Journal of Chromatography B, 877 (2009) 3405–3417

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

HPLC analysis of human erythrocytic glutathione forms using OPA and *N*-acetyl-cysteine ethyl ester: Evidence for nitrite-induced GSH oxidation to GSSG☆

Jan Thomas Michaelsen[a], Sabine Dehnert[a], Daniela Giustarini[b], Bibiana Beckmann[a], Dimitrios Tsikas[a,*]

[a] *Institute of Clinical Pharmacology, Hannover Medical School, Carl-Neuberg-Strasse 1, 30625 Hannover, Germany*
[b] *Department of Evolutionary Biology, Laboratory of Pharmacology and Toxicology, University of Siena, Siena, Italy*

ARTICLE INFO

Article history:
Accepted 29 June 2009
Available online 4 July 2009

Keywords:
N-Acetyl-cysteine ethyl ester (NACET)
Erythrocytes
Hemoglobin
Nitric oxide (NO)
Oxidation
Reduction
Thiols (RSH)

ABSTRACT

Glutathione exists in biological samples in the reduced form (GSH), as its disulfide (GSSG) and as a mixed disulfide (GSSR) with thiols (RSH). GSH is the most abundant low-molecular-mass thiol and plays important roles as a cofactor and as a main constituent of the intracellular redox status. Due to its own sulfhydryl (SH) group, GSH reacts readily with *o*-phthaldialdehyde (OPA) to form a highly stable and fluorescent isoindole derivative (GSH-OPA), which allows for sensitive and specific quantitative determination of GSH in biological systems by HPLC with fluorescence (FL) detection. In the present article we report on the utility of the novel, strongly disulfide bond-reducing thiol *N*-acetyl-cysteine ethyl ester (NACET) for the specific quantitative analysis of GSH and GSSG in the cytosol of red blood cells (RBC) as GSH-OPA derivative with FL (excitation/emission 338/458 nm) or UV absorbance (338 nm) detection. Unlike in aqueous solution, the derivatization of GSH in RBC cytosol yielded two closely related derivatives in the absence of NACET and only the GSH-OPA derivative in the presence of NACET. The HPLC method was optimized and validated for human RBC and applied to measure GSH and GSSG in RBC of healthy subjects. Basal GSH and GSSG concentrations were determined to be 2340 ± 350 μM and 11.4 ± 3.2 μM, respectively, in RBC of 12 healthy young volunteers (aged 23–38 years). The method was also applied to study the effects of nitrite on the glutathione status in intact and lysed human RBC. Nitrite at mM-concentrations caused instantaneous and considerable GSSG formation in lysed but much less pronounced in intact RBC. GSH externally added to lysed RBC inhibited nitrite-induced methemoglobin formation. Our findings suggest that nitric oxide/nitrite-related consumption rate of GSH, and presumably that of NADH and NADPH, could be of the order of 600 μmol/day in RBC of healthy subjects.

1. Introduction

In living organisms, glutathione exists in its reduced form, i.e., GSH (the tripeptide γ-glutamyl-cysteinyl-glycine), as its disulfide, i.e., GSSG, and as a mixed disulfide, i.e., GSSR, with low-molecular-mass (LMM) and high-molecular-mass (HMM) thiols (RSH). GSH is the most abundant LMM thiol in all types of cells including red blood cells (RBC) [1,2]. GSH plays many important physiological roles. For instance, GSH is a cofactor/substrate for many enzymes including GSH *S*-transferases and GSH peroxidases, and is a main constituent of the intracellular redox status [1,2]. GSH, GSSG and GSSR also occur in human blood plasma; however, their concentrations, notably those of GSH, are about three orders of magnitude lower than in cytoplasm [3–6]. The concentration of the individual glutathione species in different conditions, e.g., in health and disease, and in several matrices, e.g., in blood plasma and erythrocytes, are of particular interest, as they may allow to describe satisfactorily the glutathione and redox status of living organisms. Thus, GSH, GSSG and GSSR in peripheral human blood are potentially useful biomarkers of oxidative stress status, when measured reliably, i.e., free of artefacts [7,8].

On the basis of glutathione measurements, the utility of GSH, GSSG and GSSR as biomarkers of oxidative stress in many different organs [9–16], and the significance of GSH in the pathology of cancer and neurological diseases such as Parkinson's disease and Alzheimer's disease [16–21] have been suggested. The importance of GSH and other biomarkers of oxidative stress in human disease has been recently discussed by Dalle-Donne et al. [22]. In particular in the context of oxidative stress, reliable measurement of GSH, GSSG and GSSR is associated with particular analytical and

☆ This paper is part of the special issue "Analysis of Thiols", I. Dalle-Donne and R. Rossi (Guest Editors).

* Corresponding author. Tel.: +49 511 532 3959; fax: +49 511 532 2750.
E-mail address: tsikas.dimitros@mh-hannover.de (D. Tsikas).

1570-0232/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2009.06.043

pre-analytical difficulties which are closely related to the instability of the sulfhydryl (SH) group of GSH. It is worth mentioning that autoxidation of GSH is often a source for artifactual GSSG and GSSR formation which would erroneously pretend elevated oxidative stress [7,8,22]. For these reasons, reliable analysis of GSH, GSSG and GSSR in biological samples requires sensitive analytical methods, notably for measurement in plasma. Furthermore, it demands use of suitable procedures to avoid formation of GSSG and GSSR during blood sampling and to minimize artifactual change in the glutathione homeostasis [7,8]. This also applies to derivatization procedures which are required for improved chromatography and detection.

