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Simultaneous determination of reduced and oxidized glutathione in peripheral blood mononuclear cells by liquid chromatography–electrospray mass spectrometry

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Abstract

We developed a sensitive and specific liquid chromatography–electrospray mass spectrometric (HPLC–ESI-MS) assay for the simultaneous determination of reduced and oxidized glutathione (GSH and GSSG) in peripheral blood mononuclear cells (PBMC). Following derivatization with *N*-ethylmaleimide to prevent GSH auto-oxidation, addition of thiosalicylic acid as internal standard, and protein precipitation with cold acetonitrile, the samples were injected into a diol column, eluted with acetonitrile–1% aqueous acetic acid (25:75) and detected by the ESI-MS system. The optimized method exhibited a good detection limit for both analytes (0.01 and 0.05 μ*M* for GSH and GSSG, respectively). Good linearity was reached in the 0.01–20 μ*M* range for GSH and 0.05–20 μ*M* for GSSG. The mean recoveries of GSH and GSSG were 98.5–100.6% and 105.8–111.5%, respectively. The run-to-run repeatability for retention time and peak area was RSD% 0.06 and 1.75 for GSH and 0.18 and 2.50 for GSSG. The optimized method was applied to GSH and GSSG assay in PBMC analyzing 20 healthy individuals.

Keywords: Glutathione

1. Introduction

The tripeptide L-γ-glutamyl-L-cysteinyl-glycine, reduced glutathione or GSH, is the major low-molecular-mass thiol compound present in mammalian cells [1,2]. It is a fundamental intracellular reductant involved in the free radical scavenging system contributing to endogenous defences against oxidative and nitrosative stress [3–5]. GSH, as a substrate of glutathione-peroxidases (GSH-Px) of various natures, removes oxidizing species, producing glutathione disulfide (GSSG) [6]. The crucial role of GSH in the removal of peroxides relies on the lack of the toxicity associated with cysteine, making the compound a suitable cellular thiol reservoir [7]. Several papers have dealt with the close connection between the thiol redox state and cell proliferation demonstrating that GSH is involved in DNA and protein

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synthesis, aminoacid transport, several signal transduction pathways (some transcription factors may be either oxidized or reduced, according to the redox status of cells), gene expression and the control of apoptosis [8–12]. Physiologically, the intracellular glutathione is present in the reduced state in most tissues, accounting for more than 99% of the total amount. GSH, maintained in its reduced form by a NADH-dependent reductase, is actually the central component of a network of antioxidants and related enzymes participating in the recovery of other antioxidant activities [13,14]. Therefore, its determination provides useful information regarding the redox and detoxification status of cells [10]. Various oxidative injuries may affect the intracellular concentration of GSH and several studies have demonstrated a depletion of the reduced form during the inflammatory process or after exposure to pro-oxidants such as UV radiation [15,16]. Moreover, an impairment in the recycling of GSH renders cells particularly sensitive to ionizing radiation [17,18]. In particular, UV irradiation of biological systems induces several modifications, mostly due to the generation of reactive oxygen species (ROS), and causes the depletion of intracellular antioxidants, GSH being one of the first targets. Exposure to UV, however, is one of the therapeutic approaches to the treatment of immune-related skin diseases (e.g. psoriasis) and correlation between the generation of oxidative stress and improvement of clinical manifestations has been suggested [19].

Imbalance in the redox system has been associated with the impairment of cellular immune functions and cytokine production and the intracellular level of reduced glutathione has been reported as relevant in the initiation and progression of some lymphoid cells function, such as synthetic activity, proliferative capacity, and modality of cell death [20–23]. Therefore, the development of a sensitive and selective method for the simultaneous assaying of reduced and oxidized glutathione in immune-competent cells is extremely useful, especially in monitoring several auto-immune diseases or immune-targeted therapies. Contrarily to that for GSH, GSSG determination is difficult because of its very low concentration and ease of spontaneous oxidation of GSH to GSSG in biological extracts. Simultaneous determination of GSH and GSSG is also needed because the amount of GSH may decrease and that of GSSG may increase in response to oxidative stress. The mutual variations of GSH and GSSG concentrations are expressed by the GSSG/GSH ratio, considered to be one of the markers of oxidative stress [24].

Analytical methods used so far for the analysis of GSH and GSSG, in different biological matrices, include high-performance liquid chromatography (HPLC) coupled with post-column derivatization and spectrofluorimetric detection [25,26], fluorimetric [27–30], ultraviolet absorbance [31,32], and electrochemical detection modes [33]. Recently, oxidation and reduction of GSH and GSSG have been studied by thermospray liquid chromatography–mass spectrometry, exposing the compounds to hydrogen peroxide and mercaptoethanol [34]. ESI-MS technique is also helpful for the analysis of low-molecular-mass, including GSH, and their *S*-nitroso compounds [35]. Moreover, dansylated GSH and GSSG have been characterized by narrow bore liquid chromatography/ESI-MS [36]. Gas chromatography coupled with mass spectrometry (GC–MS) methods have also been proposed for the determination of GSH from biological samples and they rely on the preparation of volatile derivatives of the thiol-containing compounds [37]. More recently, among the separative techniques, capillary electrophoretic (CE) techniques have been reported for the simultaneous determination of GSH and GSSG from different cell types and from sub-cellular particles [38–41]. Finally, GSH can be determined, without separation, by spectrophotometric techniques [42]. These methods often suffer from poor sensitivity and selectivity and may require time-consuming sample pre-treatments such as derivatization with fluorescent reagents. Furthermore, most of the methods cannot detect GSSG, which is thus indirectly quantified by means of the GSH-reductase enzyme or reducing agents.

