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Quantitation of reduced and total glutathione at the femtomole level by high-performance liquid chromatography with fluorescence detection: application to red blood cells and cultured fibroblasts

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Abstract

A new rapid and highly sensitive HPLC method with *ortho*-phthalaldehyde (OPA) pre-column derivatization has been developed for determination of reduced glutathione (GSH) and total glutathione (GSHt) in human red blood cells and cultured fibroblasts. OPA derivatives are separated on a reversed-phase HPLC column with an acetonitrile–sodium acetate gradient system and detected fluorimetrically. An internal standard (glutathione ethyl ester) is added to facilitate quantitation. Total glutathione is determined after reduction of disulfide groups with dithiothreitol; the oxidized glutathione (GSSG) concentration is calculated by subtraction of the GSH level from the GSHt level. The assay shows high sensitivity (50 fmol per injection, the lowest reported), good precision (C.V. $<5.0\%$), an analytical recovery of GSH and GSSG close to 100%, and linearity ($r > 0.999$). This HPLC technique is very simple and rapid. Its wide applicability and high sensitivity make it a convenient and reliable method for glutathione determination in various biological samples.

Keywords: Pre-column derivatization, HPLC; Glutathione; *o*-Phthalaldehyde

1. Introduction

Glutathione (γ-L-glutamyl-L-cysteinyl-glycine) is the principal non-protein thiol compound present in most mammalian cells in millimolar concentrations. This tripeptide acts as a major bio-reducing agent. It plays an essential role in protecting cells from free radicals and reactive oxygen species and maintains an adequate intracellular redox status. Glutathione is also involved in various physiological processes such as detoxification of xenobiotics, transport of amino acids, stabilization of cell membranes and synthesis of proteins and DNA [1]. It is present in cells in two principal states: the reduced form (GSH) and the oxidized form (GSSG). Intracellular glutathione is usually maintained in the reduced state which represents more than 95% of the total content (GSH+

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GSSG). The intracellular concentration of oxidized glutathione is generally low but may increase upon oxidative stress or pathological conditions with simultaneous depletion of reduced glutathione [2–4]. Glutathione depletion has been identified in various disease processes such as diabetes [5,6], alcoholic liver diseases [7,8], Parkinson's disease [9,10], human immunodeficiency virus (HIV) infection [11], carcinogenesis and aging [12,13]. Moreover, as the GSH/GSSG ratio is considered to be an early indicator of oxidative stress and/or disease risk [14,15], it is important to measure both reduced and oxidized glutathione.

Numerous methods for glutathione analysis in biological samples have been described in the literature. Early techniques including enzymatic [16–18], fluorimetric [19,20] and colorimetric [21,22] assays have inadequate detection limits and low reproducibility. More recently, several procedures using high-performance liquid chromatography have been developed. These methods use either UV absorbance evaluation after derivatization with 5,5′-dithiobis-(2-nitrobenzoic) acid (DNTB, Ellman's reagent) [23] or with 2,4-dinitrofluorobenzene [24,25], or fluorescence measurement of monobromobimane [26–28], *ortho*-phthalaldehyde (OPA) [28–31], 1-dimethylaminonaphthalene-5-sulphonyl chloride (dansyl chloride) [32], ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (SBD-F) [33,34] or *N*-(1-pyrenyl)maleimide (NPM) [35] derivatives. Electrochemical methods of detection which do not require derivatization have also been reported [36,37]. Although all these methods are currently available for glutathione determination, most of them present complicated and time-consuming protocols, insufficient sensitivity for small samples or inability to measure oxidized glutathione.

In the present study, we report a new validated HPLC technique based on the derivatization of GSH with *ortho*-phthalaldehyde which reacts with both the sulfhydryl and the primary amino group of glutathione to form a highly fluorescent product. This method allows direct determination of reduced glutathione (GSH); total glutathione (GSHt) measurement is performed by reducing the disulfide group of oxidized glutathione (GSSG) with dithiothreitol during a pre-derivatization step. The GSSG concentration is obtained from the subtraction of the GSH from the GSHt values. This technique offers significant advantages with respect to the most commonly used procedures: simplicity, rapidity and specificity. It shows good precision, good reproducibility and high sensitivity in the femtomole range. This method is used to determine the glutathione concentration in human red blood cells and cultured human skin fibroblasts.

