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Derivatization of GSSG by pHMB in alkaline media. Determination of oxidized glutathione in blood

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ABSTRACT

Chromatographic determination of glutathione disulfide (GSSG) without any preliminary reduction has been presented using GSSG derivatization by *p*-hydroxymercuribenzoate (pHMB) in strong alkaline medium followed by the determination of GS–pHMB complex by reversed phase chromatography coupled to chemical vapour generation and atomic fluorescence detector (RPC–CVGAFS). A detection limit of 35 nM for GSSG (corresponding to 1.8 pmol) detected as GS–pHMB species was achieved based on a signal-to-noise ratio of 3 in buffer and in blood. The proposed method was applied to the determination of GSSG in whole blood and validated by the classical determination of GSSG by derivatization after reduction with dithiothreitol (DTT).

1. Introduction

Glutathione (γ-Glu-Cys-Gly, GSH) is one of the most important cellular metabolites. The functions of GSH include detoxication of xenobiotics and heavy metals, reduction of oxidation-prone protein thiols, maintenance of cellular membranes and deactivation of free radicals [1]. Its disulfide, GSSG, restores disulfide bridges and co-regulates metal content of metallothionein [2,3]. The intracellular concentration of total, reduced and oxidized GSH in human cells is often as high as 1–20 mM [4]. Under oxidative stress GSSG concentration increases, and rapidly is reverted back to GSH by the action of the enzyme glutathione reductase. Changes of the GSH/GSSG ratio are considered indices of oxidative damage [5,6].

Some analytical methods using liquid chromatography (LC) combined with different detection techniques have been developed for the analysis of GSH and GSSG. These methods are generally based on derivatization by suitable probes of reduced –SH group of GSH and of GSSG after chemical reduction [6–9]. Electrochemical methods [10–12] and LC–MS methods [13,14] have also been proposed for the direct determination of GSSG.

In 1939 Schoberl and Rambacher showed that the disulfide bond of cystine was "split" on treatment with strong alkali [15]. In 1953 Stricks and Kolthoff obtained indications that GSSG in strong alkaline media was split according to reaction (1) followed by reaction (2) [16].

$$GSSG + OH^- \leftrightarrows GS^- + GSOH \quad (1)$$

$$2\,GSOH \leftrightarrows GSH + GSO_2H \quad (2)$$

They found that reagents prevailed in reaction (1), but it was driven to completion by including $HgCl_2$, in the solution. Later, Karush et al. used alkaline solution of fluorescein mercuric acetate to determine disulfide groups in proteins and peptides [17].

Thereafter, Andersson and Berg [18] studied the kinetics of GSSG/*p*-hydroxymercuribenzoate (pHMB) reaction in the pH range 9.2–11.0 finding that (i) the stoichiometric ratio pHMB:GSSG was 3:2, in agreement with reactions (1) and (2), (ii) the kinetics were of the first order, (iii) the hydrolysis step (reaction (1)) was rate-determining and directly proportional to the concentration of OH^-, and (iv) pHMB did not participate in the disulfide cleavage and reacted with the $-GS^-$ formed in the hydrolysis. Above pH 10.6 somewhat more than 3 equiv. of pHMB appeared to react when the solution was allowed to stand for 6 h or more.

More recently, some authors proposed mercurochrom for the histochemical quantitation of protein disulfides [19].

Over the last 10 years we extensively studied the interaction between pHMB with *reduced* –SH functional groups on proteins [20–23], low molecular weight thiols [7] and nitrosothiols [24,25]

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by liquid chromatography coupled to chemical vapour generation and atomic fluorescence spectrometry (CVGAFS).

In this work we have studied the reaction of pHMB with oxidized thiols (GSSG, cystine, homocystine and selenocystine) in strong alkaline medium. We have optimized a procedure for the chromatographic determination of glutathione disulfide (GSSG) by pHMB derivatization in alkaline medium without any preliminary reduction step, followed by the determination of GS–pHMB complex by reversed phase chromatography (RPC) coupled to CVGAFS. The method has been applied to the determination of GSSG in whole blood and validated by comparing the results with those obtained by dithiothreitol (DTT) reduction method.

2. Experimental procedures

2.1. Chemicals

Analytical reagent-grade chemicals were used without further purification. pHMB (4-hydroxymercuric) benzoic acid, sodium salt (CAS No. 138-85-2, $HOHgC_6H_4CO_2Na$) was purchased from Sigma (Sigma–Aldrich–Fluka Chemical Co., Milan, Italy). 1×10^{-2} M stock solution of pHMB was prepared by dissolving the sodium salt in 0.01 M NaOH in order to improve its solubility, stored at 4 °C, and diluted freshly, just before use. The precise concentrations of pHMB solutions were determined from the absorbance at 232 nm ($\varepsilon_{232} = 1.69 \times 10^4\ cm^{-1}\ M^{-1}$).