In this study, we defined a method for the determination of both GSH and GSSG levels in human lymphocytes based on a HPLC–ESI-MS technique that couples both the high selectivity of the chromatographic separation system and the high sensitivity and specificity of the mass spectrometric detector.

2. Experimental

2.1. Chemicals and reagents

L-Glutathione (GSH, reduced form, *N*-(*N*-L-γ-glutamyl-L-cysteinyl)glycine, F.W. 307.33), glutathione disulfide (GSSG, oxidized form, L-γ-glutamyl-L-cysteinyl-glycine disulfide, F.W. 612.6), *N*-ethylmaleimide (NEM, F.W. 125.1), thiosalicylic acid (TSA, F.W. 154.2), ammonium chloride and Bradford reagent were purchased from Sigma (St. Louis, MO, USA); acetic acid and acetonitrile HPLC-grade from Merck (Darmstadt, Germany), water HPLC-grade from Carlo Erba (Milano, Italy), sterile solution of sodium chloride 0.9% from Pharmacia (Milano, Italy). Lithium ethylenediaminotetracetate was already contained in the ready-to-use tubes for venous blood collection (Becton Dickinson Vacutainer systems Europe, Meylancedex, France). The lymphocyte isolation solution Ficoll-Paque™ Research Grade was purchased from Amersham Pharmacia Biotec. AB (Uppsala, Sweden).

2.2. Standard solutions and sample preparation

2.2.1. Standard solutions

A 30.73 mg sample of GSH and 61.26 mg of GSSG were dissolved in 10 ml of 0.2% (w/v) acetic acid in ultrapure water in order to prepare 10 m*M* stock solutions. TSA stock solution was obtained dissolving 15.42 mg of the powder in a mixture solution of ultrapure acetonitrile–2% acetic acid (9:1).

Standard GSH, GSSG and TSA were prepared weekly diluting stock solutions at the desired concentrations with 0.2% acetic acid solution. NEM stock solution was made dissolving 250 mg of powder in 100 ml ultrapure water. Diluted solutions were stored in aliquots at −20°.

2.2.2. Cell preparation and extraction

For the development and validation of the assay, blood was drawn from 20 healthy volunteers. Peripheral blood mononuclear cells were obtained through Ficoll gradient separation of 5 ml whole blood, collected in Li_2EDTA containing vacutainers, deprived from plasma and washed twice with cold NaCl isotonic solution. Cell suspensions were depleted of platelets and contaminating erythrocytes by treatment with an isotonic solution of NH_4Cl. The selected PBMC were lysed in cold aqueous solution of *N*-ethylmaleimide (NEM 10 m*M*) and kept on ice for 20 min. After centrifugation, the supernatant was tested for protein content by Bradford assay and 200 μl of the remaining cell lysate were added of 100 μl of TSA 200 μ*M*. The mixture was kept on ice for other 20 min to allow the derivatization of TSA. Two hundred and fifty (250) μl of cold CH_3CN were added to precipitate the proteins, and the final volume of 1000 μl was reached adding ultrapure water. After centrifugation at 10 000 *g* for 5 min, the supernatant was injected into the HPLC–MS system.

2.3. Instrumentation

2.3.1. LC–MS apparatus

The analyses were performed with a Hewlett-Packard (Palo Alto, CA, USA) HPLC–Electrospray MS system. The series 1100 HPLC system consisted of a G1322A series degasser, a G1311A series quaternary pump and a G1313A series autosampler. The electrospray interface was connected to a single quadrupole G1946A series mass spectrometer. The HPLC–MS system was controlled, and data were processed, using a chemstation software Hewlett-Packard for the HPLC system, electrospray interface and mass spectrometer. HPLC vials, 100 μl glass inserts and PTFE screw caps were obtained from Hewlett-Packard.

The analytical column used for LC separations (250×2 mm) was packed with a Nucleosil 100-7 OH stationary phase with a particle size of 7 μm (column n. 8065552, Macherey-Nagel GmbH & Co. KG, Düren, Germany).

2.3.2. LC–MS experimental conditions

In order to optimize the MS signal, ESI-MS spectra were achieved by flow injection analysis (FIA) of 10 μ*M* solutions of GSH, GSSG, and TSA. Ten μl of standard solutions were injected automatically and driven by a 500 μl/min flow-rate of the mobile phase (acetonitrile–1% acetic acid solution (25:75). The following electrospray parameters

were kept constantly during the analysis: drying gas, N_2 (Nitrogen), flow 12 l/min, drying gas temperature 350°C, nebulizer pressure 40 p.s.i.g. Whereas the capillary and the fragmentor voltages were +4500 and +70 V, respectively, in positive polarity mode and −4500 and −70 V, respectively, in negative polarity mode. The quadrupole temperature was 100°C.

Chromatographic experiments (LC–ESI-MS) were carried out using a mobile phase composed of acetonitrile–1% acetic acid solution (25:75). The flow-rate was 500 μl/min and the total run time was 20 min. Ten μl of samples were injected into the HPLC column and eluted in the isocratic mode.