2. Experimental

2.1. Chemicals and reagents

GSH, GSSG, glutathione ethyl ester (GEE), *ortho*-phthalaldehyde (OPA), DL-dithiothreitol and metaphosphoric acid (MPA) were obtained from Sigma–Aldrich (St. Quentin Fallavier, France). Sodium acetate and sodium tetraborate were purchased from Prolabo (Fontenay-sous-bois, France). Tris(hydroxymethyl)aminomethane, sodium dihydrogen phosphate monohydrate, methanol and acetonitrile (HPLC grade) were obtained from Merck (Nogent-sur-Marne, France). All HPLC buffers were filtered through 0.22 or 0.45 μm Millipore filters (Millipore, St Quentin Yveline, France) prior to use and sparged with helium during use.

2.1.1. Standard solutions

Ten millimolar stock solutions of GSH, GSSG and GEE in 0.01 N HCl were prepared, aliquoted and stored at −20°C for 1 month. Working standard solutions were prepared daily from each stock solution by dilution in 0.01 N HCl.

2.1.2. Derivatization reagent

A 5 mg/ml OPA solution was used for derivatization. The solution was prepared by dissolving 50 mg of reagent-grade OPA in 0.5 ml methanol and diluting this to a final volume of 10 ml with 0.1 *M* sodium tetraborate, pH 9.90. This solution can be aliquoted and stored for several weeks at −20°C without any loss of activity.

2.2. Sample collection and preparation

Blood was collected by venipuncture from 10 healthy volunteers (eight women, two men; 25–53

years) into cooled tubes containing EDTA as anticoagulant and immediately transferred on ice. The blood samples were rapidly centrifuged at 1600 *g* for 5 min and plasma removed. Erythrocytes were washed twice with cold 9‰ (w/v) NaCl solution and lysed by freeze/thawing. Hemolysate (100 μl) was deproteinized by adding 400 μl of 6% MPA (w/v) and 100 μl of glutathione ethyl ester as internal standard. Precipitated proteins were removed by centrifugation (7 min, 10 000 *g*, 4°C). The resulting acid supernatant was either stored at −80°C until analysis or subjected immediately to the derivatization procedure described below.

Human skin fibroblasts were obtained from a healthy voluntary donor (woman, 30 years). Cells used in this study had a population doubling level (PDL) of 8 or 25. The PDL of a culture describes the ''age'' of the cells in terms of the number of doublings the population has undergone to reach a given point in the life of the culture. Fibroblasts were cultured in 75 cm^2 plastic flasks containing 15 ml of Minimal Essential Medium supplemented with 10% fetal bovine serum, L-glutamine (2 m*M*) and penicillin/streptomycin (100 U/ml, 100 μg/ml), and grown exponentially for 2 days at 37°C in humidified air with 5% CO_2 before cell collection. At the time of harvest, the medium was poured off and cells were rinsed with cold phosphate buffer saline (PBS). The cells from each flask were scraped with cold PBS, collected into a centrifuge tube, placed on ice, and centrifuged at 300 *g* for 7 min at 4°C. As sonication could result in an artifactual oxidation of glutathione [35], the resulting cell pellet was lysed by adding an equal volume of cold water and freeze-thawing three times (0 to −80°C). An aliquot of this cell lysate was used for protein content determination by the Lowry method [38]. The remaining lysate (100 μl) was deproteinized by adding 400 μl of 6% MPA. Precipitated proteins were removed by centrifugation (7 min, 10 000 *g*, 4°C) and the acid supernatant was either stored at −80°C until analysis or submitted immediately to the derivatization procedure as follows.