About 20 years ago, Neuschwander-Tetri and Roll [3] have reported on a highly specific and sensitive HPLC method with fluorescence (FL) detection for the measurement of glutathione in plasma and tissue. This method is based on the unique property of GSH to readily react with *o*-phthaldialdehyde (OPA) both with its amino group and with its sulfhydryl group to form a stable – for several days at 25 °C if shielded from light [3] – and highly fluorescent derivative (Fig. 1). Thus, unlike GSSG and most amino acids, the reaction of GSH with OPA does not require addition of a thiol such as 2-mercaptoethanol. By means of liquid secondary-ion mass spectrometry, Neuschwander-Tetri and Roll [3] identified the reaction product of GSH and OPA, in this article referred to as GSH-OPA, as a tricyclic isoindole derivative with a molecular weight of 405 ($C_{18}H_{19}N_3O_6S$). Analysis of GSSG and GSSR as GSH-OPA derivatives by this method requires preceding reduction of GSSG or GSSR to GSH which has been accomplished by the use of dithiothreitol (DTT) [3]. A modification of the HPLC method reported by Neuschwander-Tetri and Roll was found by us to be suitable to measure indirectly *S*-nitroso-glutathione (GSNO) in human and rat plasma [23]. Due to the isoindole moiety of the GSH-OPA derivative, UV absorbance (UV) detection at 338 nm is possible and sensitive enough – limit of detection (LOD) of about 70 nM of GSH versus 3 nM using FL detection [23] – to measure GSH in most biological samples.

Because of the unique specificity of this HPLC method for GSH and the remarkable stability of the GSH-OPA derivative [3,23], we wanted to apply this method to the measurement of GSH and GSSG in the cytosol of RBC in the frame of studies addressing potential involvement of GSH in nitric oxide (NO) and nitrite metabolism in blood. Like NO, nitrite (NO_2^-) – the autoxidation product of NO in aqueous solutions – is also oxidized in RBC by oxyhemoglobin to nitrate (NO_3^-) whereby forming methemoglobin [24] (see Eqs. (1) and (2)). It is worth mentioning that nitrite may react with deoxyhemoglobin to form methemoglobin and nitrosyl hemoglobin. It is well known that GSH in RBC may prevent hemoglobin from oxidation [25,26]. We have hypothesized that GSH may be required for the reduction of methemoglobin formed during oxyhemoglobin-catalysed nitrite oxidation in RBC. In accordance with this hypothesis, involvement of GSH in NO and nitrite metabolism in RBC could be associated with GSH consumption and GSSG formation (Eq. (3)).

$$NO + Hb[Fe^{2+}]O_2 \rightarrow NO_3^- + Hb[Fe^{3+}] \quad (1)$$

$$NO_2^- + H^+ + Hb[Fe^{2+}]O_2 \rightarrow NO_3^- + Hb[Fe^{3+}] + H_2O \quad (2)$$

$$2GSH + 2Hb[Fe^{3+}] \rightarrow GSSG + 2Hb[Fe^{2+}] + 2H^+ \quad (3)$$

HPLC analysis of aqueous GSH derivatized with OPA [3,23] revealed only one peak. Unexpectedly, analysis of lysed RBC by the same method [23] for GSH yielded repeatedly two peaks using both FL and UV detection for endogenous and externally added GSH (Fig. 2). The peak with the smaller retention time co-eluted with the GSH-OPA derivative formed using aqueous solutions of commercially available GSH. The close chromatographic and spectroscopic properties suggest that the derivative eluting behind GSH-OPA is structurally closely related to GSH-OPA that has been previously characterized by Neuschwander-Tetri and Roll [3]. In addition, we found that this derivative behaves almost identically to the GSH-OPA derivative with respect to long-term stability (data not shown). We performed a series of experiments aiming at characterizing this unknown derivative; we were able to exclude related substances including GSSG, γ-glutamyl-cysteine and homocysteine (data not shown), which are known to react with OPA in the absence of additional thiols [3]. Finally, we found that addition of other thiols such as *N*-acetyl-cysteine (NAC) and the ethyl ester of NAC, i.e., *N*-acetyl-cysteine ethyl ester (NACET), prior to OPA derivatization in analyses of GSH in RBC resulted in a single HPLC peak with the retention time of the GSH-OPA derivative. As addition of NACET to GSSG solutions yielded GSH, it was interesting to test the usefulness of NACET for GSH and GSSG measurement in RBC by HPLC with the established pre-column OPA derivatization of GSH [3,23].

In the present article we report on the utility of the novel, strongly disulfide group-reducing thiol NACET for the specific and quantitative analysis of erythrocytic GSH and GSSG as GSH-OPA derivatives by HPLC with FL and UV detection. This HPLC method was optimized and validated for human RBC glutathione. The method was applied to measure GSH and GSSG basal RBC concentrations and to study the in vitro effects of nitrite on the glutathione status in RBC of blood of healthy volunteers.

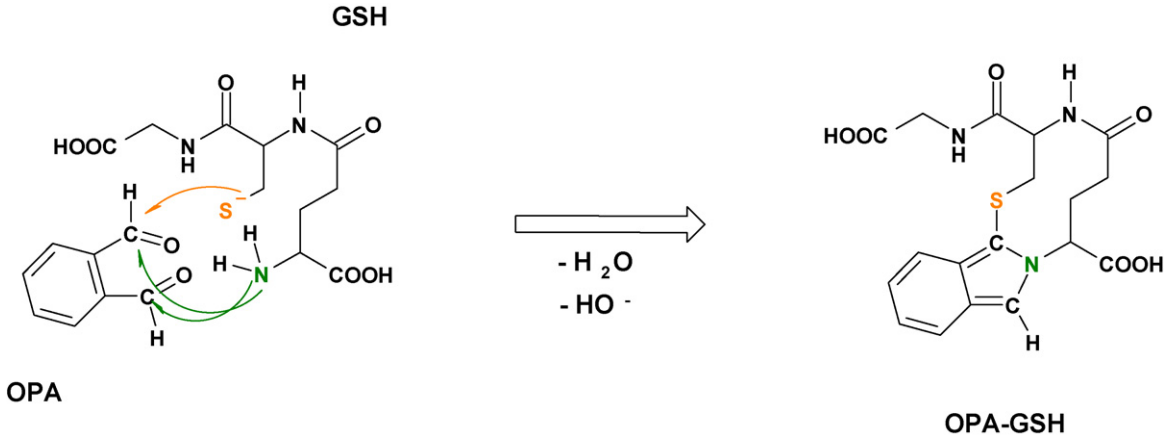


Fig. 1. Reaction of GSH with OPA to form the highly fluorescent and stable isoindole derivative OPA-GSH. Note that this reaction does not require an additional thiol such as 2-mercaptoethanol.

