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Quantitation of glutathione and its oxidation products in erythrocytes by multiple label stable isotope dilution

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ABSTRACT

A multiple-label stable isotope dilution assay for quantifying glutathione, glutathione disulfide and glutathione sulfonic acid in erythrocytes was developed. As the internal standards, [$^{13}C_3$,^{15}N]-glutathione, [$^{13}C_4$,$^{15}N_2$]-glutathione disulfide and [$^{13}C_3$,^{15}N]-glutathione sulfonic acid were used. Analytes and internal standards were detected by LC-MS/MS after derivatization of GSH with iodoacetic acid and dansylation of all compounds under study. The calibration functions for all analytes relative to their respective isotopologic standards revealed slopes close to 1.0 and negligible intercepts. As different labelings of the standards for GSH and GSSG were used, their simultaneous quantitation was possible although GSH was partly oxidized to its disulfide during analysis. The degree of this artifact formation of GSSG was calculated from the abundance of the mixed disulfide formed from unlabeled GSH and its respective standard. Thus, the detected GSSG amount could be corrected for the artifact amount. In this way, the amount of GSSG in erythrocytes was found to be less than 0.5 % of the GSH concentration. Similarly to GSSG, the detected amount of glutathione sulfonic acid was found to be formed at least in part during the analytical process, but the degree could not be quantified.

Key words: Artifact monitoring; Erythrocytes; Glutathione; Glutathione disulfide; Glutathione sulfonic acid; LC-MS/MS; Multiple isotope labeling; Stable isotope dilution assay

ABBREVIATIONS

CID collision-induced dissociation

Dansyl, 1-diaminonaphthalenesulfonyl

Dans-Cl, 1-diaminonaphthalenesulfonyl chloride

DCM, dichloromethane

ESI, electrospray ionization

GSH, glutathione

GSSG, glutathione disulfide

GSO_3H, glutathione sulfonic acid

GSH *M*+*n*, isotopologue of glutathione showing a mass increment of +*n* u compared to the mass of the GSH isotopologue consisting solely of [^{12}C], [^{1}H], [^{16}O], [^{14}N], and [^{32}S]

GSSG *M*+*n*, isotopologue of glutathione disulfide showing a mass increment of +*n* u compared to the mass of the GSSG isotopologue consisting solely of [^{12}C], [^{1}H], [^{16}O], [^{14}N], and [^{32}S]

GSO_3H *M*+*n*, isotopologue of glutathione sulfonic acid showing a mass increment of +*n* u compared to the mass of the GSO_3H isotopologue consisting solely of [^{12}C], [^{1}H], [^{16}O], [^{14}N], and [^{32}S]

HPLC-UV, high pressure liquid chromatography-ultraviolet spectrometry

IAA, iodoacetic acid

LC-MS/MS, liquid chromatography-tandem mass spectrometry

NEM, N-ethylmaleimide

PCA, perchloric acid

SD, standard deviation

SIDA, stable isotope dilution assay

INTRODUCTION

The tripeptide glutathione (γ-glutamyl-cysteinyl-glycine, GSH) plays a central role in physiology for (1) maintaining the redox status of cells along with its oxidized form GSSG, for (2) conjugation of toxic compounds, and for (3) acting as coenzyme for many enzymes such as glutathione peroxidase or glutathione dehydrogenase (ascorbate) with the latter producing ascorbate from dehydroascorbate as another coenzyme. Moreover, GSH is considered to be partly the sulfur reserve in plant seeds such as wheat kernels. Due to its vital importance for animals, GSH status in tissues is strongly regulated and a decrease in GSH has been associated with wide-spread diseases such as diabetes, cancer, AIDS or neurodegenerative disorders [1]. However, as functional tissue is hardly accessible to be analyzed, a straightforward alternative to tissue sampling is the GSH analysis of blood, which has been confirmed to reflect the status of other tissues [2-4]. Therefore, GSH quantitation in blood is very meaningful in clinical diagnosis and investigations of many diseases. In mammals, the main portion of GSH in blood circulation is located in erythrocytes. However, the percentage of GSH being present in plasma is in dispute due to differing results of several analytical studies.