3. Results and discussion

3.1. Mass spectrometric analysis optimization

For the LC–ESI-MS analysis of GSH and GSSG (without derivatization) we selected a diol stationary phase and, as mobile phase, a mixture of acetonitrile–1% acetic acid (25:75). The chromatographic conditions used allowed us to achieve the separation of both analytes in less than 15 min. Under these operating conditions the two compounds were positively charged due to their chemico-physical properties. In fact, GSH and GSSG belong to the oligopeptide family, with both basic and acidic sites that may undergo protonation or deprotonation, respectively, depending on the actual pH of the environment. In a HPLC–ESI-MS system, the selection of the mobile phase composition is of paramount importance because it must offer an acceptable compromise between good chromatographic elution and separation, analytes ionization and efficient desolvatation of charged species in the MS detector. In order to verify the usefulness of the chosen mobile phase for an optimal ESI-MS response, 10 μl of 10 μ*M* GSH and GSSG were injected separately onto the MS detector using either negative or positive detection modes. This technique is well known as flow injection analysis (FIA).

Fig. 1 displays the ESI-mass spectra of (A) GSH and (B) GSSG in the positive ion mode by using FIA analysis.

As can be observed from the ion tracks, GSH

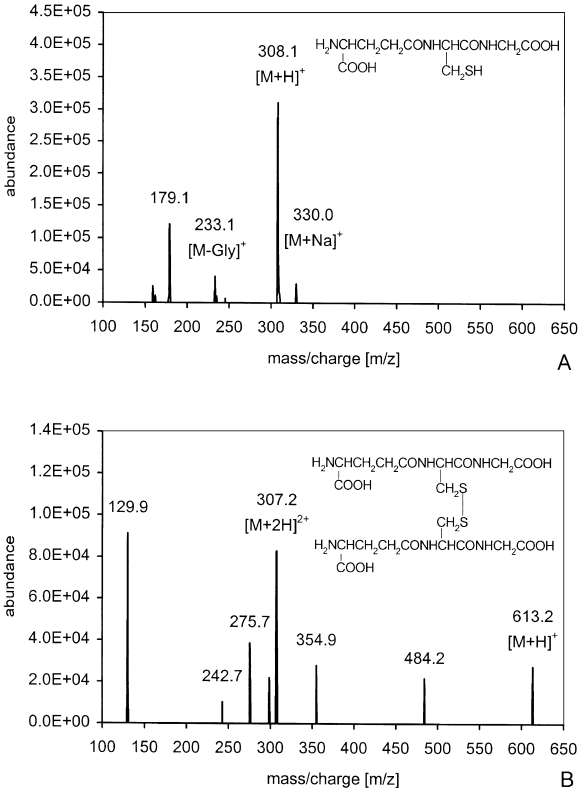

Fig. 1. Electrospray-positive ion spectra (mass/charge range m/z=100–650), generated by flow injection analysis (FIA) of 10 μ*M* GSH (A) and GSSG (B). In A and B are reported the structures of GSH and GSSG, respectively. For experimental conditions see text.

showed a base peak at m/z 308.1 corresponding to $[M+H]^+$, a parent peak at m/z 330.0 to $[M+Na]^+$, and additional peaks at m/z 179.1 and m/z 233.1 the latter consistent with the loss of the glycine moiety. GSSG exhibited a base peak at m/z 129.9 and the parent peaks at m/z 307.2 and at m/z 613.2 corresponding to $[M+2H]^{2+}$ and to $[M+H]^+$ respectively. Fig. 2 shows the ESI–Mass spectra of (A) GSH and (B) GSSG in the negative ion mode by FIA analysis. Under these conditions, GSH formed the negatively charged species with m/z 306.1, resulting from the loss of H^+, and m/z 328.2 corresponding to $[MNa-2H]^-$. In acidic conditions, GSSG showed a slight propensity to lose proton(s), since its abundance, obtained by negative detection mode, was much lower than the positive polarity, however, it

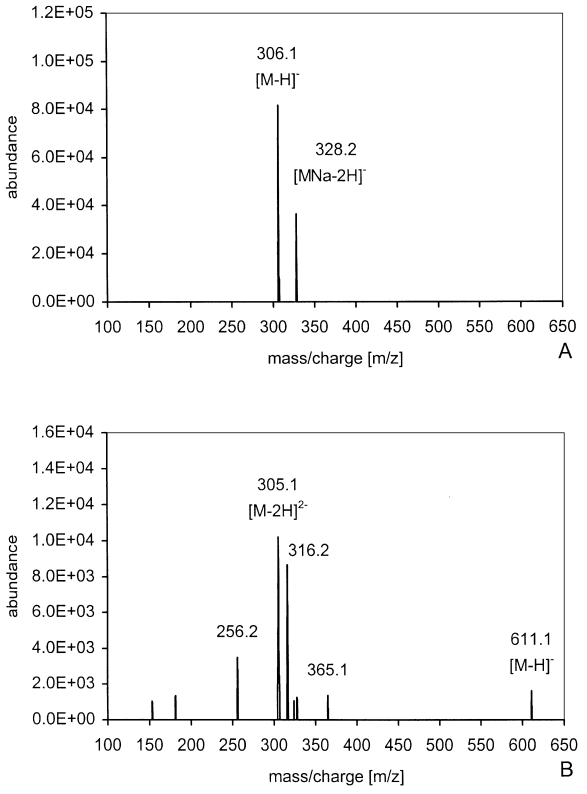

Fig. 2. Electrospray-negative ion spectra (mass/charge range $m/z = 100–650$) generated by flow injection analysis (FIA) of 10 μM (A) GSH, and (B) GSSG. For chemical structures of GSH and GSSG refer to Fig. 1A and B, respectively. For experimental conditions see text.