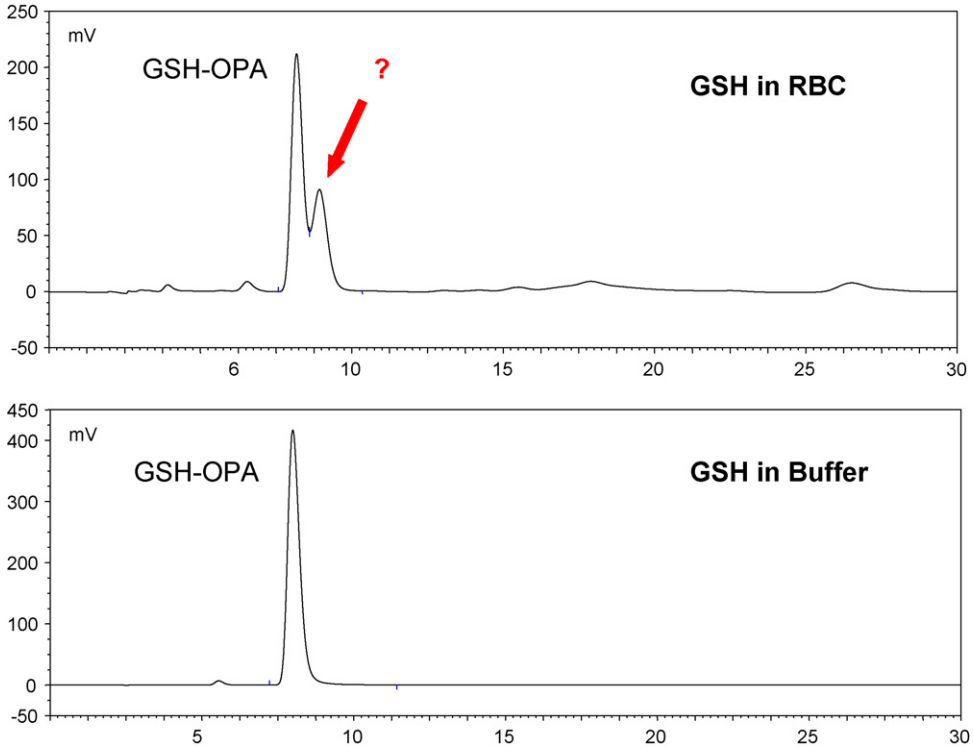


Fig. 2. HPLC chromatograms from analyses of GSH (1 mM) in borate buffer (lower panel) and of GSH in ultrafiltrate of lysed human RBC (upper panel) after derivatization with OPA. The peak eluting behind the GSH-OPA derivative (indicated by an arrow and a question mark) disappeared and the GSH-OPA peak (retention time about 8 min) increased when NACET was added to the ultrafiltrate prior to OPA derivatization (chromatogram not shown). Mobile phase A, column 1, and FL detection were used.

2. Experimental

2.1. Materials and chemicals

Glutathione, glutathione disulfide, *N*-ethylmaleimide (NEM), hydrogen peroxide (33 vol.%), borax and *o*-phthaldialdehyde were purchased from Sigma–Aldrich (Steinheim, Germany). Sodium acetate was obtained from Merck (Darmstadt, Germany). Methanol of gradient grade and ethanol were supplied by Mallinckrodt Baker (Griesheim, Germany). Sodium nitrite was purchased from Riedel-de-Haën (Seelze, Germany). Synthesis and structural characterization of *N*-acetyl-cysteine ethyl ester have been described elsewhere [27]. Vivaspin 2 Hydrosart cartridges (2 ml; cut-off, 10 kDa) for ultrafiltration were supplied by Sartorius (Göttingen, Germany). Ultrafiltration was carried out using the ultracentrifuge model RC5C from Sorvall Instruments (Waltham, MA, USA).

The OPA reagent solution was prepared in borate buffer (75 mM, pH 8), contained 36.4 mM OPA and 10 vol.% ethanol, and was stored in brown flasks at 4 °C. Stock solutions of GSH and GSSG (each 10 mM) and of NACET (100 mM) were prepared in phosphate buffered saline (PBS, 67 mM, pH 7.4), stored aliquoted at −20 °C, and used only once after thawing. Stock solutions of NEM (100 mM) and nitrite (1 M) were prepared in PBS and stored at 4 °C. Dilutions of nitrite were prepared daily in PBS directly before use in RBC experiments.

2.2. Biological samples—withdrawal of blood and generation of ultrafiltrate

Blood was drawn from the antecubital vein of healthy volunteers using EDTA-containing monovettes (Sarstedt, Germany). Immediately after collection, the blood was put in an ice bath. Blood, erythrocytes and all GSH-containing samples were stored in an ice bath and in the dark during handling to minimize GSH oxidation. Erythrocytes were separated from plasma by centrifugation (5 min, 4 °C, 800 × *g*) followed by complete decantation of plasma and a small portion of erythrocytes to avoid contamination. In all experiments erythrocytes were used unwashed. Lysis was attained by freezing the erythrocytes for at least 30 min at −80 °C, followed by slow defrosting (on ice) and finally by rapid vortex-mixing for at least 1 min with the same volume of ice-cold distilled water. Cell fragments and proteins were removed from the lysed RBC by centrifugation (30 min, 4 °C, 8000 × *g*) using Vivaspin cartridges.