The first analytical assays were based on the reaction catalyzed by glutathione reductase [5], but they often were restricted to measure the sum of oxidized and reduced GSH. Differentiation of glutathione forms required derivatization of the thiol group, which interfered with the enzyme reaction [6]. Therefore, chromatographic methods were developed with different approaches to prevent the thiol group from being oxidized. These reagents included N-ethylmaleimide (NEM) [7], iodoacetic acid (IAA) [8], 5-iodoacetamidfluorescein [9], phthalimide [10] and dithionitrobenzoate [11].

Determination of GSH and GSSG in different tissues recently has been the aim of several studies applying LC-MS. These investigations included either methods using stable isotope-labeled internal standards for dermal cells [12] and blood [13] or those using labeled internal standards in different cells [14] and the yeast Pichia pastoris [15].

However, in blood GSH oxidation can occur already during or directly after sampling, which requires careful sample preparation. Immediate cooling has to be followed by erythrocyte separation as plasma proteins have been shown to oxidize GSH [11]. A further important cleanup step is deproteinization, which may be achieved by treatment with 5-sulfosalicylic acid [7], trichloroacetic acid [11], meta-phosphoric acid [10], acetonitrile [9] or ultrafiltration [16]. Following these different procedures, erythrocytes were found to contain GSH in a range between 950 and 2440 µmol/L [9, 17]. However, the GSSG concentrations of red blood cells encompassed a significantly lower range between 3.6 and 190 µmol/L [10, 18].

Recently we developed a stable isotope dilution assay (SIDA) for accurate quantitation of total GSH in cereals [19] with the use of L-γ-glutamyl-L-[$^{13}C_3$,^{15}N]-cysteinyl-glycine as the internal standard. The method consisted of the extraction and reduction of flour with tris(2-carboxyethyl) phosphine after the addition of the internal standard, followed by protection of free thiol groups with iodoacetic acid, derivatization of free amino acids with dansyl chloride and LC-MS/MS. Therefore, the goal of the present study was to adjust this assay also to the quantitation of GSH in blood.

MATERIALS AND METHODS

Reagents

Acetonitrile Lichrosolv, formic acid (purity of 98-100%), methanol Lichrosolv, dichloromethane (distilled; DCM), glutathione (reduced), glutathione disulfide , hydrogen peroxide, lithium hydroxide, and sodium chloride were obtained from Merck, Darmstadt, Germany. Boric acid was purchased from Serva, Heidelberg, Germany. Iodoacetic acid (IAA), and perchloric acid (PCA) were obtained from Fluka, Steinheim, Germany and 1-diaminonaphthalenesulfonyl chloride (dansyl chloride; Dans-Cl) was purchased from Sigma Aldrich, Steinheim, Germany. All reagents were of p.a. or higher grade. All standard solutions and aqueous solvents were prepared with water purified by a Milli-Q system (Millipore GmbH, Schwalbach, Germany).

Standard substances

γ-Glutamyl-[$^{13}C_3$,^{15}N]-cysteinyl-glycine, -[$^{13}C_3$,^{15}N]-glutathione (GSH *M+4*; reduced, isotopic purity 90%) and γ-Glutamyl-cysteinyl-[$^{13}C_2$,^{15}N]-glycine disulfide, [$^{13}C_4$,$^{15}N_2$]-glutathione disulfide (GSSG *M+6*, isotopic purity 85 %) were prepared (chemical purity of both exceeding 90 %) and characterized as described previously [19]. Glutathione sulfonic acid (unlabeled) was purchased from Sigma Aldrich, Steinheim, Germany.

Erythrocytes

Whole blood from healthy volunteers was collected in heparinized tubes (Vacuette, Greiner bio-one, Kremsmünster, Austria). Immediately after collecting, erythrocytes were separated from plasma by centrifugation (15 min, 4 °C, about 2000 *g*). The plasma supernatant was removed; erythrocytes were washed with 0.9 % NaCl solution and centrifuged again. The supernatant was removed and the procedure

was repeated for another one to two times until the supernatant was clear. The resulting erythrocytes were analyzed immediately or stored at -80 °C until analysis.

Model Solutions

Three model solutions were prepared to evaluate isotopologic effects and detector response. For that purpose, solutions of GSH and GSH *M+4* (about 100 µg/mL each) were mixed 2:1 (by volume, mixture 1) and 1:1 (by volume, in duplicate: mixtures 2A and 2B).