was still possible to identify two peaks, the base at m/z 305.1 and a secondary one at m/z 611.1, corresponding to $[M-2H]^{2-}$ and $[M-H]^{-}$, respectively. Thus, comparing the results reported in Figs. 1 and 2, we can remark that the best response of the two analytes is given in the positive polarity.

However, in order to avoid using strong acidic solutions, to prevent the autoxidation of GSH in the sample pretreatment, condition that can generate ionization difficult to control, we investigated the possibility of protecting the thiol group of the compound studied. In this regard, we considered *N*-ethylmaleimide (NEM) as derivatizing agent, that selectively reacts with the -SH group, as indicated in the literature [24]. Moreover, several attempts were made to find an internal standard suitable for LC conditions, the positive ionization mode, and the extraction and derivatization procedures. Thiosalycilic acid proved to be the best as internal standard under the conditions used, and as GSH it possesses an -SH group prone to derivatization by NEM, forming the TSA-NEM adduct. Fig. 3A shows the chromatogram of the separation of a mixture of GSH, TSA NEM derivatives, and GSSG using the ESI-MS detector in the positive ion mode. As can be observed in Fig. 3B, a base peak at m/z 433.2 ($[M+H]^{+}$), and a minor peak with m/z 455.4 corresponding to $[M+Na]^{+}$ document the adduct formation of GSH with NEM. The TSA-NEM adduct spectrum consisted of a base peak at m/z 261.9 and a peak at m/z 302.8, the first consistent with $[MH-(H_2O)]^{+}$, and the second with $[M+Na]^{+}$ (see Fig. 3C). The mass spectrum of the peak at 14.42 min (GSSG) was the same as shown in Fig. 1B. The experimental conditions used permitted the separation of the two analytes and the internal standard within a reasonable time without interference with the derivatizing agent (NEM excess, 1.67 min).

Experiments performed in the negative ion MS detection mode were not satisfactory due to the very low GSSG signal. Therefore the positive ion detection mode was selected for further studies.

The kinetic of derivatization of GSH and TSA with NEM was followed by FIA of freshly prepared reaction mixture, detecting the disappearance of the free compound (m/z 308.1, $[GSH+H]^{+}$, free TSA is not positively chargeable at the operating conditions) and an increase in the peak derivatives (m/z 433.2 and 261.9, $[GSH\text{-}NEM+H]^{+}$ and $[TSA\text{-}NEM+H\text{-}(H_2O)]^{+}$, respectively). In the presence of a large excess of NEM (NEM/GSH molar ratio 100/1), after only 20 min the reaction was complete since the m/z 308.1 ion was no longer detected. The TSA-NEM adduct, was also formed within 20 min. As a consequence, the GSH and TSA -SH group protection realized by NEM derivatization satisfied two requirements: autoxidation prevention for both compounds, and the possibility of detecting TSA in the positive mode due to the addition of a basic group to the molecule. Therefore, TSA-NEM can be considered a suitable internal standard for the quantitative analysis of GSH-NEM and GSSG using the HPLC–ESI-MS system described above.