2.3. OPA derivatization procedures and calibration curves

The following procedures were used to measure GSSG and total GSH (tGSH). For GSSG analysis, a 50 μl aliquot of ultrafiltrates (UF) from lysed RBC was spiked with a 5 μl aliquot of a 100 mM NEM solution for 1 min to alkylate GSH. Then, a 10 μl aliquot of a 100 mM NACET solution was added. For tGSH analysis, a 50 μl aliquot of the same UF was spiked with a 10 μl aliquot of a 100 mM NACET solution without NEM addition. After 2 min of incubation at room temperature, to both sample aliquots (50 μl) borate buffer (900 μl) was added followed by addition of the OPA reagent (50 μl). Deviations from this procedure are described in the respective experiments. Samples were analyzed by HPLC immediately (i.e., after about 10 min) or after storing in a refrigerator at 8 °C. For quantification, calibration curves were prepared using solutions of GSH and GSSG in PBS, which were derivatized exactly as described above and analyzed at the same time as study samples. In addition, a freshly prepared sample of 1 mM GSH in PBS was derivatized in each experiment and served as quality control.

2.4. HPLC conditions

HPLC analyses were performed on an apparatus from Dionex (Sunnyvale, CA, USA) consisting of a pump model P680, a UV–vis

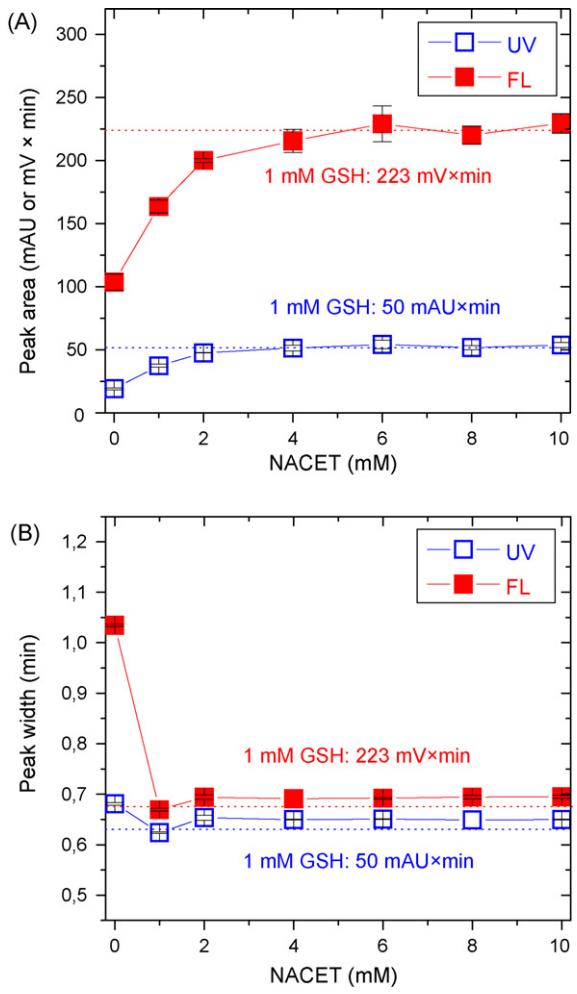


Fig. 3. Effect of NACET on the peak area (A) and peak width at baseline (B) of GSH from lysed RBC and of 1 mM GSH in borate buffer (see dotted lines and insertion). Mobile phase A and column 1 were used; effluent was detected by UV and FL detectors as described in Section 2.

detector model UVD170U and a FL detector model RF 2000 in this order. Injection was performed by a Dionex automated sample injector model ASI-100. In most experiments, the chromatographic column (250 mm × 4.0 mm i.d., packed with 5 μm particle size Nucleodur C18 gravity; referred to as *column 1*) from Macherey-Nagel (Düren, Germany), located in a Dionex thermostated column compartment model TCC-100 at 20 °C, was used. In the frame of method validation, the HPLC column 2 (100 mm × 4.0 mm i.d., packed with 5 μm particle size Nucleosil C18; referred to as *column 2*) from Macherey-Nagel was used. The HPLC system was controlled and the data were managed by the Chromeleon software.

Two mobile phases were used, which consisted of methanol–water, contained 150 mM of sodium acetate and had a pH value of 7.0 adjusted by addition of acetic acid. Mobile phase A was methanol–water (7.5:92.5, v/v) and had been used by us previously [23]. Mobile phase B contained 30 vol.% methanol and was used mainly for GSSG measurements. Isocratic runs were performed at a flow rate of 1.0 ml/min with both mobile phases and columns. The retention time of the GSH-OPA derivative on column 1 was about 8 min with mobile phase A and about 3.6 min with mobile phase B. If not otherwise specified, the injection volume was 100 μl and the running time was 20 min with both mobile phases using column 1. Using mobile phase A and column 2, the injection volume was 10 μl and the running time 5 min. In this HPLC system, the GSH-OPA derivative eluted at about 1.7 min. The UV–vis detector was set at 338 nm. Wavelengths of 338 nm for excitation and 458 nm for emission were set at the FL detector. The area of the peaks in units of mV × min in FL detection and in units of mAU × min in UV detection was used for calculations. Data were routinely collected from both detectors in all experiments. Because FL and UV detection provided almost identical results, for simplicity data from UV detection are not reported from all experiments of this work.