2.3. Derivatization procedure

The derivatization procedure was performed as previously described by Neuschwander-Tetri and Roll [29]. Briefly, 100 μl of MPA supernatant were neutralized and diluted by addition of 500 m*M* sodium phosphate, pH 7.00. Derivatization with OPA was performed by mixing 100 μl of this solution with 100 μl of OPA solution. After 5 min at room temperature, the derivatized samples were neutralized by addition of 800 μl of 500 m*M* sodium phosphate, pH 7.00. Twenty microliters were then injected into the HPLC system for reduced glutathione (GSH) determination.

Total glutathione (GSHt) was also evaluated by the present method by performing a reduction step of GSSG with dithiothreitol (DTT) before protein precipitation. Briefly, 100 μl of sample were mixed with 100 μl of 25 m*M* DTT solution and 50 μl 0.1 *M* Tris buffer, pH 8.5. After 30 min at 4°C, the proteins were precipitated by addition of 800 μl 6% MPA and removed by centrifugation (7 min, 10 000 *g*, 4°C). The MPA supernatant was either stored at −80°C until analysis or directly used for the derivatization procedure described above.

As the amount of oxidized glutathione (GSSG) cannot be determined directly using the present technique, the GSSG concentration was obtained from subtraction of the GSH from the GSHt values.

2.4. Chromatographic conditions

The HPLC separation of GSH–OPA adducts was achieved on an Inertsil C-18 reversed-phase ODS-2 silica column (250×4.6 mm; 5 μm particle size) (Chrompack, Les Ulis, France) maintained at 30°C, followed by fluorimetric detection at 420 nm after excitation at 340 nm. The HPLC system consisted of a Kontron 325 pump system (Kontron Instruments, Montigny-le-Bretonneux, France), a Kontron SFM 25 fluorescence detector and a Kontron 465 HPLC autosampler. Derivatives were eluted using an acetonitrile gradient in a 50 m*M* sodium acetate buffer, pH 6.20. The flow-rate during elution was 0.7 ml min^{-1}. The column was washed daily with acetonitrile for 60 min and stored in 100% acetonitrile overnight. The column was then re-equilibrated to the initial conditions for 60 min before the next injection. Integration of chromatograms was accomplished using KromaSystem 2000 software also purchased from Kontron. GSH and GEE peak areas were measured and the GSH/GEE ratio calculated.

A standard curve was established under the same conditions.

3. Results

3.1. Chromatographic analysis

In order to optimize the chromatographic resolution, a number of HPLC columns were tested. The best results, based on GSH and GEE separation and column life span, were obtained with a reversed-phase Inertsil ODS-2 column. Modifications were also made to the mobile phase to enhance the chromatographic resolution and increase peak sharpness. Acetonitrile had a weaker effect on retention time than methanol and was therefore more suitable for minor adjustments of chromatographic profiles. A pH of 6.20 was also selected for sodium acetate buffer to allow adequate ionization of analytes without reducing column life span.

Typical chromatograms obtained from derivatized samples are shown in Fig. 1. Peaks were identified by direct comparison of retention times with those of authentic standards and by spiking samples with standards. The retention times of glutathione and GEE (internal standard) were 3.7 and 13.4 min, respectively, with a total analysis time of 18 min. These retention times could vary between runs depending on the age and the frequency of use of the column as well as slight fluctuations in the temperature or pH of the mobile phase. Chromatograms showed excellent separation of GSH and GEE without interfering peaks. An interference appeared after DTT treatment of fibroblast lysates but the GSH peak was still easily observed. As this interference masked the GEE peak, internal standard was not added to fibroblast samples before analysis.