Synthesis of [$^{13}C_3$,^{15}N]-glutathione sulfonic acid

Synthesis of glutathione sulfonic acid [$^{13}C_3$,^{15}N]-labeled in the cysteine moiety (GSO_3H *M+4*) was performed according to [20]. Performic acid was prepared freshly before use by mixing 200 µL hydrogen peroxide (30 %, w/w) with 1.8 mL formic acid (99 %) and incubating the mixture for 1 h at room temperature. Subsequently, 200 µL methanol were added and the obtained solution was stored at -20°C until usage.
For the oxidation of the standard solution, γ-Glutamyl-[$^{13}C_3$,^{15}N]-cysteinyl-glycine (0.5 mL, 1 mg/mL) was lyophilized and treated with 200 µL of freshly prepared performic acid. The reaction mixture was incubated for 2.5 h at -10 °C, then diluted with 1 mL water and lyophilized. The reaction product was dissolved in 1 mL of 0.1 % formic acid and evaluated by HPLC-UV and LC-MS. Concentration of the obtained solution was determined by means of HPLC-UV (210 nm) and calculated from an external calibration curve obtained when injecting unlabeled GSO_3H.
According to this procedure, 0.42 mg (0.001 mmol) [$^{13}C_3$,^{15}N]-glutathione sulfonic acid with an isotopic purity of 90 % and a chemical purity exceeding 90 % was obtained.

LC-MS (ESI^+): *m/z* (%): 360 (100), 382 (70), 325 (14), 303 (12), 404 (6)

172

173 **Model experiments**

174 To evaluate a possible discrimination of one individual GSSG isotopologue or to test
175 if response curves for isotopologic didansylated GSSG are comparable, model
176 solutions 1, 2A and 2B were prepared as described above and 10 µL (model 1) or 20
177 µL (model 2A and 2B) of model solutions were partially oxidized over night for about
178 12 h at 45 °C with 300 µL boric acid/LiOH buffer (pH 8.5) that has been saturated
179 with oxygen. To stop the reaction, 25 µL IAA (1 mol/L) were added to the reaction
180 mixtures and solutions were stirred for 30 min in the dark. Subsequently 500 µL of
181 Dans-Cl (7.4 mmol/L in acetonitrile) were added to dansylate the free amino-groups
182 before detecting the derivates by LC-MS/MS.

183

184 **Sample preparation**

185 100 µL erythrocytes were transferred to a cooled 2 mL Eppendorf cap by means of a
186 multipette (Eppendorf, Wessling-Berzdorf, Germany). Subsequently, different
187 amounts (1 – 20 nmol) of isotopically labeled standards in PCA (5 %) were added.
188 Proteins were precipitated by adding 150 – 180 µL ice cold PCA (5 %), while
189 analytes remained in solution. Immediately after addition of PCA, caps were shaken
190 on a vortex mixer for 10 s to prevent agglutination. Proteins were separated by
191 centrifugation (14000 *g*, 14 min, 0 °C) and the supernatant was treated with 450 µL
192 IAA (0.1 mol/L) in a boric acid/LiOH buffer (0.5 mol/L, pH 8.5)/LiOH (1 mol/L) (2/1,
193 v/v) for 30 min at room temperature. By adding 500 µL Dans-Cl (7.4 mmol/L in
194 acetonitrile) free amino groups of carboxymethyl thiols, disulfides and glutathione
195 sulfonic acid were acylated within 1 h at room temperature. To stop the reaction,
196 dichloromethane was added to the reaction mixture, mixed well and centrifuged

(16000 *g*, 10 min, 20 °C). The aqueous supernatant was filtered (0.45 µm, Schleicher & Schuell, Dassel, Germany) and analyzed by LC-MS/MS.

LC-MS

An ion-trap mass spectrometer HCT ultra (Bruker Daltonics, Bremen, Germany) coupled with a Dionex Ultimate 3000 HPLC System (Dionex, Idstein, Germany) was used for characterization of isotopologic glutathione sulfonic acid by HPLC-UV/MS. As the stationary phase, a TSKgel Amide-80 column (2.0 x 150 mm, 5 µm particle size, 8 nm pores, Tosoh Bioscience, Stuttgart, Germany) was used. The gradient was run from 100 % acetonitrile (with 0.1 % formic acid) to 100 % water (with 0.1 % formic acid) within 20 min. The UV detector was set to 210 nm and the ion source was operated in the positive electrospray ionization (ESI^+) mode. The MS detection was run in the Ultra Scan mode, drying temperature, nebulizer, and drying gas were set to 350 °C, 35 psi and 8 L/min, respectively.