2.5. Method validation

Blood from three healthy female volunteers was collected in 9 ml EDTA monovettes and centrifuged (10 min, 4 °C, 800 × *g*). Plasma

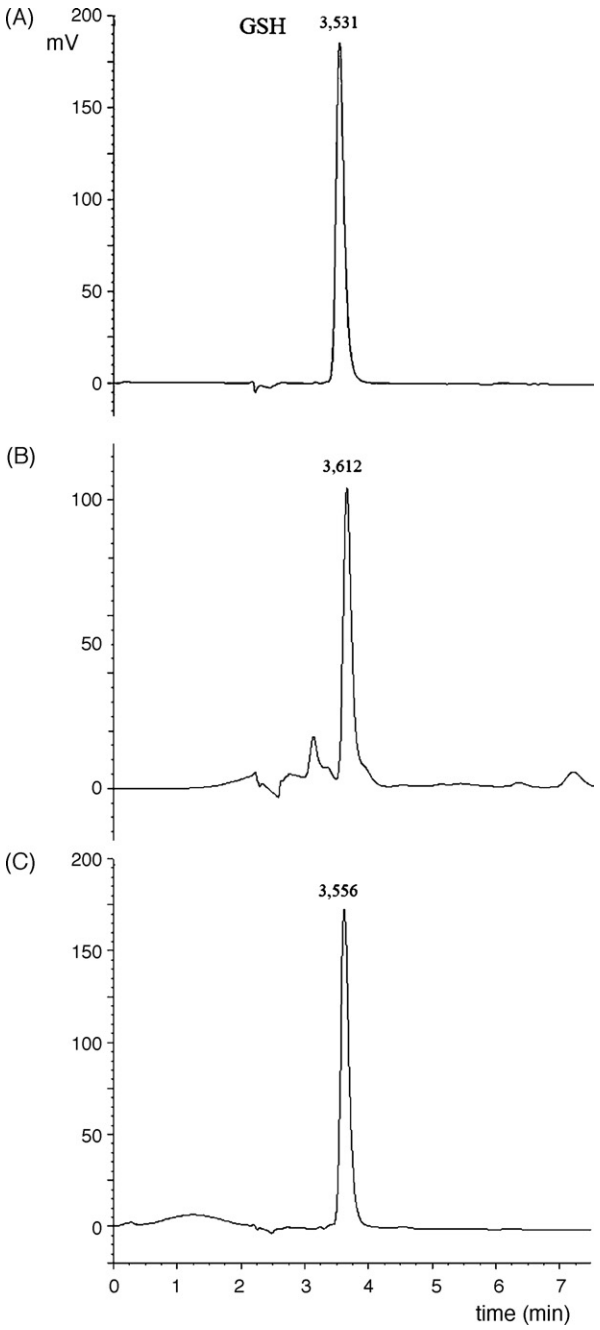


Fig. 4. Typical HPLC chromatograms from analyses of GSH (1 mM) in borate buffer (A), GSH in ultrafiltrate (50 μl) of lysed RBC in the absence of NACET (B), and of GSH (+GSSG) in ultrafiltrate (50 μl) of lysed RBC in the presence of NACET externally added (10 mM) prior to derivatization with OPA. Mobile phase B, column 1, and FL detection were used.

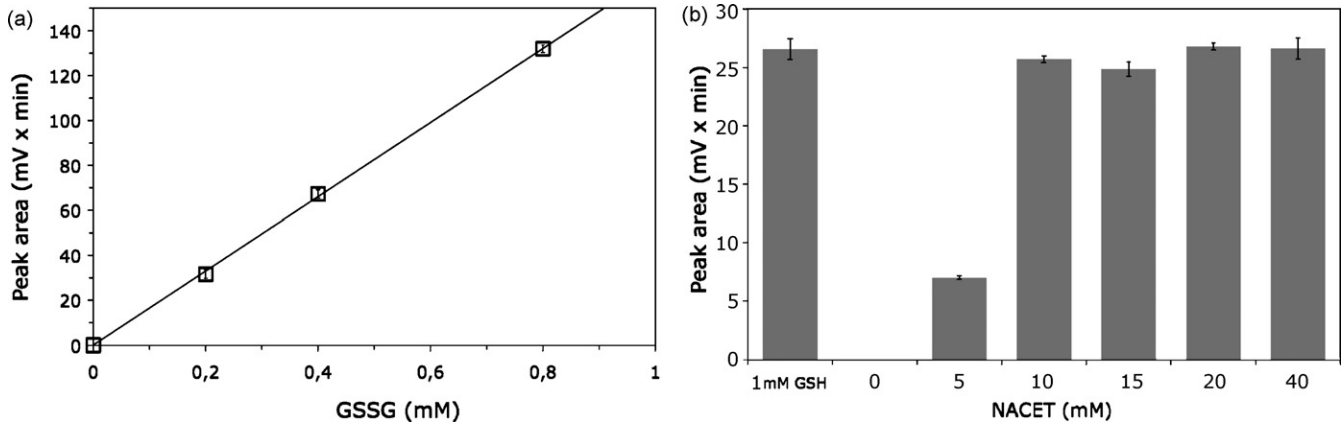


Fig. 5. NACET-induced formation of GSH from GSSG in aqueous buffered solution. (a) Aliquots of GSSG (50 μl, 0–0.8 mM) in PBS were added to borate buffer (900 μl). Samples were treated with NACET (50 μl, 20 mM), incubated for 2 min at room temperature and derivatized with OPA. (b) Aliquots of GSSG (50 μl, 0.5 mM) in PBS were incubated with different NACET dilutions (10 μl) to reach final concentrations in the range 0–40 mM. After 2 min of incubation borate buffer (900 μl) was added and derivatization with OPA was immediately performed. As a control served a 1 mM solution of GSH which was treated in the same way. Mobile phase B and column 1 were used. Injection volume was 100 μl. Data (from FL detection) are shown as mean ± SD from two independent measurements for each GSSG concentration examined.

and a small portion of the underlying erythrocytes were discarded and erythrocytes were combined, gently shaken, portioned in 500 μl aliquots, and frozen for 30 min at −80 °C. After slow thawing, aliquots (500 μl each) of distilled water were added to each sample under gentle mixing. Lysed erythrocytes were centrifuged immediately (30 min, 4 °C, 8000 × *g*). The colorless and clear UF samples were first transferred into a 12 ml polypropylene tube, and the combined and mixed UF samples were then portioned into 700 μl aliquots and stored at −20 °C until analysis. For each validation experiment one 700 μl aliquot was thawed (once only) and used immediately.