Stock solutions of GSH (G6529), cystine (30089, Cys), homocystine (H4628, HCys), oxidized glutathione (49740, GSSG), cystine (30199), oxidized HCys (H0501) and seleno-L-cystine (Fluka 09976) (Sigma–Aldrich–Fluka, Milan, Italy) were prepared in 0.1 M phosphate buffer solution (PBS) pH 7.4, 0.5 mM ethylendiaminotetracetic acid (EDTA) or 0.1 M HCl (cystine and selonocystine). In order to prevent oxidation, standard solutions of reduced thiols were prepared daily and kept cold (4 °C) and protected from light until used.

The buffer solutions were prepared from monobasic monohydrate sodium phosphate, dibasic anhydrous potassium phosphate (BDH Laboratory Supplies, Poole, England).

Dithiothreitol (DTT, D5545 SigmaUltra, ≥99.0%), N-ethylmaleimide (NEM, 04259 BioChemika Ultra, ≥99.0%, Fluka) and 2-vinylpyridine (2-VP, 132292 97%, Aldrich) were purchased from Sigma–Aldrich–Fluka and the stock solution prepared daily in MilliQ water.

Methanol for RPLC was purchased from Carlo Erba (Rodano, Milan, Italy).

Stock solutions of $NaBH_4$ (about 6.5 M) was prepared by dissolving the solid reagent (45288-2 powder, reagent grade >98.5%, from Sigma–Aldrich, Milan, Italy) into 0.3% (m/v) NaOH solution. The solutions were microfiltered through a 0.45 μm membrane and stored in a refrigerator. Diluted solutions of $NaBH_4$ (0.05 M) were prepared by appropriate dilution of the stock solutions, with the total NaOH concentration maintained at 0.3% (m/v).

The 24–26% hydrazine standard solution (53847, CAS No. 10217-52-4) was purchased from Fluka (Sigma–Aldrich–Fluka, Milan, Italy) and the optimized concentration (0.1 M) added to $NaBH_4$ solution containing 0.3% (m/v) of NaOH.

3.5 M HCl solutions were prepared with 37% (m/m) HCl (Carlo Erba, Rodano, Milan, Italy).

A working solution of Br^-/BrO_3^- was prepared by solid reagents (Carlo Erba, Rodano, Milan, Italy) (0.075 M Br^-, 0.015 M BrO_3^-) keeping an approximate Br^-/BrO_3^- 5:1 molar ratio on the basis of stoichiometry of redox reaction. Addition of a moderate excess of Br^- guaranteed a complete conversion of bromate to Br_2.

Water deionized with a MilliQ system (Millipore, Bedford, MA, USA) was used throughout.

2.1.1. Safety considerations

pHMB is toxic. Inhalation and contact with skin and eyes should be avoided. All work should be performed in a well-ventilated fume hood.

2.2. GSSG derivatization and calibration

GSSG/pHMB mixtures were prepared in 0.1 M PBS at various pH or in NaOH at various concentrations. The products of the reaction of GSSG and pHMB were studied by RPC–CVGAFS by diluting the original mixture in 0.1 M PBS pH 7.4, with or without 0.5 mM EDTA at 21 ± 1 °C in order to avoid damage of the column by basic pHs. Once the GSSG/pHMB derivatization medium was optimized, we varied GSSG/pHMB molar ratio and reaction time. In the optimized conditions GSSG was derivatized with pHMB in a molar excess between 2 and 50, in 0.1 M NaOH for 30 min reaction time at 21 ± 1 °C in a thermostatic bath. In these conditions we performed GSSG calibrations at various molar ratio (2, 4, 10, 20, 50:1 pHMB/GSSG ratio). In the same conditions we studied the derivatization of cystine, oxidized HCys and CysGly.

For the calibration experiments of GSH, GSH was derivatized by diluting the stock solution in 0.1 M PBS (pH 7.43) containing a stoichiometric amount or a moderate excess of pHMB, at 25 °C. After a reaction time ≥5 min at 21 ± 1 °C, the solution was injected in the RP chromatographic column.

The yield of GSSG derivatization was evaluated by comparing the slope of the calibration curves of GSSG with the calibration curve of GS–pHMB complex.

2.3. Human blood sampling and thiol derivatization procedures in blood

Blood was obtained from 11 volunteer donors. Venous blood was collected by puncture of an antecubital vein with a butterfly needle in resting conditions and collected into evacuated tubes containing EDTA.