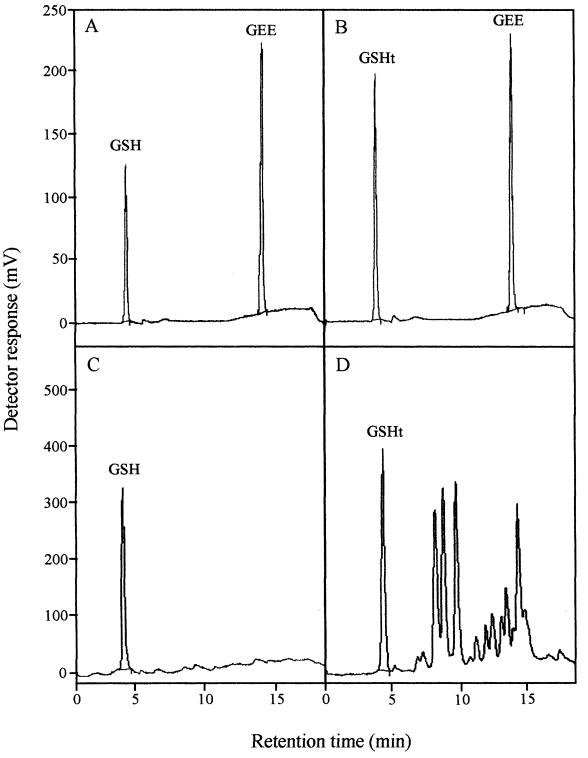

Fig. 1. HPLC profiles of a human red blood cell (A,B) and a human cultured skin fibroblast (C,D) extract. (A,C) Reduced glutathione content (GSH). (B,D) Total glutathione content (GSHt) following reduction with dithiothreitol. GEE, glutathione ethyl ester used as internal standard only for red blood cells.

3.2. Protein precipitation

We tested three different acid solutions as protein-precipitating agents: sulfosalicylic acid (SSA), perchloric acid (PCA) and metaphosphoric acid (MPA). SSA produced a large initial interfering peak which masked the GSH peak. PCA produced GSH oxidation during sample processing which was especially important for blood sample analysis: more than 15% of the GSH present was oxidized when PCA was used as deproteinizing agent (data not shown). On the contrary, a 6% MPA solution caused neither interference nor GSH oxidation and was therefore retained for deproteinization.

3.3. Reduction of disulfide moiety

To evaluate the optimum DTT concentration for a complete conversion of GSSG to GSH, we investigated the concentration–response curve for DTT in the reaction mixture. For this purpose, 0.4 m*M* GSSG was prepared in 0.01 N HCl or plasma and treated as described for total glutathione analysis with different DTT concentrations (5 to 50 m*M*). Fluorescence increased up to 20 m*M* DTT before reaching a plateau. As 50 m*M* DTT interfered with the formation of the GSH–OPA adduct, a concen-

tration of 25 m*M* was finally chosen for routine analysis. The kinetics of DTT reduction were also studied: the reaction appeared to be complete and remained unchanged up to 30 min of incubation at 4°C (data not shown).

3.4. Derivatization reaction

We have shown that derivatization of GSH with OPA is pH-dependent. By adjusting the reaction pH with NaOH, maximal fluorescence was obtained at pH 10. The fluorescence slowly decreased with a pH lower than 8 or higher than 12 (Fig. 2). Therefore, it is important for sample analysis to neutralize MPA extracts before derivatization in order to adjust the reaction pH close to 10.

3.5. Precision, recovery and linearity

Within-run precision for the method was obtained by injecting the same derivatized sample 10 times consecutively and comparing GSH peak areas. The coefficient of variation (C.V.) for the within-run precision was 4.2%. The between-run precision was determined by derivatizing and injecting the same aliquoted sample on 10 consecutive days. The C.V. for the between-run precision was 4.9%.

GSH and GSSG recovery from cells was determined by adding authentic standards to red blood cells or fibroblast lysates to approximately double the endogenous concentrations. The recovery (three separate assays) from red blood cells was 98.4±1.4% for GSH and 96.7±1.1% for GSSG, and 98.6±2.1 and 96.0±2.0%, respectively, from fibroblasts.

To determine the lower detection limit, serial dilutions of GSH were prepared and the GSH concentration giving the smallest observable derivative peak was identified: the detection limit for a signal-to-noise ratio of 3 was 50 fmol per 20 μl injected. The calibration curve for GSH showed a correlation coefficient (*r*) of 0.9998, ensuring a linear response from 0 to 32 pmol injected (data not shown).