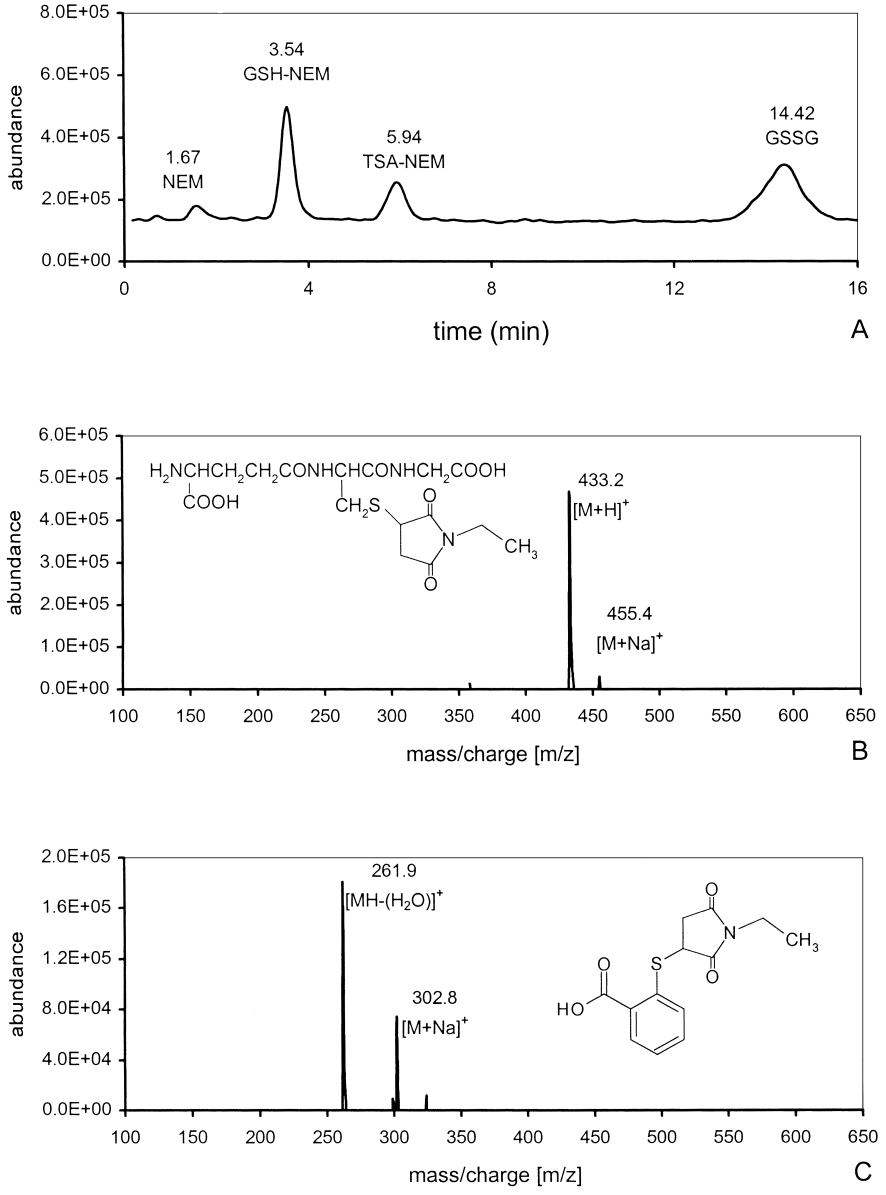

Fig. 3. (A) LC–ESI-MS analysis of a 20 μ*M* solution of GSH-NEM (3.54 min), TSA-NEM (5.94 min) and GSSG (14.42 min) with the positive ion mode mass spectrum of GSH-NEM (B) and TSA-NEM (C) (the positive ion mode mass spectrum of GSSG was the same reported in Fig. 1 B). In B and C are reported the structures of GSH-NEM and TSA-NEM derivatives, respectively. Experimental conditions: stationary phase, Nucleosil 100-7 diol (250×2 mm); mobile phase, acetonitrile–1% acetic acid solution (25:75) in isocratic elution, 500 μl/min flow-rate. The chromatogram was acquired in the scan mode in the mass/charge range 100–650. For other experimental conditions see text.

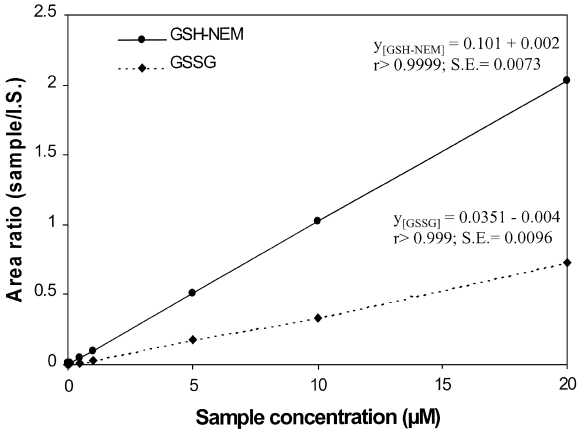

Fig. 4. Calibration curves of GSH-NEM derivative and GSSG achieved by analyzing mixtures containing the two analytes and the internal standard (I.S., TSA-NEM 20 μ*M*) by LC–ESI-MS. The separated LC zones were detected by selected ion(s) monitoring mode (SIM) (*m*/*z* 433.2 and 455.1 for GSH-NEM, 261.9 and 301.8 for TSA-NEM and 307.2 and 613.0 for GSSG). For other experimental conditions see Fig. 3 and text. S.E.=Standard Error.

3.2. Validation of the LC–ESI-MS method

For validation of the LC–ESI-MS method we studied several experimental parameters such as limit of detection (LOD), limit of quantitation (LOQ), linearity, recovery, stability and repeatability. The separated LC zones were detected by selected ion(s) monitoring mode (SIM) (*m*/*z* 433.2 and 455.4 for GSH-NEM, 261.9 and 302.8 for TSA-NEM and 307.2 and 613.2 for GSSG). The limit of detection (LOD) was assessed at 0.01 μ*M* for GSH-NEM and at 0.05 μ*M* for GSSG with a calculated signal-to-noise ratio >3. While the limit of quantitation (LOQ) was 0.05 μ*M* and 0.2 μ*M* for GSH-NEM and GSSG, respectively (signal-to-noise ratio>10). Fig. 4 depicts calibration curves of the GSH-NEM derivative and GSSG achieved by analyzing mixtures containing the two compounds and the internal standard, I.S. (TSA-NEM) in the 0.01–20 μ*M* and 0.05–20 μ*M* concentration ranges for GSH-NEM and GSSG, respectively (the TSA concentration was 20 μ*M*). The calibration curves were linear in the concentration ranges studied with correlation coefficients $r>0.9999$ for GSH-NEM and $r>0.999$ for GSSG, as obtained plotting the peak areas ratio (sample/I.S.) vs. analyte concentration. However, the correlation coefficients were also satisfactory plotting peak areas vs. analyte concentrations.