2.3.1. GSSG analysis

For the analysis of GSSG in whole blood, 500 μL of the collected blood were treated with 2-VP stock solution (400 mM final concentration) to avoid GSH interference [26], vortex, incubated 30 min at 21 °C, mixed with 500 μL of 10% trichloroacetic acid (TCA) immediately after collection and the acidified sample was centrifuged at 10,000 × *g* for 10 min at 21 °C. 100 μL of the supernatant coming from the deproteinized whole blood were diluted in 900 μL of 0.5 M NaOH and derivatized with 100 μM pHMB. After 30 min reaction time at 21 °C (thermostatic bath) the mixture was diluted 1:10 in 1 M PBS pH 7.4 and injected in the RPC–CVGAFS system.

GSSG recovery was evaluated in human whole blood by adding a known concentration of GSSG from a standard solution before any treatment.

2.3.2. Reduced GSH analysis

For the determination of free reduced GSH 500 μL of the collected blood were mixed with 500 μL of 10% TCA immediately after collection and the acidified sample was centrifuged at 10,000 × *g* for 10 min at 21 °C. 10 μL of the supernatant was diluted in 990 μL 0.1 M PBS pH 7.4, 0.5 mM EDTA and derivatized with 100 μM pHMB and injected.

2.3.3. GSH + GSSG analysis

10 μL of the TCA supernatant were diluted in 40 μL 0.1 M PBS pH 7.4, 0.5 mM EDTA, treated with 200 μL 0.2 M PBS pH 9.0, 1.0 mM EDTA, 0.5 mM DTT and incubated at 37 °C for 20 min. Then, the resulting solution was diluted with 1 M PBS pH 7.9 up to 1 mL volume and derivatized with an excess of pHMB (400 μM) in order

to complex GSH and DTT [7]. The reduction yield was evaluated applying the same procedure to a standard solution of GSSG.

2.4. Apparatus

2.4.1. Chromatographic instrumentation

An HPLC gradient pump (P4000, ThermoQuest) was coupled with a mechanical degassing system (SC1000, ThermoQuest), a Rheodyne 7125 injector (Rheodyne, Cotati, CA, USA), and a 50 μL injection loop. Sample eluted from the column passed into the CVGAFS detection system. CVGAFS detection system provided mercury-specific chromatograms. The GS–pHMB complex can be determined by CVGAFS detection in the adopted operating conditions with a detection limit of 70 nM, a precision (CV%) of 6.5% at 0.3 μM concentration level, and a 0.08–50 μM linear dynamic range [24].

2.4.2. Chromatographic conditions

The HPLC separations were carried out by a reversed phase HPLC column Hydra RP C_{18} (Phenomenex) 250 mm × 4.6 mm (silica particle size 4 μm), equipped with a Guard Cartridge KJ0-4282 Phenomenex with an isocratic elution in 99% 0.02 M PBS pH 6.0, 5 mM NaCl, 1% methanol flowing at 1 mL min^{-1} for 17 min and a gradient up to 100% methanol in 2 min to elute pHMB in excess from the column. The chromatographic run was complete in 37 min, including the column re-equilibration time. All the solutions were filtered by a 0.45 μm cellulose acetate filter (Millipore).

2.4.3. Chemical vapour generation with AFS detection

The schematic diagram and a detailed description of the continuous flow (CF) mercury chemical vapour generator modified for on line oxidation of organic mercury to inorganic Hg(II) in a miniaturized Ar/H_2 flame have been previously reported [20–24]. Reagent concentrations ($NaBH_4$, hydrazine, HCl, and Br^-/BrO_3^-), reaction coil dimension, and flow rates, as well, were optimized and reported elsewhere [27].

2.4.4. ESI-MS measurements

The ESI-MS experiments were performed on a LTQ-Orbitrap mass spectrometer manufactured by Thermo Fisher Scientific Inc. (Bremen, Germany) which was equipped with an electrospray ion source. The Orbitrap was operated at resolution setting of 30,000. Full-scan spectra over the *m*/*z* range 335–685 were acquired in the positive ion mode. The voltage on the electrospray needle was set to 3 kV. The capillary temperature was set to 300 °C. The sheath and auxiliary gases were nitrogen at flow rates of 20 and five arbitrary units, respectively. The tube lens voltage was set to 95 V, and capillary voltage was set to 39 V. Ion with *m*/*z* of 630, which correspond to the protonated molecular ion of pHMB derivatized GSSG (GS–pHMB) was extracted from the full-scan spectra. Data were processed using Xcalibur software (Thermo Fisher Scientific Inc., Bremen, Germany) version 2.0.7.