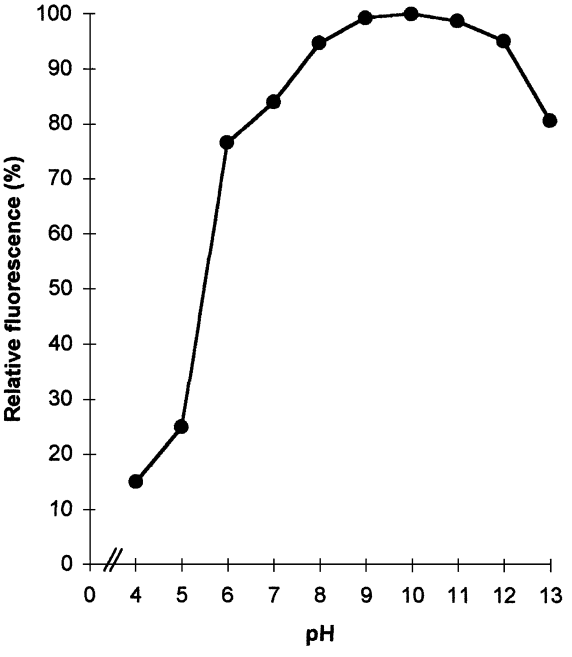

Fig. 2. Effect of pH on GSH–OPA derivative formation. Standard GSH (10 μ*M*) was incubated with OPA and the reaction pH was adjusted to the indicated pH with NaOH. The fluorescence intensity at pH 10.0 was taken as 100%.

3.6. Stability of MPA extracts and GSH–OPA derivatives

For this study, MPA extracts were stored at −80°C; an aliquot was periodically removed to determine GSH and GSSG concentrations. MPA extracts were stable for 1 month with a nearly unchanged response (Fig. 3).

The stability of GSH–OPA derivatives was also examined. The derivatives were relatively stable for 24 h when stored at 4°C in darkness (data not shown).

3.7. Quantification of glutathione forms in red blood cells and cultured fibroblasts

Table 1 shows the glutathione level of red blood cells of 10 healthy volunteers. The mean total and reduced glutathione concentrations were 2.23±0.15 and 2.04±0.14 mmol/L, respectively. The calculated oxidized fraction was 0.19±0.02 mmol/L (expressed as GSH equivalents), corresponding to 8.5% of the total glutathione content.

We measured the glutathione concentration in different fibroblast populations: ''young'' cells (PDL

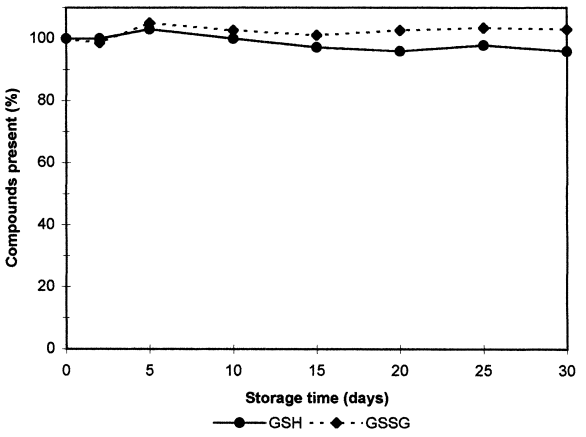

Fig. 3. Stability of MPA extracts at −80°C over 30 days. GSH and GSSG concentrations just before storage (time zero) were taken as 100%.

8) and ''old'' cells (PDL 25). Cells were collected during logarithmic growth or stationary growth. The results, presented in Table 2, show differences according to culture age and cell proliferation potential.

4. Discussion

Glutathione plays an essential role in cellular metabolism and has important functions in many biological processes, particularly in protecting cells from free radicals and reactive oxygen species [1]. Under normal conditions, glutathione is mainly found as the reduced form (GSH) and, in much smaller amounts, as the oxidized form (GSSG).