LC-MS/MS

A triple quadrupole mass spectrometer Finnigan TSQ Quantum Discovery coupled with a Finnigan Surveyor Plus HPLC System (Thermo Electron Corporation, Waltham, USA) was used for LC-MS/MS analysis. The stationary phase was a Synergi HydroRP C_{18} column (2.0 x 150 mm, 4 µm particle size, 8 nm pores; Phenomenex, Aschaffenburg, Germany), which was equipped with a C_{18} guard column (Phenomenex). For separation of GSH and GSSG derivatives, gradient elution (flow rate: 0.2 mL/min) was employed with aqueous 0.1% formic acid (solvent A) and 0.1% formic acid in acetonitrile (solvent B). Initial conditions were 100% A and were raised to 100% B within 25 min. The gradient mixture was maintained at this composition for 5 min and then returned back to initial conditions within 1 min. Before

each injection, the column was equilibrated for 15 min. Injection was carried out at the full loop mode and injection volume was 10 µL. The LC eluate from 0 to 13 min and from 21 min to the end of gradient was directed into waste. The effluent between 13 and 21 min was introduced into the mass spectrometer, which was operated in the ESI^+ mode with a spray needle voltage of 3.7 kV. The temperature of the capillary was 300 °C, and the capillary offset was set to 35 V. The sheath and auxiliary gas were adjusted to 35 and 10 arbitrary units, respectively. The collision cell was operated at a collision gas (argon) pressure of 6.7×10^{-2} Pa and source CID (collision-induced dissociation) was used with the collision energy set at 12 V. On both mass filter quadrupoles, the peak width was adjusted to 0.7 full width at half maximum, the scan time for each transition was 0.2 s, and the scan width was 0.7 amu. During method development, the unlabeled derivatized compounds were subjected to LC-MS/MS recording full scans of the products to find the two most intense and specific product ions for selected reaction monitoring (SRM). To determine GSH, GSSG and GSO_3H in erythrocytes within one run, different collision energies in quadrupole 2 were tested using SRM mode to obtain signals for GSSG and GSO_3H with maximum intensity and to obtain signals of GSH within the linear range of the detector. The results of the optimization are summarized in Table 1.

Spiking experiments with GSH

To evaluate GSSG formation during sample preparation, spiking experiments were performed. In the first set of experiments, different amounts of GSH (in 5 % PCA) were added to erythrocytes, before preparing them for analysis as described above. To 100 µL of a mixture of erythrocytes from different volunteers, 5 – 35 µL of a GSH solution (1.0 mg/mL in 5 % PCA) were added. After addition of GSSG *M+6* (5 µL, 2.2 mg/mL) and GSH *M+4* (20 µL, 1.1 mg/mL), proteins were precipitated by the addition

of 140 – 170 µL ice-cold PCA (5 %). Subsequently, the mixture was centrifuged (14000 g, 14 min, 0 °C) and the supernatant was treated with IAA and Dans-Cl as described above and analyzed by LC-MS/MS.

In the second series of tests, higher levels of GSH (in 5 % PCA) were added than in the first experiment. Furthermore in this attempt, GSH (20 µL, 1.7 – 4.5 mg/mL) and labeled standard substances GSSG *M+6* (5 µL, 2.2 mg/mL) and GSH *M+4* (20 µL, 1.3 mg/mL) were premixed with ice-cold PCA (5 %, 155 µL) and stored cool until the addition of 100 µL of erythrocytes. After centrifugation, the supernatant was derivatized and analyzed as described above.

Standardization

For determination of response factors, solutions of unlabeled and labeled GSH, GSSG and GSO_3H, respectively, in 0.1% formic acid were mixed in seven molar ratios between 0.1 and 9. The derivatization procedure was performed as described above. After LC-MS/MS analysis, calibration curves of area ratios in relation to molar ratios were obtained. From the molar ratios, the added amounts of labeled standards, and the weighted samples, the molar concentrations of the analytes in the samples were calculated (Table 2).