3. Results and discussion

3.1. pHMB/GSSG reaction in alkaline medium

Sulphydryl group of GSH reacts quantitatively with a moderate excess of pHMB in stoichiometric ratio of 1:1 at room temperature in 0.1 M PBS, pH 7.4, 0.5 mM EDTA in less than 2 min and the complex is stable 48 h at room temperature and for months at −20 °C [24]. Fig. 1 (curve a) shows the RPLC–CVGAFS chromatograms of the GS–pHMB complex, which elutes at 12.8 ± 0.1 min. The peak eluting with the dead volume of the column was present also injecting the pHMB in the same buffer solution and it was likely due to a non-covalent complex of pHMB with EDTA. The pHMB excess eluted as a broad peak between 15 and 30 min and it did not interfere with the analysis.

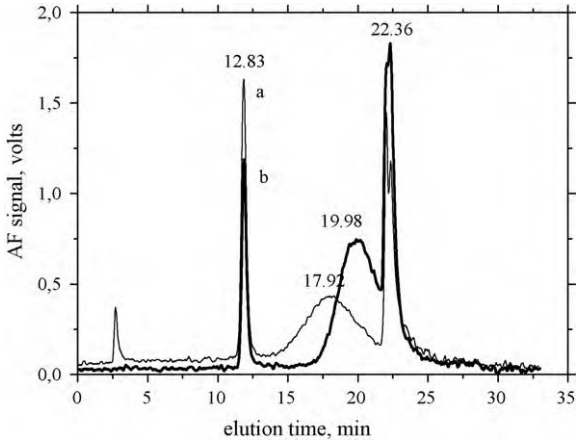

Fig. 1. AF chromatograms of GS–pHMB complex (5 μM GSH, 20 μM pHMB, trace a, thin line) in 0.1 M PBS pH 7.4, 0.5 mM EDTA, and GSSG–pHMB mixture (2 μM GSSG, 20 μM pHMB final concentration, trace b, bold line) in 0.1 M PBS pH 7.4. GSSG–pHMB mixture was prepared in 0.1 M NaOH at 21 °C and diluted 20 times after 30 min incubation time in 0.1 M PBS pH 7.4 before injection. AF = atomic fluorescence.

At pH 7.4 we found that oxidized glutathione (GSSG) did not react with pHMB. However, by mixing GSSG and pHMB in a basic incubation medium (0.1–1 M NaOH), we found by RPC–CVGAFS analysis a peak having the same retention time of GS–pHMB complex (Fig. 1, curve b). GSSG (2 μM) and pHMB (20 μM) were mixed in 0.1 M NaOH at 21 °C and diluted 20 times after 30 min incubation time in 0.1 M PBS pH 7.4. This procedure avoided the injection of basic solution in the RPC column.

The peaks observed in the GSSG/pHMB mixture chromatogram had the same retention time of GS–pHMB complex. This suggested that in 0.1 M NaOH GSSG gave GS^-, and GS^- formed the GS–pHMB complex.

This reaction was studied as a function of pH of medium. Fig. 2 shows the concentration of GS–pHMB in GSSG/pHMB mixtures (1:2 molar ratio) after 30 min reaction time at 21 °C in 0.1 M PBS solutions at various pH (6–12) and in 1 M NaOH (pH 14). The values have been normalized with respect to the *plateau* value obtained

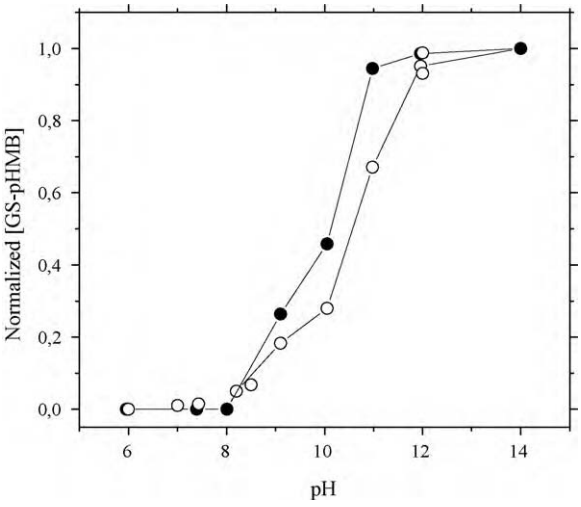

Fig. 2. GSSG/pHMB reaction as a function of pH of medium. Normalized GS–pHMB concentrations (calculated on the basis of the area of 12.1 min peak and sensitivity factor of GS–pHMB calibration curve) are reported on the *y*-axis. Operating conditions: GSSG/pHMB mixture prepared in 0.1 M PBS buffer at the indicated pH or 1 M NaOH (pH 14) with (open circles) or without 0.5 mM EDTA (filled circles), incubated 30 min at 21 °C and diluted 20 times in 0.1 M PBS pH 7.4 before injection.