Table 1
Concentration (mmol/L) of different glutathione forms in red blood cells obtained from 10 healthy volunteers

Subject	GSH	GSSG	GSHt	GSH/GSSG	% GSSG
1	2.15	0.20	2.35	10.75	8.51
2	2.07	0.18	2.25	11.50	8.00
3	1.99	0.19	2.18	10.47	8.72
4	1.92	0.18	2.10	10.67	8.57
5	2.06	0.18	2.24	11.44	8.04
6	2.14	0.21	2.35	10.19	8.94
7	1.94	0.21	2.15	9.24	9.77
8	1.92	0.18	2.10	10.67	8.57
9	1.90	0.15	2.05	12.67	7.32
10	2.34	0.20	2.54	11.70	7.87
Mean±SD	2.04±0.14	0.19±0.02	2.23±0.15	10.93±0.94	8.43±0.67

Table 2
Concentration (nmol/mg protein) of different glutathione forms in cultured human skin fibroblasts obtained from a healthy volunteer[a]

Stage of fibroblast culture	Population doubling level (PDL)	GSH	GSSG	GSHt	GSH/GSSG	% GSSG
Logarithmic growth	8	20.24±0.90	0.85±0.15	21.09±1.00	24.34±4.32	4.04±0.63
Post-confluency	8	17.53±0.34	0.73±0.19	18.26±0.86	24.01±3.66	3.99±0.55
Logarithmic growth	25	16.48±0.27	0.83±0.09	17.31±1.08	19.86±4.04	4.79±0.21
Post-confluency	25	14.41±0.54	0.76±0.11	15.17±1.09	18.96±3.18	5.01±0.13

[a] Values are expressed as mean±standard deviation of six separate analyses.

However, GSH and GSSG levels can significantly change upon oxidative stress or pathological conditions, so determination of the GSH/GSSG ratio provides useful information about the redox status of cells [2–4]. Therefore, assays for glutathione determination should be able to measure both GSH and GSSG and be sensitive to small amounts of GSSG. Correct measurement of glutathione is difficult because of the instability of GSH in aqueous solution: GSH auto-oxidation can easily occur during sample preparation, resulting in an overestimation of the GSSG level [14]. Consequently, assays must be rapid, specific and sensitive enough in order to ensure accurate measurements.

Quantification of glutathione in biological samples requires appropriate extraction, separation from other thiols or related compounds and a detection procedure. Several methods have been described for glutathione determination: the majority of assays involve separation of glutathione or its derivative by HPLC followed by UV [23–25], fluorescence [26–35] or electrochemical detection [36,37]. Limits of detection are typically in the pico- to femtomole range. We have retained an HPLC system including pre-column derivatization with *ortho*-phthalaldehyde (OPA) and fluorescence detection since it provides efficient separation and higher sensitivity. Derivatization of glutathione with OPA is rapid (complete within 5 min), non-enzymatic and highly selective: OPA reacts with both the sulfhydryl and the primary amino group of glutathione, affording excellent selectivity with regard to other amino acids and peptides.

The HPLC procedure reported here was performed as described previously by Neuschwander-Tetri and Roll [29] with some modifications: by replacing SSA with MPA as deproteinizing agent and methanol with acetonitrile as eluting solvent, we have abolished the large interfering peak on the solvent front in the chromatogram and increased peak sharpness, resolution and the sensitivity limit. The sensitivity limit is also influenced by the presence of reagent impurities or other cellular thiols which result in multiple small interfering peaks. For this reason, we optimized sample preparation and HPLC conditions (HPLC column, mobile phase) to avoid such interference and increase specificity: the resulting high chromatographic resolution together with the linearity of the standard curve at low concentration ($r > 0.999$) are the major reasons for the low detection limit compared to published data (Table 3).

