Short communication

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Determination of different forms of aminothiols in red blood cells without washing erythrocytes

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ABSTRACT: Detection and quantification of different aminothiols forms (reduced and total) in biological fluids are important for the investigation of oxidative stress-related diseases and cell homeostasis study. The aim of this study was to optimize a HPLC method in order to determine both reduced and total thiol forms in red blood cells (RBC) at low temperature without washing erythrocytes. Analytical recoveries for total and reduced thiols were 91.6–98.5 and 94.9–98.2% respectively. The relative standard deviations intra-assay for total and reduced thiols were 1.14–3.64 and 0.83–2.3% respectively and the relative standard deviations inter-assay for total and reduced thiols were 1.12–3.54 and 0.84–2.03%, respectively. This method allows specific analysis of the aminothiol state inside the RBC, as a model of intracellular metabolism functioning.

Keywords: red blood cells; reduced and total aminothiols; redox state; HPLC

Introduction

The redox-state is a critical determinant of cell functions and homeostasis, and any major imbalances can cause severe damage or cell death. Various redox active compounds are implicated in those processes, among them the biological thiols (SH-) containing amino acids known as aminothiols, which play numerous physiological roles.

Erythrocytes are rich in protein-SHs and antioxidant enzymes and have glutathione (GSH)-redox recycling to protect tissues from prolonged oxidative stress elicited by reactive oxygen species (Valko *et al*., 2007). Therefore, thiol compounds in plasma can be used as an index of a peripheral response of the organism to external stimuli that may impact on the redox state, whereas erythrocytes may be a model of the intracellular response (Valko *et al*., 2007; Powers and Jackson, 2008).

Characterization of the biochemical profile of the different forms of aminothiols (reduced and total forms) in biological fluids may be relevant both in clarifying the mechanisms underlying the oxidative stress-related diseases and in monitoring the response to possible drug/food supplement treatments (Pastore *et al*., 2003; Camera and Picardo, 2002).

Several chromatographic methods for the determination of thiols have been described in the literature; however, all of these methodologies show some disadvantages, such as equipment costs, complex protocols and sample processing, long run times and also simultaneous quantification of thiols and/or complicated parameter validation for high-throughput routine clinical or research purposes. Therefore the choice of method is crucial to obtain relevant data in a short time. In this paper a method able to determine reduced and total aminothiols forms was optimized, in red blood cells (RBC) at low temperature without erythrocyte washing. This method was applied for the assessment of oxidative stress through thiols measurement in human plasma and RBC in order to understand the interplay between the forms of thiols in the different compartments, both in the redox homeostasis maintenance and in the oxidative stress conditions. The balance among glutathione forms plays a central role as a hallmark and a potential biomarker of several diseases (Petrillo *et al*., 2013; Dhawan *et al*., 2011; Martín *et al*., 2004).

Experimental

Chemicals

L-cystine, cysteinylglycine, DL-homocystine, L-glutathione, oxidized and reduced forms; 4-fluoro-7-sulfamoylbenzofurazan (ABD-F) and Tris-(2-carboxyethyl)-phosphine hydrochloride (TCEP) were purchased from Sigma (St. Louis, MO, USA). Methanol, acetic acid, sodium acetate, boric acid, sodium hydroxide, trichloroacetic acid (TCA) and ethylenediaminetetra acetic acid (EDTA) were purchased from Merck (Darmstadt, Germany). Bi-distilled and deionized water (18 MΩ of conductance) were obtained from Milli Q System, Millipore (Vimodrone, Milan, Italy). All chemicals were HPLC grade or of the highest purity available.

Instrumentations and chromatographic conditions

We used an HPLC pump Varian Pro Star model 240 (Varian, Palo Alto, CA, USA), coupled to an auto-sampler Varian Pro Star model 410 with a Peltière temperature control (Varian, Palo Alto, CA, USA) and an FP 1520 Jasco fluorescence detector with a 150 W xenon lamp (Jasco Europe, Cremella,

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Abbreviations used: ABD-F, 4-fluoro-7-sulfamoylbenzofurazan; EDTA, ethylenediaminetetra acetic acid; RBC, red blood cells; TCA, trichloroacetic acid; TCEP, Tris-(2-carboxyethyl)-phosphine hydrochloride.

Lecco, Italy). The fluorimetric detector was set at excitation and emission wavelengths of 390 and 510 nm respectively, and a bandwidth of 18 nm. Chromatograms were automatically integrated using dedicated software, Varian STAR Chromatography Workstation (Varian Palo Alto, CA, USA). Analyte separation was performed on a 5 μm Discovery C_{18} analytical column (250 × 4.6 mm i.d, Supelco, Sigma-Aldrich) at room temperature. The mobile phases consisted of 0.1 M acetate buffer, pH 4.0–methanol, 81:19 (v/v). Aminothiols were separated over 10 min at a flow rate of 1.0 mL/min. The units of measurement are micromolar.