Very good results were obtained for run-to-run repeatability of retention time, peak area and concentration of GSH-NEM, TSA-NEM and GSSG.

The data shown in Table 1 were achieved by injecting a standard solution containing 10 μ*M* of GSH and GSSG and 20 μ*M* of internal standard after derivatization with NEM six times, in order to test the within-day variation of retention time, peak area and peak area ratios (sample/I.S.).

Day-to-day variation of the method was assessed by analyzing two standard mixtures containing 5 and 10 μ*M* of both GSH and GSSG and 20 μ*M* of TSA injected on different days ($n=6$). Day-to-day accuracy for the standard solution at the concentration of

Table 1
Retention time, peak area and peak area ratio (sample/I.S.) repeatability in GSH and GSSG analysis[a]

Injection Number	GSH-NEM			TSA-NEM		GSSG		
	R.T. (min)	P.A. ($\times 10^7$)	$A_S/A_{I.S.}$	R.T. (min)	P.A. ($\times 10^7$)	R.T. (min)	P.A. ($\times 10^7$)	A_S/A_{IS}
1	3.60	3.27	0.98	5.93	3.34	14.42	1.09	0.33
2	3.60	3.35	0.98	5.93	3.42	14.44	1.12	0.33
3	3.60	3.42	0.98	5.93	3.49	14.49	1.04	0.30
4	3.60	3.29	0.98	5.93	3.36	14.43	1.11	0.33
5	3.60	3.32	0.97	5.93	3.42	14.44	1.09	0.32
6	3.60	3.27	0.97	5.94	3.36	14.42	1.11	0.33
Mean	3.60	3.32	0.98	5.93	3.36	14.44	1.09	0.32
Std. Dev.	0.00	0.06	0.00	0.00	0.06	0.03	0.03	0.01
RSD%	0.06	1.75	0.40	0.03	1.67	0.18	2.50	3.72

R.T.=retention time; P.A.=peak area; $A_S/A_{I.S.}$=peak area ratio (Sample/I.S.).
[a] For experimental conditions see text.

5 μ*M* was found to be 102.2 and 104.1% for the concentration of GSH and GSSG, respectively, whereas that for the standard solution at the concentration of 10 μ*M* was found to be 99.6 and 100.3%, respectively. The precision of the day-to-day analyses, expressed as RSD%, was calculated as 1.6 and 2.1% for GSH and GSSG concentration, respectively, in the 5 μ*M* standard solution, and 1.9 and 2.9%, respectively, in the 10 μ*M* standard solution.

The recovery of the method was verified analyzing lymphocyte lysate samples after the addition of three known amounts of GSH and GSSG standard solutions and subjected to the previously described derivatization procedure. The theoretical concentrations of the spiking GSH and GSSG standards were 1, 5 and 10 μ*M*. For each point of the recovery testing we analyzed three aliquots coming from different subjects. The mean recovery and the standard deviations for the three spiking amounts of GSH were 99.3%±4.9, 98.5%±4.3, 100.6%±3.8, respectively, and those for GSSG were 105.8%±10.1, 108.2%±9.6 and 111.5%±8.6, respectively.

3.3. Assay of GSH and GSSG in peripheral blood mononuclear cells

The optimized LC–ESI-MS method described above was applied to peripheral blood mononuclear cells (PBMC) to monitor GSH and GSSG content. Samples of 20 healthy volunteers were collected, treated as described in the experimental section, and analyzed, revealing the presence of both GSH and GSSG. The average level found for GSH and GSSG was 74.4±21.2 nmol/mg of proteins and 0.9±0.6 nmol/mg of proteins respectively. Table 2 reports the concentrations of GSH and GSSG in the supernatants obtained as described in the sample preparation section, expressed as μ*M*, and the concentration of the same analytes in the cell lysates, corrected for the protein content and expressed as nmol/mg proteins. The amount of GSH measured in PBMC was in accordance with values reported in the literature [22], whereas the detected levels of GSSG in supernatants were comprised between the LOD and the LOQ assessed for this analyte, with the exception of sample 5.

Table 2
Levels of GSH and GSSG in lymphocytes from 20 healthy individuals

	GSH		GSSG	
	μ*M*[a]	nmol/mgP	μ*M*[a]	nmol/mgP
1	13.9±0.08	69.0	0.056±0.004	0.28
2	7.27±0.05	53.2	0.052±0.002	0.38
3	7.99±0.06	79.5	0.128±0.009	1.27
4	9.02±0.08	92.8	0.214±0.015	2.20
5	28.9±0.19	74.5	0.598±0.029	1.54
6	18.6±0.09	54.0	0.061±0.004	0.18
7	14.9±0.08	68.4	0.142±0.009	0.65
8	4.75±0.02	64.3	0.089±0.006	1.21
9	12.5±0.08	97.9	0.212±0.011	1.66
10	31.8±0.20	94.8	0.418±0.019	1.24
11	4.75±0.03	26.1	0.054±0.002	0.30
12	22.4±0.15	91.8	0.324±0.017	1.33
13	12.9±0.09	98.8	0.229±0.014	1.75
14	7.06±0.05	63.2	0.058±0.004	0.52
15	9.34±0.07	81.2	0.127±0.007	1.10
16	8.08±0.05	91.2	0.112±0.005	1.26
17	3.45±0.03	39.9	0.052±0.002	0.60
18	13.6±0.10	93.9	0.134±0.006	0.92
19	24.1±0.14	56.1	0.054±0.003	0.13
20	14.2±0.08	98.1	0.053±0.002	0.37

[a] Analytes concentration in supernatants prepared as described in the experimental section. The values are reported as means of three determinations±Std. Dev.

