. 3d, e, f, shows the results obtained on the fresh collected tissues and despite the total GSH (Fig. 3d) value are the same using both methods, and in both conditions, the GSSG concentrations (Fig. 3e) are lower in the fresh collected tissues in particular in the brain. PSSG (Fig .3f) had a great decrease in concentration in fresh samples and the levels were lower than the detection limit of DTNB assay in two out of three cases.

The majority of studies on GSH transport and metabolism have been done in liver due to its high concentration of GSH, result of an equilibrium between the synthesis and the efflux into bile and blood circulation.

Analysis of GSH was performed in liver and, as for the other tissue samples, the concentration obtained with the two methods were comparable and despite the absence of statistical significance it is possible to see a slightly difference between the fresh samples and the snap frozen ones, in both assays (Fig. 4a).

GSSG instead (Fig. 4b) showed a decrease of about 3 times comparing the fresh samples with the snap frozen ones. It was possible to measure the concentration changes with DTNB and fluorescent-based method with high accuracy due to the high concentration of total glutathione in liver.

Detection of PSSG in fresh liver (Fig. 4c) was not possible using the DTNB assay however there was a lower difference between fresh and snap frozen values of PSSG with respect to the values of the other tissues (Fig. 3).

An aliquot of the fresh homogenized liver was treated with 10 mM diamide, a compound known to increase protein *S*-glutathionylation [29], for 20 min and only in the fluorescent method we were able to measure the expected increase of PSSG.

3.4. Total GSH measurement in human plasma

Measurement of GSH in plasma samples has been used for clinical purposes [30–34] and samples usually present very low amounts of GSH. We tested the substrate specificity and sensitivity of our new method by measuring total GSH in plasma from healthy blood donors. For each plasma sample a sample-background was run in parallel to remove all the possible unknown interference in the reduction of Di-E-GSSG. The fluorescence increase over time removed by the sample background, was plotted and the concentration of GSH was calculated from the standard curve run at the same time as the samples.

The levels of total GSH measured ranged from 1 µM to 7 µM (Fig. 5a) with values in accordance with previously reported data [7,35]. The values with higher GSH content could be due to partial hemolysis but we cannot exclude individual variations among the subjects.

A new set of samples was pooled three by three to have 5 new plasma samples and we divided each in two sub-aliquots. As it can be seen in Fig. 5b using the same amount of plasma (20 µl), it was not possible to determine a reliable result with the DTNB-method. This would also be explained by the fact that the total GSH measured in the fluorescent assay includes both the GSSG and the mixed disulfide form with Cys (CSSG) and PSSG. It is known that thiols in plasma have very low stability and are rapidly oxidized [36]. However exploiting the catalytically features of Grx we are able to measure all the disulfide formed by GSH (CSSG, GSSG, PSSG) without interference of CSSC (Fig. 1b).

4. Discussion

Glutathione plays several important roles in cellular physiology, such as the maintenance of redox status and signaling through protein *S*-glutathionylation.

Glutathione is present at high concentrations in the intracellular environment and changes in the GSH/GSSG ratio may be considered an indirect measure of intracellular redox status. Moreover the focus on redox regulation via protein *S*-glutathionylation has increased during the past decade both in physiological and pathological conditions such as diabetes, neurodegenerative diseases, and cancer [37–39]. Considering the importance of glutathione in the cell physiology, it is not surprising that several methods have been developed to measure it. Different approaches has been used such as colorimetric assays, detection by HPLC coupled to mass spectrometers and fluorescence [40–43]. The most widely used is the colorimetric assay using DTNB because it is easy to perform and accurate in the majority of the samples tested. However in specific context such as plasma or PSSG, more