After immediate thawing the 700 μl UF was divided into 50 μl aliquots, and 50 μl aliquots of a 20 mM solution of NACET were added. To these samples various volumes of a 10 mM GSH solution as well as of distilled water were added resulting in a final volume of 120 μl each. Thereafter, 900 μl aliquots of borate buffer and 50 μl aliquots of the OPA reagent were added to 100 μl aliquots of the samples and mixed by vortexing. From these solutions 250 μl aliquots were transferred into glass vials and samples were analyzed by means of the autosampler (injection of 200 μl aliquots with column 1 and 10 μl with column 2). Each series of validation samples was analyzed at the same time as two 1 mM GSH standard samples serving as quality control.

2.6. Kinetics of GSSG reduction by NACET

GSSG was dissolved in 0.1 M Na^+/K^+ phosphate buffer, pH 7.4, and a 1 ml aliquot of a 0.1 mM GSSG solution was incubated at room temperature with 1 mM NACET (final concentration from a 100 mM stock in water). At 0 min, 5 min, 15 min, 30 min and 60 min, 10 μl aliquots were taken, diluted with 90 μl of 50 mM Na^+/K^+ phosphate buffer, pH 7.4, and incubated with 1 mM monobromobimane {mBrB, Calbiochem (La Jolla, CA, USA), 40 mM stock in methanol} for 10 min in the dark at room temperature. Samples were then acidified with 3 μl of 37 vol.% HCl and analyzed by HPLC as described elsewhere [7]. A Zorbax Eclipse C18 column (Agilent Technologies,

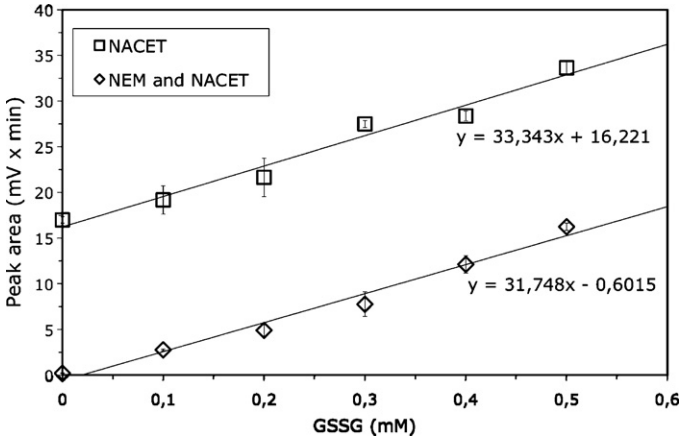


Fig. 6. Effect of NEM and NACET on the HPLC analysis of GSSG in PBS in the presence of GSH. All samples (50 μl) contained GSH at a fixed concentration (1 mM) and various concentrations of GSSG (0–0.5 mM). To one series of samples, NEM (5 μl, 100 mM) was added, whereas the second series was treated with distilled water (5 μl). After an incubation time of 2 min all samples were treated with NACET (10 μl, 100 mM), incubated for further 2 min at room temperature followed by immediate OPA derivatization. Mobile phase B and column 1 were used. Injection volume was 100 μl. Data (from the FL signal) are shown as mean ± SD from two independent incubations for each GSSG concentration examined.

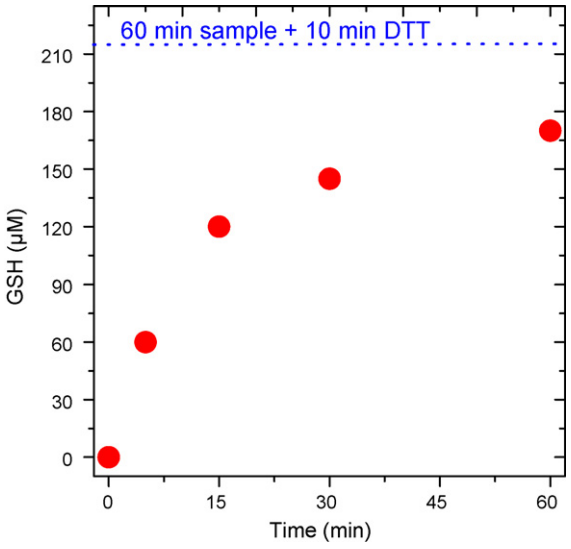


Fig. 7. Kinetics of NACET (1 mM)-induced conversion of GSSG (0.1 mM) to GSH in 0.1 M phosphate buffer (pH 7.4) at room temperature. At the specified times, samples were taken, and 1:10 (v/v) dilutions with 50 mM phosphate buffer (pH 7.4) were incubated with 1 mM mBrB and analyzed for GSH by HPLC with FL detection as described elsewhere [7]. After 60 min of incubation, the sample was treated with dithiothreitol (DTT) and incubated for 10 min prior to derivatization with mBrB and HPLC analysis as described elsewhere [7].

Table 1
Inter-assay validation of the HPLC method for GSH in pooled ultrafiltrate from lysed human RBC.