Table 3
Selection of some of the reported HPLC methods for detecting reduced glutathione

Method	LOD[a]	Ref.
Electrochemical detection	16.2 pmol	[36]
	1 pmol	[37]
Pre-column derivatization with monobromobimane	20 pmol	[26]
	6.25 pmol	[28]
	<2 pmol	[27]
Pre-column derivatization with dansyl chloride	1 pmol	[32]
Pre-column derivatization with *N*-(1-pyrenyl)maleimide	100 fmol	[35]
Post-column derivatization with *ortho*-phthalaladehyde (OPA)	100 fmol	[31]
Pre-column derivatization with *ortho*-phthalaladehyde (OPA)	12.5 pmol	[28]
Pre-column derivatization with *ortho*-phthalaladehyde (OPA) Indirect measurement (GSHt−GSSG)	50 fmol	[30]
Direct measurement (GSH)	50 fmol	This work

[a] Limit of detection.

Our method offers numerous advantages compared to currently used HPLC techniques for glutathione analysis. It is specific and very sensitive with a lower detection limit of 50 fmol per 20 μl injected allowing quantitative measurement of glutathione in about 400 red blood cells or 6000 fibroblasts. Satisfactory analytical precision of the method (within-day C.V. 4.2%, between-day C.V. 4.9%) was obtained without including the internal standard, and the analytical recovery of GSH was above 98%. These results make this technique a convenient and reliable tool for glutathione determination. Other advantages of the method are simplicity and rapidity. With regard to sample preparation, our procedure requires a few steps of no longer than 50–60 min and fluorescent derivatives are eluted from the column in 20 min. This technique appears to be significantly quicker than other HPLC procedures coupled with pre- or post-column derivatization. Furthermore, OPA derivative stability allows automated sampling.

Conditions for sample collection, treatment and

storage represent a critical factor in glutathione determination in biological samples. As GSH is unstable in aqueous solution, GSH auto-oxidation can rapidly occur during sample preparation, leading to overestimation of the GSSG level and erroneous conclusions on the pathophysiological changes of the glutathione level. So, the accurate measurement of GSH and GSSG levels in biological samples is dependent on the prevention of GSH auto-oxidation during sample processing. GSH oxidation can be especially significant in blood sample analysis. This oxidation process can be blocked by injecting directly into the collection tube suitable agents such as *N*-ethylmaleimide (NEM) [27,30], 2-vinyl pyridine (2-VP) [17,35] or iodoacetic acid (IAA) [14,25]. These agents trap thiols and block their oxidation but make GSH unavailable for the derivatization reaction and HPLC analysis. We observed that storage of red blood cells or the fibroblast pellet at −80°C resulted in a rapid decrease in the amount of GSH with a simultaneous increase in the GSSG level (data not shown). In order to prevent possible loss of GSH during sample processing, acid deproteinization was performed as quickly as possible after sample collection and acid supernatants were immediately analyzed or stored at −80°C until analysis, thus ensuring sample stability. In our study, freezing acid supernatants for as long as 1 month gave good reproducibility compared to samples analyzed immediately after preparation. To improve assay precision, we immediately injected GSH–OPA adducts; nevertheless, stability studies showed no detectable loss of GSH when derivatives were stored in the dark at 4°C for 24 h. Using these sample processing procedures, complete recovery of GSH added in known amounts to actual samples was obtained for red blood cells and fibroblasts.

GSSG measurement can be performed either directly or indirectly. In the latter case, the GSSG concentration is calculated by subtraction of the GSH from the GSHt level obtained after reduction of the disulfide moiety. We prefer this method for two reasons. (i) Direct determination of oxidized glutathione is possible by using a high reaction pH (pH 12.00) to derivatize GSSG with OPA as reported by Lenton et al. [31]: this approach allows simultaneous measurement of both GSH and GSSG. However, this reaction pH is not convenient for glutathione measurement: in fact, we have shown that the reaction of GSH with OPA is pH-dependent and that the optimal pH for the derivatization reaction is between 9.5 and 10 as previously reported by Yan et al. [28]. In the present study, we used a reaction pH of 10. OPA does not react with GSSG under these working conditions but this reaction pH ensures complete reaction between GSH and OPA and increases the precision of the measurement. (ii) Direct measurement of GSSG can also be performed by blocking reduced glutathione with NEM, 2-VP or IAA. Oxidized glutathione is then reduced and measured by formation of an OPA derivative. Paroni et al. [30] used this method to determine the GSSG concentration with a detection limit similar to ours (50 fmol). However, their technique excludes the possibility to measure GSH and requires additional steps to inactivate or remove excess blocking agent which can react with GSH produced after GSSG reduction, giving erroneously low GSSG values. These additional steps make this approach laborious and time-consuming.