Calculation of glutathione disulfide in erythrocytes formed during analysis

Concentration of GSSG M+4 was calculated from the following equation curve:

$$c(A) = \left(m \cdot \frac{A(A)}{A(S)} + t \right) \cdot c(S)$$

c(A) = Concentration of analyte in erythrocytes
c(S) = Concentration of internal standard in erythrocytes
A(A) = Area analyte m/z 1083 →1008
A(S) = Area internal standard m/z 1085 →1007
m = slope of response curve for didans GSSG

M+6 (table 1)

t = y intercept of response curve didans GSSG *M+6* (table 1)

A statistical distribution of GSSG isotopologues formed from GSH and GSH *M+4* was assumed to calculate their abundances P from the following equations:

P(GSH) + P(GSH *M+4*) = 100 % results in GSSG distributions:

$P(GSSG) = P(GSH)^2$

P(GSSG *M+4*) = 2 * P(GSH) * P(GSH *M+4*);

$P(GSSG\ M+8) = P(GSH\ M+4)^2$

Hence, a factor f_t for the theoretical distribution of $GSSG_{formed}$ / GSSG *M+4* was calculated from areas of GSH and GSH *M+4* in the characteristic mass traces according to the following equation:

$f_t = GSSG_{formed}$ / GSSG *M+4* = A(GSH) / 2 * A(GSH *M+4*)

Glutathione disulfide formed during analysis ($GSSG_{formed}$) was calculated then by multiplication of the GSSG *M+4* concentration with the factor f_t of the theoretical distribution of GSSG / GSSG *M+4*.

Method validation

As there was no material available which was similar to erythrocyte matrix and did not contain the analyte GSH, the limit of detection (LOD) was derived from LOD calculated for cereal flour [19] considering the different sample dilution.

Intra-day precison was evaluated by measuring GSH, GSSG and GSO_3H in the erythrocytes of one volunteer in triplicate.

For measuring the recovery, erythrocytes were spiked (each in triplicate) with three different amounts of GSH and analyzed by SIDA.

295

RESULTS AND DISCUSSION

Quantitation of glutathione by stable isotope dilution assays

The glutathione present in erythrocytes is susceptible to degradation and oxidation due to the occurrence of hemoglobin, iron, and various enzymes. Therefore, even careful sample preparation under exclusion of oxygen is not likely to prevent oxidation of GSH. For similar challenging analytes such as folates [21] or thiol containing odorants [22], SIDAs have proven their superiority over other alternatives. Therefore, we decided also to use this methodology for quantitation of GSH in erythrocytes by application of [$^{13}C_3$,^{15}N]-GSH labeled in the cysteine moiety. This standard already has been used for quantitation of total GSH in cereals after derivatization with IAA and dansylation [19]. In the latter study we reported that IAA was preferred as thiol protection agent over NEM as the former does not react with amino groups. Nevertheless, in a recent study the suitability of NEM for analyzing GSH in blood was shown [23]. In our SIDA for cereals we dansylated the analytes to enhance retention in HPLC and sensitivity in LC-MS. However, [$^{13}C_3$,^{15}N]-GSH would not allow for simultaneous quantitation of GSSG as the amount of GSH isotopologues oxidized to GSSG would be unknown. Moreover, we also observed reduction of GSSG to GSH upon some extraction conditions, which would not be recognized when using identically labeled GSH and GSSG as applied in the studies of Haberhauer-Troyer et al. [15] and Harwood et al. [14]. Therefore, we decided to use a label for GSSG that can be distinguished from the label resulting from the oxidized labeled GSH standard. In contrast to this, the otherstrategy for a SIDA recently was reported by Haberhauer-Troyer et al. [15], who used the same label for GSH and GSSG, but calculated the degree of oxidized GSH by monitoring the mixed disulfide formed from unlabeled GSH and the labeled GSH internal standard.

However, this approach would not allow to quantify simultaneously GSH and GSSG and to differentiate oxidation of GSH from reduction of GSSG.