Day	Detector Fl UV	*y*-Axis intercept (mV × min) (mAU × min)	Slope ([mV × min]/mM) ([mAU × min]/mM)	*R*	RSD (%) (min/max)
HPLC column 1, mobile phase A, 200 μl injection volume					
1	FL	49.8 (0.2)	183 (0.5)	0.99999	0.1/3.7
2	FL	49.3 (0.3)	185 (0.6)	0.99998	0.1/4.1
3	FL	46.8 (0.1)	182 (2.1)	0.99973	0.1/4.2
4	FL	50.7 (2.1)	176 (3.9)	0.99900	0.3/3.8
5	FL	49.4 (2.6)	174 (2.4)	0.99961	0.2/2.6
1	UV	25.7 (0.1)	96.2 (0.2)	0.99999	0.2/3.8
2	UV	25.5 (0.2)	96.9 (0.2)	0.99999	0.1/14
3	UV	24.1 (0.1)	95.3 (1.3)	0.99963	0.3/6.0
4	UV	25.5 (1.3)	93.3 (1.8)	0.99924	0.6/3.7
5	UV	24.3 (0.3)	95.1 (0.8)	0.99987	0.4/2.6
HPLC column 2, mobile phase A, 10 μl injection volume					
1	FL	1.54 (0.05)	6.92 (0.12)	0.99934	0.9/3.7
2	FL	2.03 (0.04)	8.04 (0.05)	0.99993	0.7/11
3	FL	2.07 (0.31)	8.19 (0.42)	0.99480	0.5/6.1
4	FL	2.28 (0.16)	7.62 (0.16)	0.99914	0.5/6.3
5	FL	2.36 (0.21)	9.25 (0.21)	0.99896	1.4/14
1	UV	0.90 (0.01)	3.49 (0.04)	0.99969	0.1/3.3
2	UV	1.08 (0.01)	4.07 (0.01)	0.99999	0.1/15
3	UV	1.08 (0.05)	4.29 (0.03)	0.99992	0.1/5.3
4	UV	1.31 (0.09)	3.81 (0.07)	0.99929	0.2/4.9
5	UV	1.16 (0.05)	4.12 (0.03)	0.99986	0.1/5.1

Milan, Italy) was used for chromatographic separation. Solvent A was sodium acetate 0.25 vol.%, pH 3.09; solvent B was acetonitrile. Elution conditions: 0–5 min: solvent A–solvent B (94:6, v/v); 5–10 min: linear gradient from 6% to 10% solvent B. A constant flow rate of 1.2 ml/min was applied. Detection was performed at 390 nm excitation and 480 nm emission wavelengths. After incubation with NACET for 60 min, a 100 μl aliquot of the remaining sample was incubated for 10 min with 2 mM dithiotreitol (DTT, 50 mM stock solution in water) and then reacted with 6 mM mBrB for 10 min in the dark at room temperature, acidified and analyzed by HPLC as described above. An Agilent 1100 series was used to carry out analyses.

2.7. Biomedical applications

Approval from the local Ethics Committee of the Medical School of Hannover was obtained for studies in which blood from healthy volunteers was taken for measurement of basal concentrations of glutathione in RBC as well as for in vitro studies.

2.7.1. Measurement of glutathione in RBC of healthy volunteers

Total glutathione (tGSH) and GSSG were measured in freshly isolated RBC from 7 females and 5 males aged between 23 and 38 years. These subjects were apparently healthy and did not receive any medication for at least two weeks prior blood donation. In most experiments, blood from only one person was analyzed per day.

2.7.2. Effect of nitrite on glutathione in RBC of healthy volunteers

To examine the effect of nitrite on GSH in intact RBC, erythrocytes isolated from freshly generated blood (anticoagulated with EDTA) were combined with the same volume of PBS and gently shaken. Thus prepared blood was portioned into 500 μl aliquots which were then incubated at 37 °C in a water bath under gentle shaking. To these samples, 20 μl aliquots of nitrite solutions of various concentrations in PBS were added under rapid vortex-mixing to reach final added nitrite concentrations in the blood in the range 0–20 mM. After an incubation time of 30 min in the water bath, samples were first transferred into an ice bath and then centrifuged (5 min, 4 °C, 800 × *g*). Glutathione was analyzed by HPLC in UF samples from lysed RBC as described above. The same experiment was performed in parallel by adding nitrite to lysed RBC. After incubation for 30 min in the water bath, incubation mixtures were ultrafiltered and glutathione was analyzed in UF samples.

To investigate the effect of nitrite on RBC glutathione as a function of the incubation time, 7 ml of RBC suspended in PBS (1:1, v/v) or 7 ml of lysed erythrocytes (RBC-distilled water, 1:1, v/v) were incubated at 37 °C in a water bath. To these samples, each a 140 μl aliquot of $NaNO_2$ solution (1 M) in PBS was added under gentle shaking. At various times after nitrite addition, each 250 μl aliquots were taken and processed immediately, including lysis, ultrafiltration, derivatization and HPLC analysis. As controls served samples from intact and lysed erythrocytes which were incubated without external addition of nitrite. These samples were prepared in parallel and handled in the same way.

3. Results

3.1. HPLC analysis of GSH in buffer and lysed erythrocytes

As mentioned above, we found that GSH from lysed RBC reacted with OPA to produce two closely eluting derivatives with similar FL and UV properties, unlike GSH from buffer which yielded apparently a single derivative, i.e., GSH-OPA (see ref. [23]). The formation of two OPA derivatives was evident by using mobile phase A that contained considerably less methanol than mobile phase B, i.e., 7.5 vol.% versus 30 vol.%, and allowed partial separation of the peaks which was maximum on newly used HPLC columns (Fig. 2). Addition of NACET to the UF prior to OPA derivatization resulted in a concentration-dependent decrease of the later-eluting peak and in a concentration-dependent increase of the GSH-OPA peak (see below). Interestingly, chromatographic resolution decreased quite rapidly with increasing number of analyses. For this reason we used the peak area and the peak width at baseline to evaluate the effect of NACET on erythrocytic GSH analysis as OPA derivative.