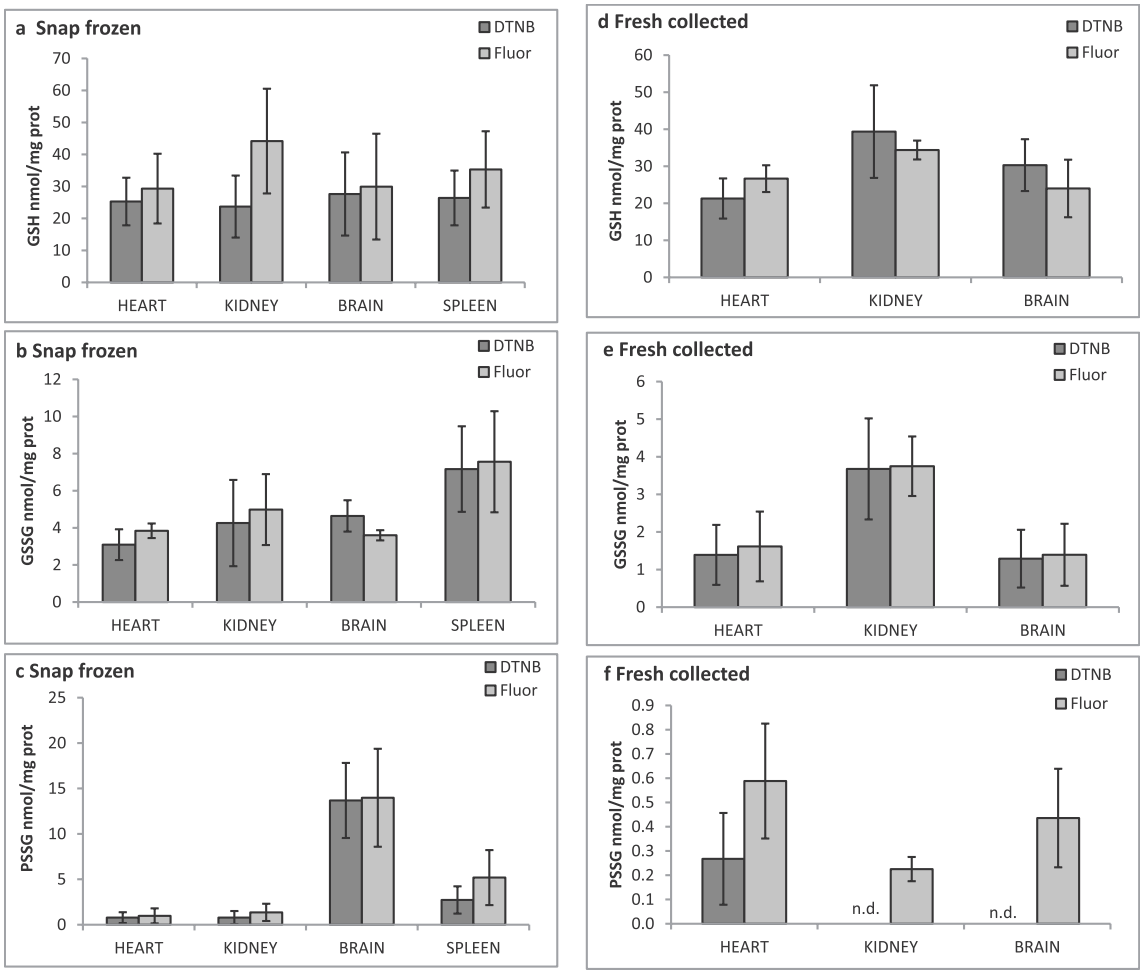

Fig. 3. Levels of total GSH and GSSG in different mice tissues. Total GSH (a), GSSG (b) and PSSG (c) were measured in snap frozen tissues. Each sample was divided in two and analyzed in parallel following the different methods. No significant differences were obtained comparing the DTNB-based assay and our fluorescent-based method (n = 4).

Total GSH (d), GSSG (e) and PSSG (f) were measured in fresh collected tissues. The results shows that for glutathione measurement, snap frozen tissue stored at −80 °C must be handle with extra careful due to artefact oxidations. The DTNB assay was not sensitive enough to measure the level of PSSG in fresh collected tissues.

sensitive techniques must be used.

Here we have developed and characterized a comparably rapid and easy to perform fluorescence-method to measure GSH, GSSG and PSSG. Importantly the sensitivity limit of our method is lower than the colorimetric one allowing the measurement of GSH from plasma and from glutathionylated proteins in complex samples.

The method is based on the Grx assay previously developed by our group [17,18]. It consists in the enzymatic reaction of Grx keeping the GSH from the samples as limiting factor for the kinetic, using as substrate, the fluorescent compound Di-E-GSSG.

The conjugation of two molecules of eosin to glutathione disulfide (Di-E-GSSG) shows very low fluorescence that increases, up to 20 times, after its reduction to E-GSH. Remarkably Di-E-GSSG is a very poor substrate for GR but it is a well-recognized substrate by Grx.

We wanted to compare the efficacy and sensitivity of our method against the standard DTNB-based assay. To achieve this target we used three different samples, from the easiest to the more challenging: cell lysate, mice tissue and human plasma. We approached stepwise the low molecular weight thiols and then the PSSG.