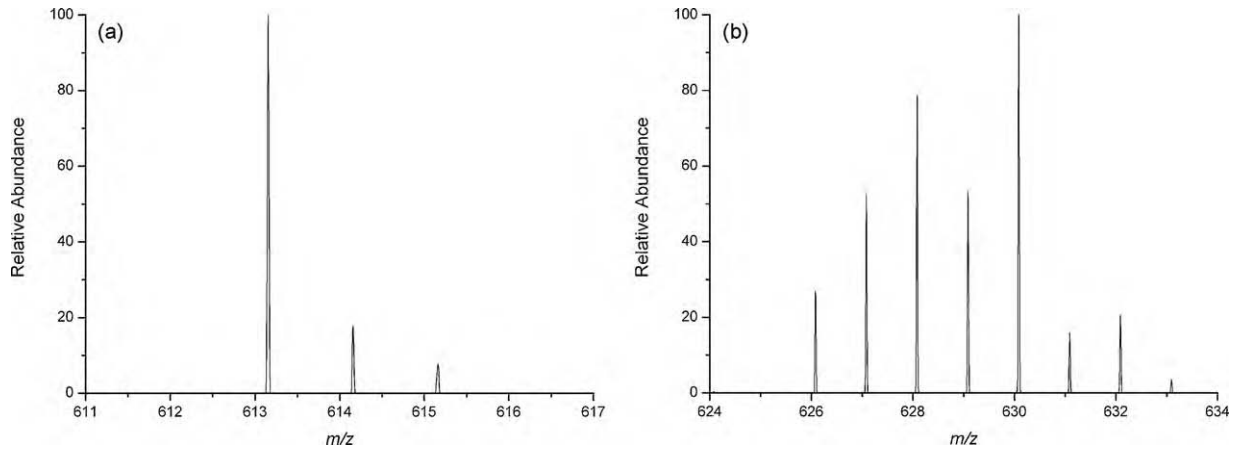

Fig. 3. Mass spectra of GSSG (a) and GS–pHMB (b) using the Orbitrap at resolution 30,000.

between pH 12 and 14. GS–pHMB concentration values were calculated on the basis of the area of 12.8 min peak and sensitivity factor of GS–pHMB calibration curve. Experiments were performed with or without EDTA in the medium in order to evaluate a possible catalytic effect of adventitious metals.

For pH ≤ 8 no signal was observed. For pH > 8 the GS–pHMB signal increased and reached a *plateau* for pH > 12. The *plateau* value ranged between 77 and 95% (inter-day variability: 83 ± 6% mean value ± SD, $N = 12$ experiments performed during 12 different days) of expected GS–pHMB concentration considering that 1 mole of GSSG gives 2 moles of GS–pHMB. The *plateau* value was reached in 5 min and GS–pHMB concentration did not change for 3 h (investigated time). The presence of EDTA in the reaction medium slightly shifted the curve toward higher pH by about 0.5–1 pH unit, indicating that adventitious metals actually catalyze the reactions (1) and (2).

The reaction of 5 μM GSSG was studied as a function of pHMB/GSSG molar ratio (30 min reaction time at 21 °C) and temperature (30 min reaction time, 2:1 pHMB/GSSG molar ratio). We found that for molar ratios <1 GS–pHMB signal linearly increased up to 85.1 ± 1.5% of the expected value for the complete derivatization of 5 μM GSSG (data not shown for brevity). The first linear part of the curve showed an intersection point with the *plateau value* at pHMB/GSSG molar ratio = 1.5 ± 0.09, which corresponded to a 3:2 stoichiometric ratio, in agreement with literature data [18].

We performed calibration curves of GSSG obtained by reaction of GSSG with pHMB in 0.1 M NaOH at different pHMB/GSSG molar ratio (2:1, 4:1, 10:1, 20:1, and 50:1) followed by RPC–CVGAFS analysis. The concatenate fitting of data gave a slope of 0.229 ± 0.003 ($R^2 = 0.9947$), which corresponds to 92 ± 9% of the double of the slope of GS–pHMB calibration curve (0.124 ± 0.001, $N = 5$, $R^2 = 0.9996$). This result indicates that (i) the yield of GSSG derivatization by pHMB in alkaline medium is 92 ± 9%, in agreement with the data obtained at different pHMB/GSSG molar ratio and (ii) the dynamic range for GSSG determination is linear in the investigated range 0.08–50 μM (injected concentration). The yield of the reaction did not change in the 20–37 °C range investigated (data not shown for brevity).

The advantage of the proposed method is its ability in the determination of GSSG with good sensitivity and without the need of thiolic or not thiolic reducing agents to give reduced–SH groups. The detection limits in RPC–CVGAFS were 70 nM for GSH and 35 nM for GSSG (injected concentration of GSH and GSSG in buffer), based on a signal-to-noise ratio of 3. GSH and GSSG species were detected as GS–pHMB complex.