Standard preparation

Individual stock solutions of Cystine (Cys), Cysteineglycine (CysGly), Homocysteine (Hcy) and Glutathione (GSH) oxidized and reduced forms were prepared from separate weighs of each compound dissolved in HCl 1 M at 600 μM final concentrations. All individual stock solutions were stored at −80°C to simulate the storage conditions of physiological samples. Standard mixes containing all four aminothiols were prepared at the beginning of each validation experiment by appropriate dilution with HCl 1 M.

Sample pre-treatment

Whole blood was collected by venipuncture into EDTA tubes and plasma was immediately separated from the cellular portion by centrifugation at 4000***g*** for 2 min at 4°C to prevent thiol oxidation. Immediately after discarding plasma and the buffy coat, aliquots of RBC were transferred to test tubes and frozen in liquid nitrogen. Each sample was stored at −80°C until analysis. Immediately before analysis the RBC were thawed, diluted 1:2 (v/v) with distilled water and re-frozen in liquid nitrogen to achieve the membrane rupture. For reduced RBC aminothiols the samples were thawed, diluted 1:2 (v/v) in TCA (100 g/L with 1 mM EDTA) and centrifuged at 14,000***g*** for 10 min at 4°C before sample preparation.

For total aminothiols, RBC samples or calibration standard curve were first reduced with TCEP and subsequently diluted 1:2 (v/v) in TCA (100 g/L with 1 mM EDTA) and centrifuged at 14,000***g*** for 10 min at 4°C before sample preparation.

Sample preparation

To 100 μL of each supernatant obtained were added 10 μL of 0.4 M NaOH, 70 μL of 1 M borate buffer pH 11 containing 4 mM EDTA, 30 μL of 1 M borate buffer pH 9.5 containing 4 mM EDTA, and 10 μL of 10 g/L ABD-F in borate buffer (1 M pH 9.5 containing 4 mM EDTA). Samples were incubated at 4°C for 90 min and then 10 μL were injected onto the HPLC system for analysis

Method validation

Method validation was carried out according to the FDA guidelines (US Food and Drug Administration, 2001) including the linearity, sensitivity, accuracy, precision, recovery, carryover and stability. For stability the samples of standard and RBC were stored at −80°C.

Three different whole blood samples were centrifuged at 4000***g*** to separated the RBC. Then the RBC were subjected to three washing cycles with saline solution. Before the washings and after each subsequent single washing, an aliquot of each sample was frozen in order to verify the variation of thiols concentration during the washing of the RBC.

Results and discussion

Representative chromatograms of standard solutions, human total and reduced plasma and human total and reduced RBC from healthy volunteers are depicted in Fig. 1. Retention times for Cys, CysGly, Hcy and GSH were approximately 5.4, 6.1, 7.1 and 8.6 min, respectively. The peaks of interest were well separated without interfering compounds. We have reported in previous work that the presence of internal standard decreases the accuracy of the analysis, so in this method we did not use the internal standard (Accinni *et al.*, 1998).

In order to test the linearity of the method we injected increasing concentration of standard solutions. Ten standard concentration of different mix oxidized thiols concentrations ranging from 0.59 to 300 μM for Cys, from 0.12 to 60 μM for Hcy and from 0.24 to 120 μM for CysGly and GSH were processed