In cell lines, where the concentration of GSH is in mM range, total GSH showed no significant differences across methods. This means that the fluorescent-method is comparable with the DTNB assay. Moreover, we used different cell lines and their GSH content was in accordance with previous data published in the literature. GSSG measurements accounted for 10–20% of total GSH in samples using both assays.

After checking in cell lines that the DTNB and fluorescent-methods were equivalent we wanted to measure the glutathionylation profile of tissue samples. We tested different conditions to check if the variations observed across both methods and laboratories can also be due to the sample storage and manipulation.

All the organs used presented comparable amount of total GSH, except for the liver, which showed higher concentration. This difference is expected because in the liver GSH is utilized for detoxification of different molecules and xenobiotics and it is produced to be exported in towards other parts of the body such as bile and blood [44]. No statistically significant differences were observed between methods using tissues samples where the amount of GSH and GSSG was abundant confirming the cell results about the comparability of the two assays for total GSH and GSSG measurement.

Furthermore PSSG concentration was analyzed and in snap frozen samples (Figs. 3c and 4c) the value obtained were similar comparing the DTNB and fluorescent assay results. However comparing the data with fresh tissues, it is clear that the previous values are most likely artefacts due to sample manipulation. The effect is particularly visible in brain tissue.

It was possible to measure with small variation the PSSG content in fresh collected samples only using the fluorescent-method (Fig. 3f).

Liver analysis using our fluorescent assay shows low levels of PSSG

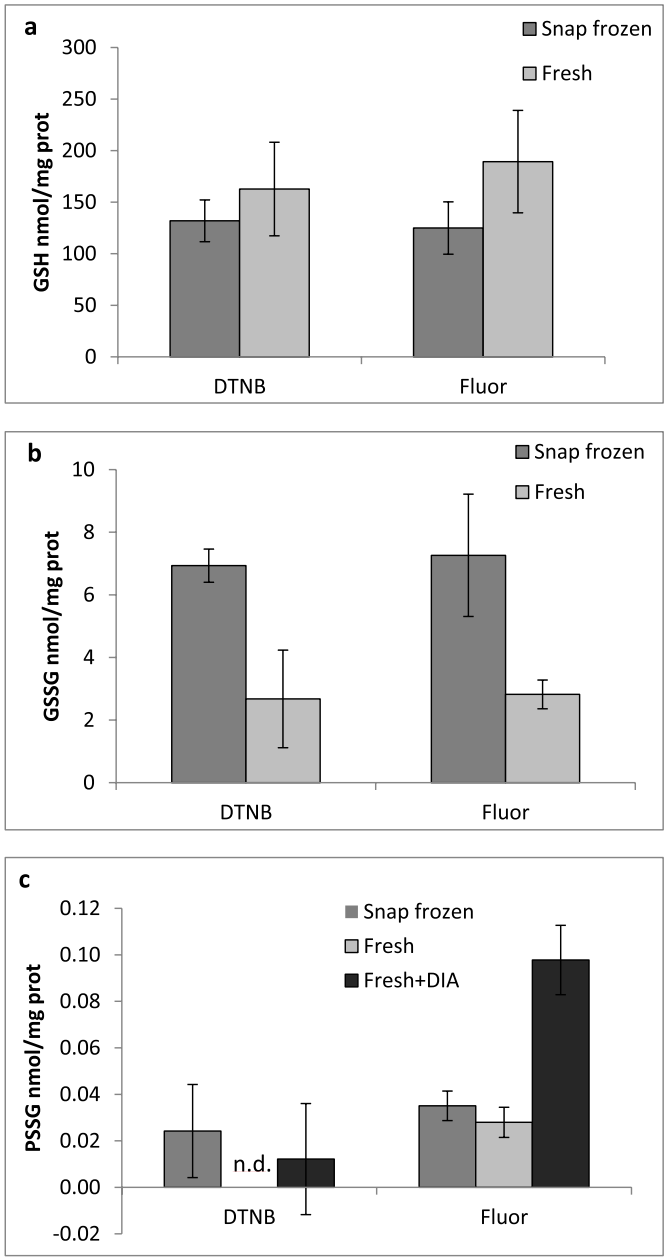

Fig. 4. Levels of total GSH and GSSG in mice liver. Total GSH (a), GSSG (b) and PSSG (c) were measured in snap frozen liver. Each sample was divided in two and analyzed in parallel following the different methods. No significant differences were obtained comparing the DTNB-based assay and our fluorescent-based method when the samples were in relatively high concentration (total GSH and GSSG) but at very low concentration (PSSG) the DTNB-method is not sensitive enough to give a reliable result (n = 4).

