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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 (GSH), glutathione disulfide (GSSG), 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 various 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. Similar 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.

The tripeptide glutathione (γ-glutamylcysteinylglycine; GSH)[1] plays a central role in physiology for (1) maintaining the redox status of cells along with its oxidized form GSSG, (2) conjugation of toxic compounds, and (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. Because of its vital importance for animals, GSH status in tissues is strongly regulated and a decrease in GSH has been associated with widespread diseases such as diabetes, cancer, AIDS, and neurodegenerative disorders [1]. However, as functional tissue is hardly accessible to be analyzed, a straightforward alternative to tissue sampling is the analysis of blood GSH, 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 present in plasma is in dispute because of 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 measuring 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 various 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 various cells [14] and the yeast *Pichia pastoris* [15].

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[1] *Abbreviations used:* dansyl, 1-diaminonaphthalenesulfonyl; Dans-Cl, 1-diaminonaphthalenesulfonyl chloride; 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; SIDA, stable isotope dilution assay.

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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]. After 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] cysteinylglycine 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 to the quantitation of GSH in blood.

Materials and methods

Reagents

Acetonitrile Lichrosolv, formic acid (purity 98–100%), methanol Lichrosolv, dichloromethane (distilled), 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). 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, Schwalbach, Germany).

Standard substances

γ-Glutamyl-[$^{13}C_3$,^{15}N]cysteinylglycine([$^{13}C_3$,^{15}N]glutathione; GSH *M*+4; reduced, isotopic purity 90%) and γ-glutamylcysteinyl-[$^{13}C_2$,^{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.

Erythrocytes

Whole blood from healthy volunteers was collected in heparinized tubes (Vacuette; Greiner Bio-One, Kremsmünster, Austria). Immediately after collection, 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 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 fresh 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 was added and the obtained solution was stored at −20 °C until use.

For the oxidation of the standard solution, γ-glutamyl-[$^{13}C_3$,^{15}N]cysteinylglycine (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 and 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 (positive electrospray ionization (ESI^+)): *m*/*z* (%) 360 (100), 382 (70), 325 (14), 303 (12), 404 (6).

Model experiments

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

Sample preparation

One hundred microliters of erythrocytes was transferred to a cooled 2-ml Eppendorf cap by means of a multipette (Eppendorf, Wessling-Berzdorf, Germany). Subsequently, various amounts (1–20 nmol) of isotopically labeled standards in PCA (5%) were added. Proteins were precipitated by adding 150–180 μl ice-cold PCA (5%), whereas analytes remained in solution. Immediately after addition of PCA, caps were shaken on a vortex mixer for 10 s to prevent agglutination. Proteins were separated by centrifugation (14,000*g*, 14 min, 0 °C) and the supernatant was treated with 450 μl IAA (0.1 mol/L) in a boric acid/LiOH buffer (0.5 mol/L, pH 8.5)/LiOH (1 mol/L) (2/1, v/v) for 30 min at room temperature. By adding 500 μl Dans-Cl (7.4 mmol/L in acetonitrile) free amino groups of carboxymethyl thiols, disulfides, and glutathione sulfonic acid were acylated within 1 h at room temperature. To stop the reaction, dichloromethane was added to the reaction mixture, mixed well, and centrifuged (16,000*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