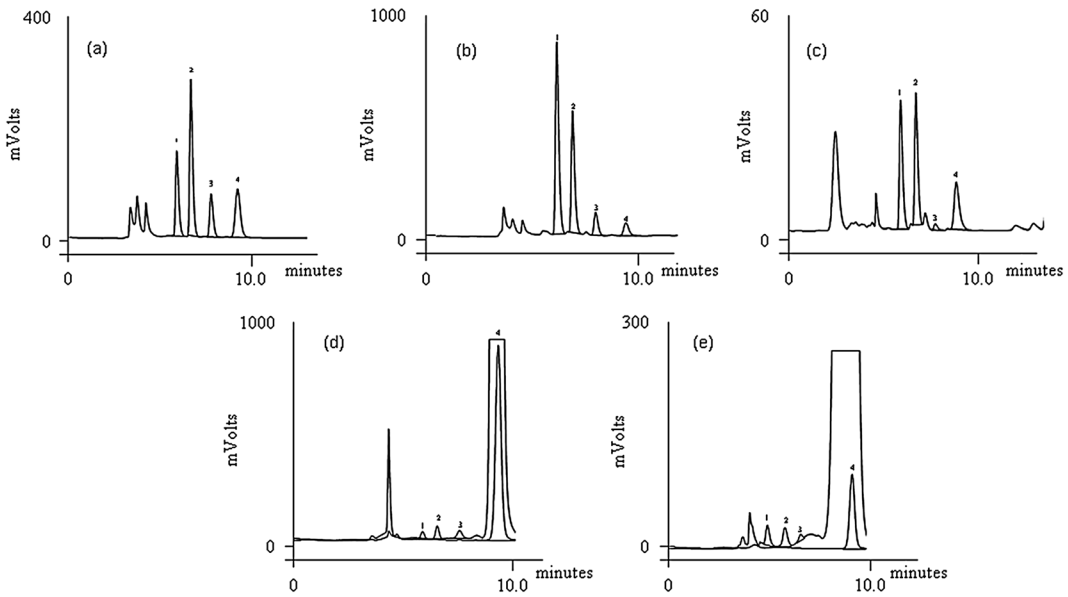

Figure 1. Representative chromatograms of: standard solution (a) containing 75 mM of Cys (peak 1). 30mM of CysGly (peak 2), 15mM of Hcy (peak 3) and 30 mM of GSH (peak 4); human plasma total (b) and reduced (c) thiols and human RBC total (d) and reduced (e) thiols from healthy volunteers. Peak 1 was Cys, peak 2 was CysGly, peak 3 was Hcy and peak 4 was GSH. Standard mixture and sample were prepared and analyzed as described in the text. The peak 4 of human RBC is eluted after dilution 1:20 (v/v) with borate buffer pH 9.5. Injection volumes of all sample were 10 μ L.

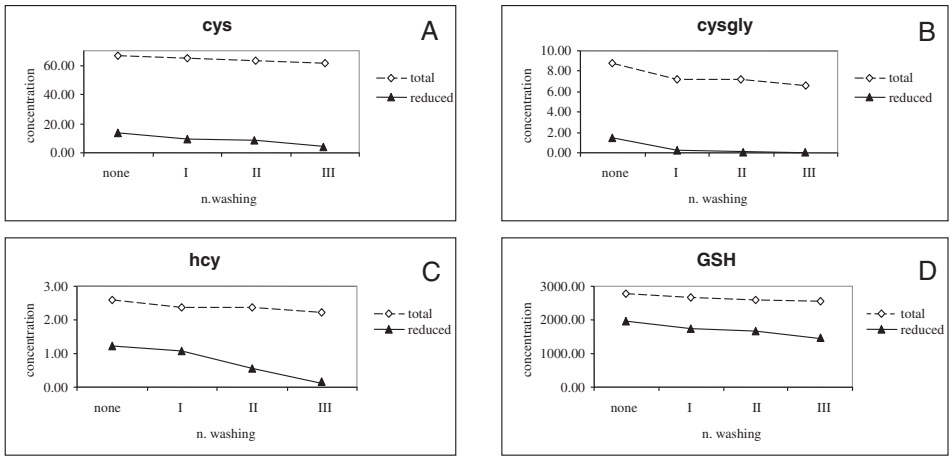

Figure 2. Variation of RBC total (◇) and reduced (▲) thiols concentrations in different washes. Panel A: variations in the RBC Cys concentrations. Panel B: variations in the RBC CysGly concentrations. Panel C: variations in the RBC Hcy concentrations. Panel D: variations in the RBC GSH concentrations.

as described above. The calibration curve for each aminothiol were obtained by linear regression analysis of the aminothiol peak area plotted against the nominal aminothiol concentration expressed in micromolar. For Cys the linearity curve was $y = 28{,}904x + 34{,}559$, $r = 1$; for CysGly it was $y = 133{,}637x + 74{,}539$, $r = 0.999$; for Hcy it was $y = 99{,}109x + 10{,}686$, $r = 1$; and for GSH it was $y = 88064x + 24873$, $r = 1$.

For accuracy and precision, pools of RBC were collected and frozen in liquid nitrogen as described above. Ten replicates of total and reduced RBC samples were made for two days followed by analysis of 10 replicates on the third day, for the determination of inter-day precision. Intra-day precision were instead determined from $n = 10$ replicate samples on day 3. Inter-assay and intra-assay coefficients of variation were $<4\%$ (Table 1).