Fig. 5 shows the chromatogram of the GSH and GSSG assay in the PBMC sample from a healthy volunteer treated as reported in the experimental section.

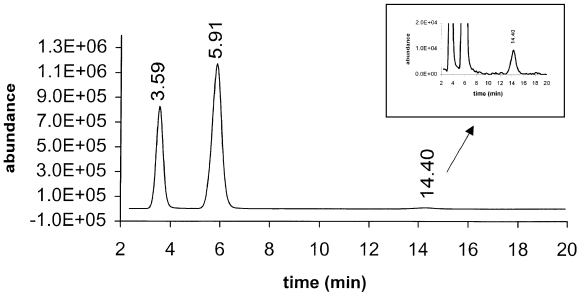

Fig. 5. Reconstituted chromatogram of the assay of GSH-NEM and GSSG in biological sample (PBMC) obtained by LC–ESI-MS. See Fig. 4 for the ions selected for the analysis of the LC. The conditions of the chromatographic separations are reported in the text and in Fig. 3.

Table 3
Stability of GSH and GSSG in 5 μ*M* standard solution and sample extracts at different storage temperatures (expressed as percentage of variation with respect to the analyte concentration at time = 0).[a]

		24 h		48 h		72 h		120 h		168 h	
		GSH (%)	GSSG (%)	GSH (%)	GSSG (%)	GSH (%)	GSSG (%)	GSH (%)	GSSG (%)	GSH (%)	GSSG (%)
Room T°	std 5 μ*M*	101.20	97.46	91.38	79.06	88.58	70.06	81.96	60.47	78.96	59.49
	PBMC 1	96.37	99.78	89.62	88.89	84.88	77.78	79.74	88.89	74.50	66.67
	PBMC 2	79.95	100.10	63.95	91.67	63.12	91.67	61.04	83.33	60.47	66.67
+4°C	std 5 μ*M*	99.40	100.06	99.80	99.60	99.20	99.60	99.20	97.60	92.83	91.42
	PBMC 3	97.72	100.04	94.60	97.75	98.57	100.00	94.68	93.26	82.03	85.39
	PBMC 4	100.57	99.88	100.76	100.00	100.38	100.00	101.38	100.00	90.06	81.82
−20°C	std 5 μ*M*	100.20	101.80	99.60	101.40	100.60	99.80	99.40	95.79	99.60	97.39
	PBMC 5	98.04	98.02	99.05	100.00	99.46	97.03	96.01	97.03	93.99	96.04
	PBMC 6	100.55	103.60	102.26	105.41	100.28	103.60	104.52	107.21	94.77	97.30

[a] PBMC 1–6 = peripheral blood mononuclear cells samples from patients 1–6. For the experimental conditions refer to the text.

3.4. Stability

To test the stability of the GSH-NEM derivative and GSSG, three groups, each constituted by a 5 μ*M* standard solution and two extracted from two different donors, were maintained: the first at room temperature, the second at +4°C and at −20°C the third, up to 7 days. Table 3 reports the amounts of GSH and GSSG determined in the three groups and shows the effect of the different storage temperature on the stability of the standard solutions and the extracted samples. As can be observed in Table 3, the six samples analyzed exhibited a very good stability of the GSH and GSSG compounds up to 24 h at any studied temperature with the exception of sample PBMC 2. Increasing the storage period, we can observe a decrease of GSH and GSSG concentration that was less evident at −20°C.

4. Conclusions

In this study, we have shown that the separation and quantification of GSH and GSSG in peripheral blood mononuclear cells can be easily achieved using LC–ESI-MS. The analysis of the two compounds studied was carried out, after derivatization of GSH with *N*-ethylmaleimide (NEM) and protein precipitation with cold acetonitrile, using a diol stationary phase. The analytes were eluted in less than 15 min and the composition of the mobile phase (acetonitrile–1% acetic acid, 25:75) was compatible with the detection system. The method described provides the specificity associated with the ESI-MS detection mode, enhanced by an excellent and reproducible chromatographic separation and a constant ionization pattern of GSH and TSA NEM adducts and GSSG, as proven by the slight inter-day and intra-day variability of both retention time and peak areas. Moreover, the great sensitivity displayed offers the possibility of disposing even small amounts of biological specimens. A good level of sensitivity is particularly critical for the detection of GSSG which is physiologically present only in very low amounts, accounting for nearly 1% of the GSH levels. Due to these aspects of specificity, reproducibility and sensitivity, associated with a valid extraction procedure, the assay developed has been demonstrated itself to be applicable to complex matrices such as biological samples.

References

[1] A. Meister, M.E. Anderson, Ann. Rev. Biochem. 52 (1983) 711.
[2] A. Meister, Science 220 (1983) 472.
[3] A. Jain, J. Martensson, E. Stole, P.A. Auld, A. Meister, Proc. Natl. Acad. Sci. U.S.A. 88 (1991) 4656.
[4] A. Meister, Cancer Res. 54 (1994) 1969s.