Taking these considerations into account, a differently, six-fold labeled GSSG generated from [$^{13}C_2$,^{15}N]-GSH labeled in the glycine moiety was chosen. Taking into account possible oxidation as well as reduction or thiol/disulfide exchange of the GSH isotopologues, for reduced GSH the occurrence of three isotopologues (unlabeled GSH *M+0*; [$^{13}C_2$,^{15}N]-GSH *M+3*; [$^{13}C_3$,^{15}N]-GSH *M+4*) and for GSSG the occurrence of six isotopologues (unlabeled GSSG *M+0*; [$^{13}C_2$,^{15}N]-GSSG *M+3*; [$^{13}C_3$,^{15}N]-GSSG *M+4*; [$^{13}C_4$,$^{15}N_2$]-GSSG *M+6*; [$^{13}C_5$,$^{15}N_2$]-GSSG *M+7*; [$^{13}C_6$,$^{15}N_2$]-GSSG *M+8*) was conceivable. Therefore, we tested in preliminary experiments whether the isotopologues could be differentiated by LC-MS/MS. For GSH, the fragmentation of the carboxymethyl-dansyl GSH resulted mainly in two products, (1) upon loss of 2-aminoacetic acid and (2) of 2-carbonylaminoacetic acid, both from the C-terminus (Fig. 1). The isotopologues shown in Fig. 1 reveal a mass difference of at least 3 u either in the precursor or the product ions.

For didansylated GSSG, MS/MS fragmentation revealed the most intense fragment by loss of one glycine as depicted in Fig. 2. However, differentiation of the isotopologues is more difficult than for GSH as the mass differences between the possible isotopologues are smaller. Moreover, due to the higher number of atoms present in the dansylated GSSG molecule, natural isotopes of carbon, sulfur, oxygen and nitrogen are likely to cause interferences between the single isotopologues. When reacting the single GSH isotopologues GSH *M+0*, [$^{13}C_2$,^{15}N]-GSH *M+3*, and [$^{13}C_3$,^{15}N]-GSH *M+4* in model experiments separately to mixed GSSG isotopologues, in particular significant interferences for the GSSG isotopologues [$^{13}C_3$,^{15}N]-GSSG *M+4*, [$^{13}C_5$,$^{15}N_2$]-GSSG *M+7*, and [$^{13}C_6$,$^{15}N_2$]-GSSG *M+8* were observed. As shown in Table 3, the natural isotopologues with an additional mass of 1 u of [$^{13}C_2$,^{15}N]-GSSG

M+3+1, of [$^{13}C_4$,$^{15}N_2$]-GSSG *M+6+1* and of [$^{13}C_5$,$^{15}N_2$]-GSSG *M+7+1* interfered with [$^{13}C_3$,^{15}N]-GSSG *M+4*, [$^{13}C_5$,$^{15}N_2$]-GSSG *M+7*, and [$^{13}C_6$,$^{15}N_2$]-GSSG *M+8*, respectively, each by giving signal abundances of about 50 % of the respective major isotopologue.

This could be particularly crucial for monitoring the GSH oxidation during analysis, as this is reflected by the formation of [$^{13}C_3$,^{15}N]-GSSG *M+4* and [$^{13}C_6$,$^{15}N_2$]-GSSG *M+8* generated by reaction of the labeled standard [$^{13}C_3$,^{15}N]-GSH *M+4* with itself or with unlabeled GSH. Therefore, the respective overlap with natural isotopologues of GSSG *M+3* and GSSG *M+7* has to be considered.

Synthesis and mass spectrometry of glutathione sulfonic acid

GSO_3H has been described as oxidation product from GSH and GSSG when reacted with oxidants such as hydroperoxide [20]. Therefore, blood was also analyzed for this possible oxidation product of GSH.

[$^{13}C_3$,^{15}N]-labeled glutathione sulfonic acid was prepared by oxidation of [$^{13}C_3$,^{15}N]-GSH with performic acid according to Henschen [24]. For quantitation by LC-MS/MS, GSO_3H was dansylated using the same conditions as for GSH and GSSG. As selective transition, the simultaneous loss of glycine and cysteine was chosen. Thus, for the labeled GSO_3H isotopologues, all labels were lost and the product ion at *m/z* 363 is the same for all labeled and unlabeled GSO_3H isotopologues (Figure 3). However, the labeled compounds still could be distinguished from the unlabeled compound by their different precursor ion masses.

Response curves of derivatized GSH, GSSG and GSO_3H isotopologues

To enable calculation of the molar ratio of analyte to the respective internal standard from the area ratios, calibration mixtures of GSH and [$^{13}C_3$,^{15}N]-GSH *M+4* were

carboxymethylated, dansylated, and analyzed by LC-MS/MS. For GSSG and GSO_3H, respective calibration mixtures were measured after dansylation. The response equations A (labeled standard) / A (analyte) = R_F* m(labeled standard)/ m (analyte)+b are given in Table 2. All response curves revealed linearity in ratios between 0.1 and 10, which defined the working range of the developed SIDAs.