Analytical recovery was performed by adding human RBC to three different concentrations of mix oxidized or reduced forms standard solution. Samples were treated as described above. Six replicates unspiked (control) and spiked of total and reduced RBC samples were analyzed using calibration curves. The recovery was calculated by subtracting the concentration of unspiked samples from the spiked samples concentration. The value obtained was divided by the concentration of standard and multiplied by 100. Measured concentrations were defined as 100%. Means, standard deviations and relative standard deviations were calculated (Table 2).

The absolute detection limit was determined as three times the baseline noise level. The samples of standard and RBC were measured in order to test the stability of the total and reduced samples for three months and no changes were noted in the concentration of the samples assayed. The fluorescent adduct samples was stable for 24 h if stored at 4°C and 48 h if stored at −20°C (data not shown).

The preservation of the original reduced state in plasma and especially in RBC appears to be the most delicate step in the sample preparation after collection. This is due to fast kinetics of oxidation of SH groups. In fact, during the first hour after collection, reduced thiols undergo a significant decrease if stored at room temperature (Anderson *et al*., 1995) and even if stored at lower temperatures (data not shown). Also, the temperature during the formation of fluorescent adducts for the fluorimetric detection of thiols may play a key role in the formation of methodological artifacts. In fact, if the reaction occurs at high temperatures (usually at 60°C) the reduced thiol forms and the oxidized ones react with each other, generating mixed disulfides (Santa *et al*., 2006). Other authors demonstrated that ABD-F (4-aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole) is a preferred derivatization reagent for the accurate determination of the reduced-form thiols in samples containing the oxidized-form thiols with respect to 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate (Otani *et al*., 2011).

Table 1. Intra- and inter-day precision for all four aminothiols in red blood cells (RBC)

	Total aminothiols in RBC				Reduced aminothiols in RBC			
	Intra-day[a]		Inter-day[b]		Intra-day[a]		Inter-day[b]	
	μM	RSD (%)	μM	RSD (%)	μM	RSD (%)	μM	RSD (%)
Cys	36.29 ± 1.32	3.64	36.32 ± 1.29	3.54	3.72 ± 0.09	2.3	3.73 ± 0.07	1.85
CysGly	5.79 ± 0.07	1.14	5.79 ± 0.06	1.12	0.72 ± 0.01	1.79	0.71 ± 0.01	2.03
Hcy	2.83 ± 0.08	2.91	2.82 ± 0.08	2.75	0.65 ± 0.01	1.04	0.65 ± 0.01	0.91
GSH	1977.3 ± 52.2	2.64	1985.9 ± 44.0	2.22	1059.7 ± 8.7	0.83	1061.1 ± 9.0	0.84

[a]Ten control replicates on day 3.
[b]Ten replicates for each form for two days followed by analysis of 10 replicates on day 3.

Table 2. Recovery of all four thiols from human RBC ($n = 6$)

	Concentration (μM)				
	Total aminothiols in RBC	Spiked	Measured (mean ± SD)	%	RSD (%)
Cys	43.23 ± 0.60	75.00	117.1 ± 0.6	98.5 ± 0.8	0.9
		18.75	61.4 ± 0.2	97.1 ± 1.0	1.0
		9.38	52.3 ± 0.2	94.7 ± 1.6	1.6
CysGly	5.57 ± 0.06	30.00	33.39 ± 0.06	92.8 ± 0.19	0.2
		7.50	12.7 4 ± 0.06	95.7 ± 0.7	0.8
		3.75	9.00 ± 0.02	91.6 ± 0.4	0.5
Hcy	3.02 ± 0.03	15.00	17.73 ± 0.09	98.1 ± 0.6	0.6
		3.75	6.70 ± 0.02	98.2 ± 0.6	0.6
		1.88	4.9 ± 0.01	98.3 ± 0.6	0.7
GSH	2149.4 ± 1.2	600.00	2739.8 ± 3.5	98.4 ± 0.6	0.6
		150.00	2293.5 ± 1.3	96.1 ± 0.9	1.0
		75.00	2222.9 ± 6.0	98.1 ± 0.8	0.8
	Reduced aminothiols in RBC				
Cys	4.17 ± 0.1	75.00	77.8 ± 0.7	98.2 ± 0.9	1.0
		18.75	22.3 ± 0.2	96.6 ± 1.0	1.0
		9.38	13.4 ± 0.1	98.2 ± 1.3	1.4
CysGly	1.17 ± 0.01	30.00	30.3 ± 0.1	97.2 ± 0.4	0.4
		7.50	8.5 ± 0.1	97.1 ± 1.0	1.0
		3.75	5.0 ± 0.1	96.5 ± 1.2	1.2
Hcy	1.10 ± 0.05	15.00	15.5 ± 0.1	96.2 ± 0.9	1.0
		3.75	4.7 ± 0.02	96.5 ± 0.7	0.7
		1.88	2.9 ± 0.04	95.2 ± 2.4	2.5
GSH	1186.4 ± 15.7	600.00	1760.5 ± 7.5	95.7 ± 1.3	1.3
		150.00	1328.8 ± 2.1	94.9 ± 1.4	1.5
		75.00	1259.1 ± 1.0	96.9 ± 1.4	1.5