Inter- and intra-day precision calculated as CV% were determined for standards of GSSG in buffer. Intra-day CV% was 5%. It was calculated by performing $N = 5$ replicate measurements at 1 μM GSSG concentration level. Inter-day CV% was 7%, calculated on $N = 12$ experiments performed during 12 different days.

ESI-MS experiments using Orbitrap at 30,000 resolution confirmed that the product of GSSG/pHMB reaction at basic pH is GS–pHMB complex. Fig. 3 shows mass spectra of GSSG (A) and GS–pHMB (B).

The method for the identification of GS–pHMB complex has been described elsewhere [28]. Typically the mass spectral profile of GSH reacted pHMB was identical to that of GSSG reacted with pHMB in a basic environment.

The molecular formula of GS–pHMB is $C_{17}H_{22}N_3O_8HgS$ ($m/z = 630$), and the molecular formula of GSSG is $C_{20}H_{33}N_6O_{12}S_2$ ($m/z = 613$). We have typically measured them within 3 ppm of the expected masses.

The reaction of cystine, homocystine and selenocystine with pHMB in 0.1 M NaOH in the operating conditions optimized for GSSG reaction was studied by RPC–CVGAFS.

Table 1 shows the percents of derivatization of disulfides by pHMB in alkaline medium, in neutral conditions and in the presence of chemical reducing agent DTT calculated on the basis of the peak areas.

Cystine and homocystine were not reduced by pHMB when prepared in 0.1 M PBS (pH 7.4) and only 17% homocystine was reduced in NaOH. Meanwhile, only 10% of selenocystine was reduced at neutral pH and nearly half was reduced in NaOH.

RPC–CVGAFS was also used to check the purity of disulfide standards. Disulfide standard solutions of Cys, HCys, GSH and SeCys (at a concentration of 50 μM each) were analysed after pHMB derivatization with or without N-ethylmaleimide (NEM), a blocking agents of –SH groups [29], in the reaction medium at pH 7.4. Not significant difference between intensity of RS–pHMB peaks with and without NEM were found, indicating that the percentage of free thiols in disulfides standard solutions was below the instrumental detection limit.

Table 1
Percentage of derivatization of disulfides by pHMB in neutral and alkaline solutions, and in the presence of DTT.

	Percentage of derivatization[a]		
	In 0.1 M PBS pH 7.4	In 0.1 M NaOH	In 0.5 mM DTT
Cystine	0	0	62 ± 6[b]
Homocystine	0	17 ± 2[b]	66 ± 7[b]
GSSG	0	77 ± 6[b]	99 ± 5[b]
Selenocystine	10 ± 1[b]	55 ± 6[b]	36 ± 4[b]

[a] RPC–CVGAFS operating conditions: pHMB/RSSR 2:1, 30 min, 21 °C.
[b] SD, $N = 3$ replicates.

Table 2
Values[a] of GSSG (by pHMB direct derivatization and after DTT reduction), GSH, (GSH + GSSG) (obtained after DTT reduction) and GSSG % in TCA-deproteinized blood samples.

Sample	$GSSG_{pHMB}$ (μM)	$GSSG_{DTT}$ (μM)	GSH (μM)	$(GSH+GSSG)_{DTT}$ (μM)	% GSSG
1[b]	94 ± 9[c]	102	625	829	14
2	184	181	880	1243	17
3	187	200	473	873	28
4	253	257	947	1461	21
5	205	206	1167	1578	15
6	118	147	849	1143	12
7	116	120	821	1060	12
8	115	121	651	893	15
9	196	172	990	1335	17
10	110	127	684	939	14
11	92	118	624	860	13

[a] Triplicate analysis was performed for each analysis. CV% ranged between 5 and 7%.
[b] Sample analysed by analyte addition techniques.
[c] Calculated on the basis of intercept = 0.182 ± 0.017, slope = 0.194 ± 0.014, R^2 = 0.9769, N = 4.

3.2. Control of GSH interference

In view of the application of GSSG/pHMB reaction for the analytical determination of GSSG in human blood, we tested NEM and 2-vinylpyridine (2-VP), another blocking agent of –SH groups [26], to eliminate GSH interference. 100 μM GSH in 0.1 M PBS pH 7.4, 0.5 mM EDTA were spiked with 12 mM NEM or 400 mM 2-VP. After 5 min incubation for NEM and 30 min for 2-VP the solutions were diluted 1:10 in 0.1 M NaOH and 100 μM pHMB and, after 30 min incubation time at 21 °C, diluted 20 times and injected into the RPC–CVGAFS system. While in the GSH solution treated with NEM, 3% of total GSH was complexed by pHMB, the GSH solution treated with 2-VP did not give any signal, showing that 2-VP is a good blocking agent also at basic pHs.