Formation and detection of GSSG isotopologues from oxidation of GSH isotopologues or thiol/disulfide exchange

In order to calculate the extent of GSH oxidation from the occurrence of mixed disulfide GSSG *M+4*, model solutions with different ratios of GSH isotopologues were partly oxidized with oxygen and the obtained areas were compared with the theoretical distribution assuming statistical combination and identical MS/MS response of all GSSG isotopologues. The results of the trials using two different mixtures and the second performed in duplicate are listed in Table 4. The difference of the calculated versus the observed distribution was less than 7 %, thus showing no systematic deviation. Therefore, the GSSG formed during analysis could be calculated from the observed GSSG *M+4* area and the observed ratio of GSH and GSH *M+4* that were still present in their reduced form.

Quantitation of glutathione, glutathione disulfide and glutathione sulfonic acid in erythrocytes

To prevent formation of possible artefacts, analysis of erythrocytes started with immediate cooling and centrifugation followed by deproteinization using ice-cold 5 % PCA. Preliminary experiments have shown that the application of the latter acid gave a clearer supernatant in comparison to 10 % meta-phosphoric acid or 10 % sulfosalicylic acid.

The developed assays for GSH, GSSG, and GSO_3H in erythrocytes were applied to a set of six healthy volunteers. The LC-MS/MS chromatograms revealed unambiguous signals of the analytes and their respective standards as shown in Fig. 4.

The contents of GSH, GSSG and GSO_3H were found to range between 1.33 and 2.21, between 0.168 and 0.313 mmol/L and between 13 and 25 µmol/L, respectively. Thus, the GSH content measured in the erythrocytes of our volunteers was well in the reported range between 0.95 and 2.44 mmol/L [9, 17] (see also below).

Quantitation of glutathione disulfide formed during analysis

Interestingly, the molar ratios GSSG/GSH and GSO_3H/GSH for all subjects were within a much smaller range of 12.7 to 15.3 % and 0.6 to 1.2%, respectively. This suggests that significant amounts of GSSG and GSO_3H might have been formed during analysis. For GSSG, this assumption was confirmed by detection of GSSG *M+4* that reflected selectively the amount of $GSSG_{formed}$ from endogenous GSH and its internal standard GSH *M+4* during sample preparation. Upon consideration of equal MS/MS responses of all GSSG isotopologues and a statistical formation of the mixed GSSG from the GSH isotopologues, the respective amounts of $GSSG_{formed}$ were calculated. However, the standard deviation of $GSSG_{formed}$ was very high and in some cases would have exceeded the total GSSG found due to random errors. Thus, an exact quantitation of $GSSG_{formed}$ could not be performed in these trials. This would require isotopologues with a higher degree of labeling to prevent spectral overlap. However, the amounts found for $GSSG_{formed}$ were in the same order of magnitude as total GSSG, which confirmed the assumption that the major part of or all GSSG detected can be assigned to artifact formation during analysis.

In contrast to GSSG, GSH formed from reduction or thiol/disulfide exchange was below 1 % of the total GSH content as was found by measurement of the GSH *M+3* trace. The formation of this isotopologue would have originated from the GSSG *M+6* isotopologue added as standard for GSSG. Therefore, the data found for GSH in erythrocytes were accurate and confirmed the data found in previous publications [9, 17].

Spiking experiments to clarify the amount of glutathione disulfide formed during analysis and to deduce the initial disulfide content in erythrocytes

As $GSSG_{formed}$ could not be quantified from GSSG *M+4* due to imprecision, we decided to quantitate the artefactual GSSG from spike experiments. In a first set of assays, we added three different GSH amounts ranging from 0.171 and 0.684 mmol/L. GSH, total GSSG and $GSSG_{formed}$ were quantified as described before and gave the results shown in Table 5.

For these studies, the ratio between GSSG and GSH was quite similar for all levels of added GSH. This result was the same as in the first trials without addition of GSH and pointed to a constant amount of $GSSG_{formed}$ during analysis. Within these addition experiments, the standard deviation of the calculated $GSSG_{formed}$ was acceptable and allowed to calculate the apparent initial GSSG content from the difference between GSSG measured and $GSSG_{formed}$. Thus the initial GSSG content of around 0.03 - 0.04 mmol/L was found to be only one sixth to one fourth of the total GSSG measured.