Table 3. Mean total and reduced aminothiols in plasma and RBC from 102 healthy volunteers

	Cys	CysGly	Hcy	GSH	Cys	CysGly	Hcy	GSH
	Total aminothiols in plasma (μM)				*Reduced aminothiols in plasma (μM)*			
Women	229.8 ± 40.6	34.6 ± 8.3	9.2 ± 2.0	10.9 ± 1.8	9.5 ± 1.7	2.4 ± 0.6	0.16 ± 0.04	2.4 ± 0.6
Men	218.6 ± 45.4	35.3 ± 8.5	10.3 ± 2.3	11.6 ± 2.3	9.5 ± 1.8	2.7 ± 0.6	0.18 ± 0.06	2.3 ± 0.7
All together	224.4 ± 43.1	35.0 ± 8.4	9.7 ± 2.2	11.3 ± 2.1	9.5 ± 1.7	2.55 ± 0.6	0.18 ± 0.05	2.4 ± 0.7
	Total aminothiols in RBC (μM)				*Reduced aminothiols in RBC (μM)*			
Women	29.5 ± 21.4	4.2 ± 3.6	2.5 ± 1.6	1989.5 ± 715.6	4.7 ± 4.1	0.73 ± 0.58	0.75 ± 0.69	1182.3 ± 534.7
Men	30.6 ± 14.3	5.3 ± 3.3	2.7 ± 1.4	2069.0 ± 707.1	3.4 ± 2.7	0.86 ± 0.56	0.79 ± 0.71	1220.5 ± 548.6
All together	30.1 ± 18.2	4.7 ± 3.5	2.6 ± 1.5	2027.8 ± 709.2	4.1 ± 3.5	0.79 ± 0.57	0.77 ± 0.71	1200.6 ± 539.1

Age 45.9 ± 12.0 (women 46.7 ± 10.7, $n = 53$; men 45.1 ± 13.2, $n = 49$) and body mass index 22.7 ± 2.7 (women 22.2 ± 2.6, $n = 53$; men 23.2 ± 2.7, $n = 49$).

We have presented an improved pre-analytical procedure, especially designed to minimize several pitfalls that frequently influence the determination of those aminothiols with low concentrations in certain compartments (e.g. Hcy and CysGly blood concentrations).

To avoid a significant decrease of reduced thiols (Anderson *et al.*, 1995), in our procedure the blood is immediately centrifuged at 4°C after collection, to separate the compartments avoiding exchanges. This protocol allows possible interference in the analysis to be avoided. For the same reason, we did not proceed with the washing of the RBC, but immediately collected the RBC after removing the buffy coat.

Avoiding this step, we can dose aminothiols inside the RBC without both the plasma interference obtained if we measure the aminothiols in whole blood, and the important alterations in the aminothiols pattern that we noticed when the washing step was applied (Fig. 2A–D). In this way we can analyze specifically the aminothiol state inside the RBC, as a model of intracellular metabolism functioning and also in plasma, as indicators of the peripheral response of the organism.

Differently from other validated methods, this method can be used to investigate the relationships between Hcy, Cys, CysGly and GSH not only in cell but also in plasma. By the described method, total and reduced aminothiols forms were investigated in RBC and in the plasma of 102 healthy volunteers. The average values obtained are shown in Table 3.

This study has been fully validated as a simple, sensitive and reproducible HPLC method for total and reduced thiols both in the intracellular compartment (RBC) and extracellular space (plasma). The method does not utilize an internal standard, as previously discussed (Accinni *et al*., 1998), and demonstrates good RBC and plasma recovery and an excellent sensitivity. It is suitable for clinical study to support both the monitoring and investigation of redox interplay between the different forms of aminothiols in physiological and pathophysiological conditions.

This simple method also allows study of the metabolism of the different forms of circulating aminothiols as a marker of both oxidative stress and antioxidative defence capacity. The high sensitivity of the method can be used to monitor clinical trials (studies) based on nutritional supplementation to verify the efficacy and antioxidant capacity of many molecules. The method additionally allows study of the different forms of aminothiols in the different compartments simultaneously, to discern their respective roles and to select appropriate therapies.

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