3.3. Analytical application: determination of GSSG, GSH and DDT-reduced GSH (GSH + GSSG) in human blood

GSSG determination method based on pHMB derivatization in alkaline medium was applied to acid deproteinized whole blood. The recovery of GSSG was 85 ± 7%, calculated on the basis of the slope of standard addition curve and GSSG calibration curve. For GSSG calibration, standard solutions were treated as blood samples, i.e. diluted in 0.5 M NaOH, treated with 100 μM pHMB (30 min at 21 °C), diluted 1:10 in 1 M PBS pH 7.4 and injected.

The method was validate by determining GSSG in 11 sample of human whole blood by direct derivatization with pHMB in alkaline medium ($GSSG_{pHMB}$) and by pHMB derivatization after chemical reduction with DTT ($GSSG_{DTT}$) (reduction yield 90 ± 5%). $GSSG_{DTT}$ value was calculated by subtracting GSH value from (GSH + GSSG) value obtained after DTT reduction, and dividing by 2. The correlation plot of the results obtained by the two methods in TCA-deproteinized blood samples (Fig. 4) gave a slope of 0.96 ± 0.027 with R^2 = 0.9911. Table 2 summarizes the values of GSSG (by pHMB direct derivatization and after DTT reduction), GSH, (GSH + GSSG) (obtained after DTT reduction) and GSSG % (GSSG % = 100 × [GSSG]/([GSH] + [GSSG])).

The GSH and GSSG concentrations (Table 2) were in agreement with the range of GSH and GSSG concentrations in human plasma [30].

Inter- and intra-day precision (CV%) in TCA-deproteinized blood sample was 6 and 8%, respectively. These data are based on triplicate assays on each of 3 days of dilutions of TCA-deproteinized blood sample as described in the text.

The reactivity of pHMB with other oxidized species of GSH was not investigated in this report. Indeed, while it is known that Cys residues in the proteins can be oxidized to sulfenic (SOH) or sulfinic acids (SO_2H) species [31], currently no reports have been published on the existence *in vivo* of GSH as stable sulfenic or sulfinic species. Nitrosothiols (RSNOs) could also react with pHMB at basic pH. However, due to their low stability at basic pH, this method is not recommended. Suitable blood sampling conditions are also required to preserve the stability of RSNOs in plasma sample [24]. Therefore, we can assess that the method proposed is specific for RSSR species.

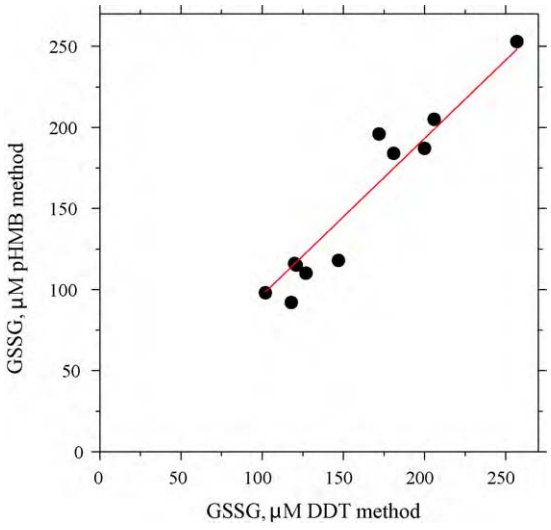

Fig. 4. Correlation plot of GSSG determined in human whole blood by direct derivatization with pHMB in alkaline medium and by pHMB derivatization after chemical reduction with DTT (slope = 0.96 ± 0.027, R^2 = 0.9911, N = 11).

4. Conclusions

GSSG can be directly derivatized by pHMB in strong alkaline medium with a 3:2 pHMB/GSSG stoichiometric ratio and the GS–pHMB complex determined by RPC–CVGAFS. For pH > 12 (typically in 0.1 M NaOH) GSSG was converted into GS–pHMB with a yield of 83 ± 6% in a reaction time of 5 min and GS–pHMB concentration did not change for 3 h (maximum investigated time). The detection limits of 35 nM GSSG (injected concentration, 1.8 pmol injected amount) in buffer standard solution and TCA-deproteinized blood samples derivatized and diluted as reported were achieved based on a signal-to-noise ratio of 3. The dynamic linear range was 0.08–50 μM and the coefficient of variation was 6.5% at 0.3 μM concentration level. The proposed method was applied to the determination of GSSG in whole blood deproteinized by TCA. Recovery of GSSG spiked to whole blood was 85 ± 7%, based on the analyte addition technique. GSSG determination by pHMB was validated by the classical determination of GSSG after DTT reduction. The correlation plot of the results obtained by the

two methods in TCA-deproteinized blood samples gave a slope of 0.96 ± 0.027 with $R^2 = 0.9911$.