In order to verify this result, we performed a second set of experiments with a higher addition level of GSH and a more gentle sample preparation by adding the standards dissolved in the ice-cold protein precipitation agent.

The results shown in Table 6 revealed a lower GSSG content than in the previous experiment (Table 5) and pointed to the significant influence of sample preparation. The apparent GSSG content calculated from the difference of GSSG measured and $GSSG_{formed}$ and finally was found to be as low as 0.010 mmol/L in the sample without addition showing a rather small SD of 0.003 mmol/L. Obviously the GSSG content in erythrocytes is below 0.5 % of the GSH content, which is in agreement with the literature describing a physiological range of the GSSG:GSH ratio between 1:100 and 1:1000 [25].

The generation of GSSG as an artifact during analysis already was described by Srivasta and Beutler [18] and Rossi et al. [26] and quantified by Haberhauer-Troyer et al. [15], who found that 10 to 20 % of the observed GSSG was formed during analysis. This degree depended on the extraction conditions, which is in accordance with our findings. When comparing our results with reports from the literature, the GSSG/GSH ratio in blood ranged from 0.1 % [14] to 10 % [13] and pointed also to the effects of extraction and underlined the need for compensation for artifact formation. Reaction with NEM followed by acidification with TCA [23] recently was found appropriate to prevent from GSSG formation during analysis. However, the SIDA approach allows to monitor GSH oxidation during analysis. The single label approaches performed by Harwood et al. [14] and Haberhauer-Troyer et al. [15] both were suitable as the former authors did not have to consider artifact formation due to application of NEM and cold ethanol. In contrast to this, Haberhauer-Troyer et al. evaluated artifact GSH oxidation by measuring mixed GSSG isotopologues similarly to this study. However, the simultaneous quantitation of GSH and GSSG was not possible as the internal standards contained the same label and the exact amount of GSSG could not be calculated as the total amount of labeled GSSG was unknown. Moreover, the double label approach presented here allows the quantitation of

formed GSSG from all ratios of GSH and its internal standard by simple calculations. Furthermore, our method offers the advantage to discover also a possible reduction of GSSG to form GSH as an artifact.

Quantitation of glutathione sulfonic acid

GSO_3H was quantified analoguously to GSH and GSSG by using [$^{13}C_3$,^{15}N]-GSO_3H as the internal standard. The later was synthesized by oxidation of [$^{13}C_3$,^{15}N]-GSH using performic acid. Unlabeled GSO_3H and its labeled isotopologue revealed clear signals in the blood extracts of the volunteers. The calculation of the amounts in the extract revealed an amount of only around 1% of the respective GSH concentration. Interestingly, this percentage was quite similar for all volunteers studied. In analogy to the GSSG analyzed, we assume that this percentage at least in part is formed by oxidation of GSH and GSSG during analysis as model experiments revealed formation of GSO_3H from both GSH and GSSG at the analytical conditions used. However, a quantitation by considering the fraction formed from both GSH and GSSG would require a third different labeling for GSO_3H, which was not available. Therefore, the occurrence of physiological GSO_3H in blood cannot be excluded and has to be subject to further studies. The formation of another GSH oxidation product, glutathione sulfonamide, recently has only been detected in cells treated with the strong oxidant hypochlorous acid [14]. Therefore, the formation of oxidation products besides GSSG seems not very likely under physiological conditions.

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590 LEGENDS TO THE FIGURES

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592 Fig.1. MS/MS fragmentation of isotopically labeled dansylated carboxymethyl-GSH
593 derivatives; GSH unlabeled, GSH *M+3* and GSH *M+4*.
594 Fig.2. Fragments of isotopically labeled didansylated GSSG derivatives; GSSG
595 unlabeled, GSSG *M+3*, GSSG *M+4*, GSSG *M+6*, GSSG *M+7* and GSSG *M+8*.
596 Fig.3. MS/MS fragmentation of isotopically labeled dansylated GSO_3H derivatives;
597 GSO_3H unlabeled, GSO_3H *M+3* and GSO_3H *M+4*.
598 Fig.4. LC-MS/MS chromatogram of derivatized erythrocytes showing the mass
599 traces of the derivatives of GSO_3H, GSH and GSSG along with their labeled
600 internal standards.

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