To eliminate these problems, we measured reduced glutathione (GSH) and total glutathione (GSHt) after treatment with DTT as reducing agent. We verified that GSSG reduction was complete with 25 m*M* DTT solution. The GSSG concentration was then calculated by subtraction of the GSH from the GSHt level. The validity of GSSG determination was demonstrated by the successful recovery of authentic GSSG (>96%).

To demonstrate the applicability of our HPLC method, we measured the glutathione level in red blood cells and fibroblasts. We chose these two cellular types for their different characteristics: contrary to fibroblasts, red blood cells contain a high level of glutathione and proteins and they present severe oxidative conditions. For the two cellular types, the GSH peak was easily observed in the chromatogram without an interfering peak from other thiols. However, interference appeared in fibroblast extracts after DTT treatment. These interfering peaks may be due to either impurities or products obtained after reduction of disulfide groups, or the lower dilution of fibroblast extracts compared to red blood cells during sample processing.

The mean GSH and GSSG concentrations for red blood cells were 2.04±0.14 and 0.19±0.02 mmol/L,

respectively, without a distinction between sexes. The oxidized form represents 8.5% of the total glutathione content. This value is similar to that reported by Inal et al. [39] and Yoshida [24] (8.1 and 8.6%, respectively). We believe that this high GSH oxidation rate in blood extracts may be due to the high concentration of oxyhemoglobin present in red blood cells [24,40]. To prevent GSH auto-oxidation, we hemolysed red blood cells with 1,10-phenanthroline, an iron chelating agent, as described by Yoshida but this compound was inefficient. Finally, we minimized GSH oxidation by collecting blood into tubes placed on ice, lysing red blood cells by rapid freeze/thawing and performing acid deproteinization as quickly as possible.

For cultured human skin fibroblasts, the glutathione content appeared to be associated with cell proliferation potential: cellular glutathione concentrations were higher when cells were in logarithmic growth and decreased when cultures approached stationary growth or post-confluency. Such results have been reported by Takahashi et al. [41]. We also observed that a decrease in cellular glutathione occurred in ''old'' (PDL 25) compared to ''young'' (PDL 8) fibroblast cultures. Mean GSH and GSSG concentrations were 21.09 ± 0.9 and 0.85 ± 0.15 nmol/mg protein, respectively, for ''young'' fibroblasts collected in logarithmic growth. These results are in agreement with published reports [42,43]. The mean GSH concentration for ''old'' cells collected in logarithmic growth was 16.48 ± 0.27 nmol/mg protein. This decrease of GSH content may be due to various causes: decreased synthesis, increased degradation, increased efflux to the extracellular space or to complex formation that cannot be detected by the method used in this study. Moreover, the persistence of a stable GSSG concentration associated with a decrease in the GSH concentration in ''old'' cells may also be due to the oxidative process related to aging.

5. Conclusion

We have developed a specific HPLC technique to measure glutathione concentrations in biological samples. This method offers some advantages with respect to existing HPLC procedures such as ease of use, rapidity of analysis, good reproducibility and particularly higher sensitivity. Its wide applicability makes it a convenient and reliable tool for glutathione determination in various biological specimens. This method is well suited for studies on the metabolism and the biological roles of glutathione in cellular homeostasis and pathophysiological situations.

6. Nomenclature

HPLC	high-performance liquid chromatography
GSH	reduced glutathione
GSSG	oxidized glutathione
GSHt	total glutathione
GEE	glutathione ethyl ester
OPA	*ortho*-phthalaldehyde
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
MPA	metaphosphoric acid
SSA	sulfosalicylic acid
PCA	perchloric acid
C.V.	coefficient of variation
PDL	population doubling level

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