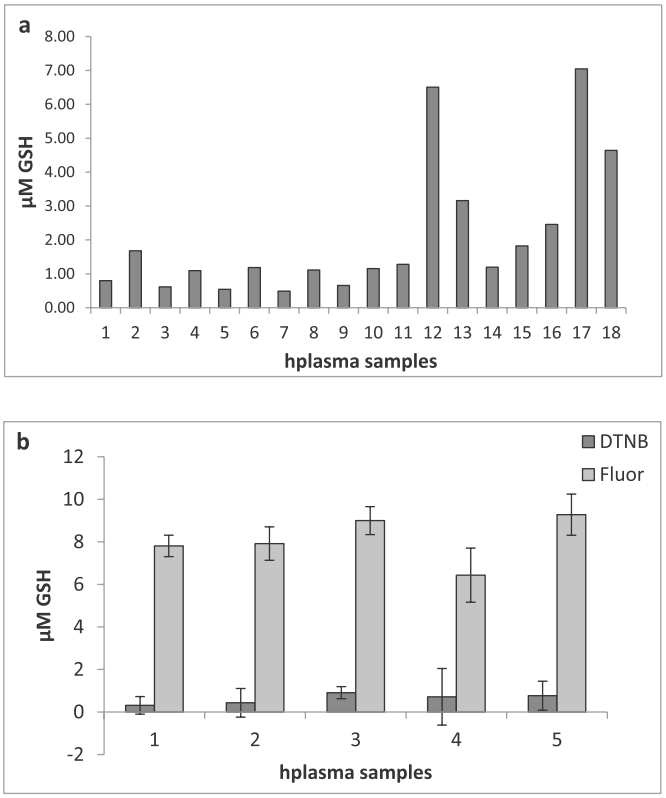

Fig. 5. Levels of total GSH in human plasma. Total GSH was measured in human plasma from different donors (a). Fresh set of samples were pooled 3by3, divided in two aliquot and analyzed in parallel following the different methods. Significant differences were obtained comparing the DTNB based assay and our fluorescent-based method. The DTNB method is not sensitive enough to measure the glutathione in plasma.

in physiological condition and an increasing concentration, as expected, after diamide treatment. It was not possible to obtain the same results using the DTNB method.

Therefore, data comparing snap frozen and fresh samples underlined first, the importance of a careful handle of samples in order to avoid artefact oxidation during storage and manipulation; and second the high sensitivity of the fluorescent assay to measure PSSG in physiological conditions.

Finally we approached the plasma samples. Even though it is not an exact estimation to determine oxidative stress in tissues from blood samples, this may be the least invasive sample one could get. GSH content in plasma can pose a problem when it comes to detection because its range is low (usually between 1 and 10 µM) and the presence of mixed disulfide could represent a problem of underestimation depending of the approach used. Here we showed that using our fluorescent-method it was possible to measure total GSH in plasma with a concentration in the expected range (Fig. 5) opening the possibility to use our method in clinical studies. Moreover another advantage is the possibility to store the plasma and be able to measure the total GSH content considering also the different disulfides forms that are rapidly formed during the sample preparation and storage.

As seen in Figs. 2–4, the measurements were comparable between methods when the samples had high concentration of glutathione, vice versa for plasma samples with low concentration, we were able to detect total GSH including all form of GSH oxidation (GSSG, CSSG and PSSG), only using our fluorescence-based method proving the high sensitivity. The values obtain are in line with the literature where more sophisticated techniques were used.

The major strategies utilized to measure protein *S*-glutathionylation consist in three main approaches: (i) labelling GSH; (ii) immunoblotting using antibodies against GSH; (iii) break the disulfide bond with GSH and label these now free SH groups.

However, some important disadvantages limit the use of these methods such as lack of applicability in vivo, low antibody specificity and sensitivity of the detection overall.

In conclusion, considering this context we optimized a method to measure the GSH in low concentration as in plasma and derived from glutathionylated proteins. Based on the results the fluorescent-method can be considered a very useful tool to measure glutathione in particular for clinical studies when the samples are in small amounts and the concentration of GSH is very low.

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