[5] S. Luperchio, S. Tamir, S.R. Tannebaum, Free Radic. Biol. Med. 21 (1996) 519.
[6] R. Brigelius-Flohe', Free Radic. Biol. Med. 27 (1999) 952.
[7] J. Vina, G.T. Saez, D. Wiggins, A.F. Roberts, R. Hems, H. A Krebs, Biochem. J. 212 (1983) 39.
[8] I.A. Cotgreave, R.G. Gerdes, Biochem. Biophys. Res. Commun. 242 (1998) 1.
[9] A.P. Arrigo, Free Radic. Biol. Med. 27 (1999) 936.
[10] T.P. Dalton, H.G. Shertzer, A. Puga, Annu. Rev. Pharmacol. Toxicol. 39 (1999) 67.
[11] D. Voehringer, Free Radic. Biol. Med. 27 (1999) 945.
[12] R.S. Fernandes, T.G. Cotter, Biochem. Pharmacol. 48 (1994) 675.
[13] H. Sies, Free Radic. Biol. Med. 27 (1999) 916.
[14] O.W. Griffith, Free Radic. Biol. Med. 27 (1999) 922.
[15] J.A. Bush, V.C. Ho, D.L. Mitchell, V.A. Tron, G. Li, Photochem. Photobiol. 70 (1999) 329.
[16] M.T. Leccia, M.J. Richard, F. Joanny-Crisci, J.C. Beani, Eur. J. Dermatol. 8 (1998) 478.
[17] L. Mazur, Mutat. Res. 468 (2000) 27.
[18] S. Kojima, S. Matsumori, H. Ono, K. Yamaoka, Anticancer Res. 19 (1999) 5271.
[19] J. Krutmann, A. Morita, J. Invest. Dermatol. 4 (1999) 70.
[20] W. Droge, K. Schulze-Osthoff, S. Mihm, D. Galter, H. Shenk, H.P. Eck, S. Roth, H. Gmunder, FASEB J. 8 (1994) 1131.
[21] M. Picardo, S. Passi, in: J.D. Bos (Ed.), Free Radicals in Skin Immune System (SIS), CRC Press, Boca Raton-New York, 1997, p. 207.
[22] R.W. Pero, M.W. Anderson, G.A. Doyle, C.H. Anna, F. Romagna, M. Markowitz, Cancer Res. 50 (1990) 4619.
[23] J.D. Peterson, L.A. Harzenberg, K. Vasquez, C. Waltenbaugh, Proc. Natl. Acad. Sci. U.S.A. 95 (1998) 3071.
[24] Y. Morel, R. Barouki, Biochem. J. 342 (1999) 481.
[25] K.J. Lenton, H. Therriault, J.R. Wagner, Anal. Biochem. 274 (1999) 125.
[26] C. Parmentier, P. Leroy, M. Wellman, A. Nicolas, J. Chromatogr. B 719 (1998) 37.
[27] C.-S. Yang, S.-T. Chou, L. Liu, P.-J. Tsai, J.-S. Kuo, J. Chromatogr. B 674 (1995) 23.
[28] C.C. Yan, R.J. Huxtable, J. Chromatogr. B 672 (1995) 217.
[29] J.-L. Luo, F. Hammarkvist, I.A. Cotgreave, C. Lind, K. Andersson, J. Warnerman, J. Chromatogr. B 670 (1995) 29.
[30] A.P. Senft, T.P. Dalton, H.G. Shertzer, Anal. Biochem. 280 (2000) 80.
[31] T. Yoshida, J. Chromatogr. B 678 (1996) 157.
[32] M.A. Raggi, R. Mandrioli, G. Casamenti, D. Musiani, M. Marini, Biomed. Chromatogr. 12 (1998) 262.
[33] J. Lakritz, C.G. Plopper, A.R. Buckpitt, Anal. Biochem. 247 (1997) 63.
[34] J.C. Deutsch, C.R. Santhosh-Kumar, J.F. Kolhouse, J. Chromatogr. A 862 (1999) 161.
[35] D. Tsikas, M. Raida, J. Sandmann, S. Rossa, W.G. Forssmann, J.C. Frolich, J. Chromatogr. B 742 (2000) 99.
[36] D.E. Hammermeister, J. Serrano, P. Schmieder, D.W. Kuehl, Rapid Commun. Mass Spectrom. 14 (2000) 503.
[37] P. Capitan, T. Malmezat, D. Breuillè, C. Obled, J. Chromatogr. B 732 (1999) 127.
[38] G. Piccoli, M. Fiorani, B. Biagiarelli, F. Palma, L. Potenza, A. Amicucci, V. Stocchi, J. Chromatogr. A 676 (1994) 239.
[39] M.W. Davey, G. Bauw, M. Van Montagu, J. Chromatogr. B 697 (1997) 269.
[40] C. Muscari, M. Pappagallo, D. Ferrari, E. Giordano, C. Capanni, C.M. Caldarera, C. Guarnieri, J. Chromatogr. B 707 (1998) 301.
[41] M.A. Raggi, R. Manadrioli, C. Sabbioni, F. Mongiello, M. Marini, S. Fanali, J. Microcol. Sep. 10 (1998) 503.
[42] J. Viña, J. Sastre, M. Asensi, L. Packer, Methods Enzymol. 251 (1995) 237.