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References

[1] H. Sies, Free Radical Biol. Med. 27 (1999) 916–921.
[2] W. Maret, Proc. Natl. Acad. Sci. U.S.A. 91 (1994) 237–241.
[3] L.J. Jiang, W. Maret, B.L. Vallee, Proc. Natl. Acad. Sci. U.S.A. 95 (1998) 3483–3488.
[4] A. Meister, M.E. Anderson, Annu. Rev. Biochem. 52 (1983) 711–760.
[5] A. Pastore, G. Federici, E. Bertini, F. Piemonte, Clin. Chim. Acta 333 (2003) 19–39.
[6] P. Monostori, G. Wittmann, E. Karg, S. Túri, J. Chromatogr. B 877 (2009) 3331–3346.
[7] E. Bramanti, C. Vecoli, D. Neglia, M.P. Pellegrini, G. Raspi, R. Barsacchi, Clin. Chem. 51 (2005) 1007–1013.
[8] K. Kusmierek, G. Chwatko, R. Głowacki, E. Bald, J. Chromatogr. B 877 (2009) 3300–3308.
[9] E. Bald, G. Chwatko, R. Głowacki, K. Kusmierek, J. Chromatogr. A 1032 (2004) 109–115.
[10] A. Safavi, N. Maleki, E. Farjami, F.A. Mahyari, Anal. Chem. 81 (2009) 7538–7543.
[11] W. Zhang, F.L. Wan, W. Zhu, H. Xu, X. Ye, R. Cheng, L.-T. Jin, J. Chromatogr. B 818 (2005) 227–232.
[12] T. Vovk, M. Bogataj, R. Roskar, V. Kmetec, A. Mrhar, Int. J. Pharm. 291 (2005) 161–169.
[13] E. Camera, M. Rinaldi, S. Briganti, M. Picardo, S. Fanali, J. Chromatogr. B 757 (2001) 69–78.
[14] R.L. Norris, G.K. Eaglesham, G.R. Shaw, M.J. Smith, R.K. Chiswell, A.A. Seawright, M.R. Moore, J. Chromatogr. B 762 (2001) 17–23.
[15] A. Schoberl, P. Rambacher, Chem. 538 (1939) 84.
[16] W. Stricks, M. Kolthoff, Anal. Chem. 25 (1953) 1050.
[17] F. Karush, N.R. Klinman, R. Marks, Anal. Biochem. 9 (1964) 100–114.
[18] L.-O. Andersson, G. Berg, Biochim. Biophys. Acta 192 (1969) 534–536.
[19] G. Nöhammer, G. Desoye, Histochem. Cell Biol. 107 (1997) 383–390.
[20] E. Bramanti, S. Lucchesini, A. D'Ulivo, L. Lampugnani, R. Zamboni, M. Spinetti, G. Raspi, J. Anal. Atom. Spectrom. 16 (2001) 166–171.
[21] E. Bramanti, C. Sortino, C. Lomonte, M. Onor, R. Zamboni, G. Raspi, A. D'Ulivo, Talanta 63 (2004) 383–389.
[22] E. Bramanti, C. Lomonte, M. Onor, R. Zamboni, G. Raspi, A. D'Ulivo, Anal. Bioanal. Chem. 380 (2004) 310–318.
[23] E. Bramanti, C. Lomonte, A. Galli, M. Onor, R. Zamboni, G. Raspi, A. D'Ulivo, J. Chromatog. A 1054 (2004) 285–291.
[24] E. Bramanti, K. Jacovozzi, L. D'Ulivo, C. Vecoli, R. Zamboni, Z. Mester, A. D'Ulivo, Talanta 77 (2008) 684–694.
[25] E. Bramanti, V. Angeli, Z. Mester, A. D'Ulivo, Talanta 79 (2009) 554–555.
[26] O.W. Griffith, Anal. Biochem. 106 (1980) 207–212.
[27] A. D'Ulivo, E. Bramanti, L. Lampugnani, R. Zamboni, Spectrochim. Acta B 56 (2001) 1893–1907.
[28] Y. Rao, B. Xiang, E. Bramanti, A. D'Ulivo, Z. Mester, J. Agric. Food Chem. 58 (2010) 1462–1468.
[29] J.D. Gregory, J. Am. Chem. Soc. 77 (1955) 3922–3923.
[30] E. Camera, M. Picardo, J. Chromatogr. B 781 (2002) 181–206.
[31] C.E. Paulsen, K.S. Carroll, Chem. Biol. 5 (2010) 47–62.