Fig. 3 shows that addition of NACET in the concentration range of 0–6 mM to the UF prior to OPA derivatization resulted in a

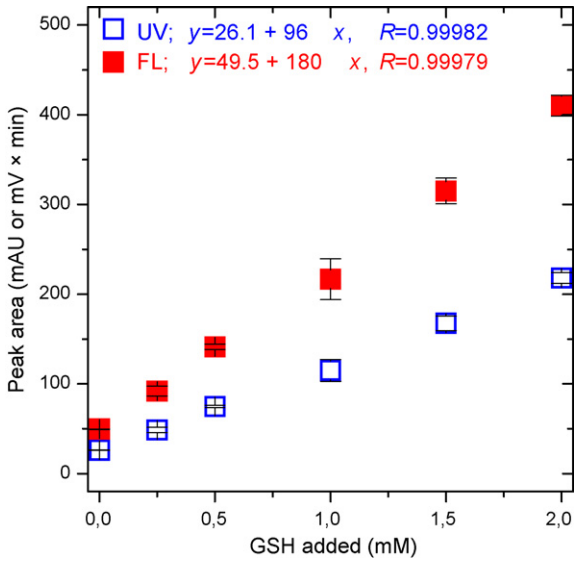


Fig. 8. Results from intra-assay validation of the HPLC method for GSH in pooled ultrafiltrate generated from freshly obtained human RBC which had been lysed with distilled water (1:1, v/v). Ultrafiltrate (100 μl aliquots) was treated with NACET (100 μl, 20 mM). After 2 min of incubation 100 μl aliquots of the resulting solutions were derivatized with OPA as described in Section 2. HPLC column 1 and mobile phase A were used. Data are shown as mean ± SD from two independent analyses for each GSH concentration examined. For experimental details see Section 2.5.

concentration-dependent increase of the GSH-OPA peak using both detectors. Addition of NACET at a final concentration of 1 mM (the lowest concentration tested) resulted in a clear reduction of the GSH peak width from FL detection (by about 30%), which did not change further at higher NACET concentrations. Peak width reduction was also noticed using the UV detector. The extent of reduction of the UV peak was considerably smaller, in part because the mobile phase passed first the UV detector. The maximum peak areas achieved using NACET were almost equivalent to those obtained from 1 mM GSH in distilled water, PBS and borate buffer. Considering a dilution factor of 2 for lysed RBC, these findings suggest that the concentration of total glutathione, i.e., of GSH + GSSG, in the RBC used in this experiment was about 2 mM. In consideration of this finding we used mobile phase B, which resulted in apparently complete co-elution of the otherwise partially separated peaks. HPLC chromatograms from the analysis of erythrocytic glutathione under these conditions are shown in Fig. 4. This figure shows that addition of NACET to the UF prior to OPA derivatization increased the GSH-OPA peak considerably.

3.2. HPLC analysis of GSSG using NACET, NEM and OPA

Derivatization of aqueous solutions of GSSG with OPA did not result in a peak within the relevant retention time window (chromatogram not shown). Addition of NACET to aqueous solutions of GSSG prior to OPA derivatization resulted in formation of the GSH-OPA peak. The peak area observed depended linearly upon the concentration of GSSG in the relevant range of 0–0.8 mM (Fig. 5A). NACET-induced formation of GSH from GSSG (0.5 mM) depended upon the NACET concentration and was maximum at final NACET concentrations ≥10 mM (Fig. 5B). Parallel derivatization of GSH (1 mM) with OPA indicated that NACET concentrations of 10, 15, 20, and 40 mM caused almost complete conversion of GSSG to GSH (Fig. 5B). These findings suggest that NACET used in excess over GSSG reduces the disulfide bond to produce quantitatively GSH. We also found that NACET did not interfere with the HPLC analysis of GSH as GSH-OPA derivative. For instance, derivatization of aqueous GSH (1 mM) resulted in very similar peak areas both in the absence and in the presence of 10 mM NACET both from the FL signal (99.9 ± 2.5 mV × min versus 95.1 ± 4.0 mV × min, $n = 2$) and from the UV signal (51.6 ± 1.7 mV × min versus 53.0 ± 0.6 mV × min, $n = 2$).

For specific measurement of GSSG in UF from lysed RBC, complete elimination of GSH prior to reduce GSSG to GSH is absolutely required in this method. We addressed this issue in a further experiment by simulating the conditions prevailing in RBC UF regarding GSH and GSSG concentrations. Fig. 6 and the almost identical slope

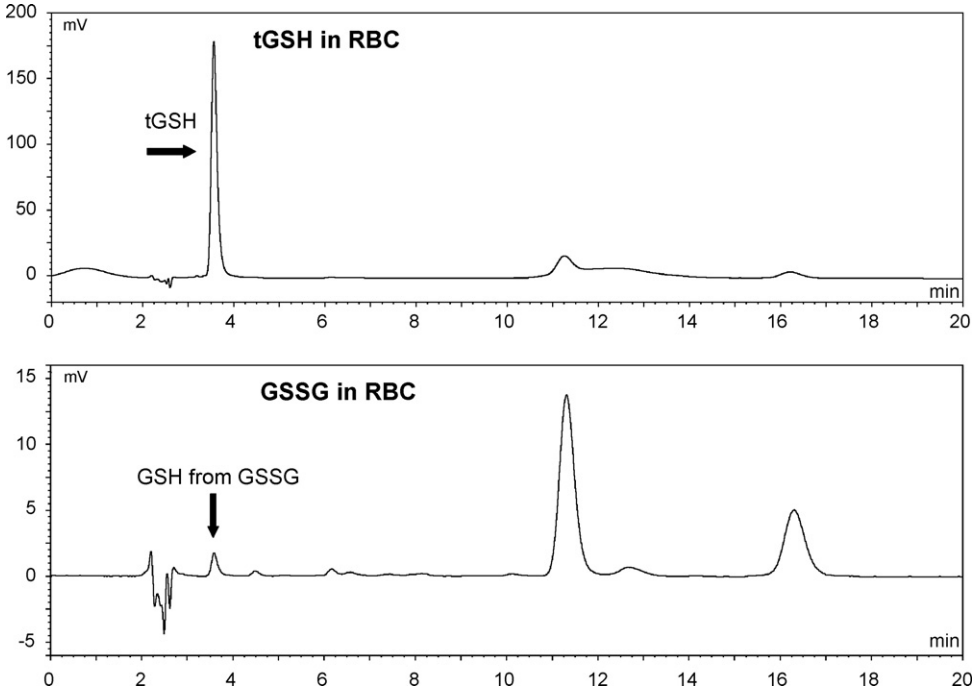


Fig. 9. Representative chromatograms from the HPLC analysis of total glutathione (tGSH; upper panel) and GSSG (lower panel) in RBC of a healthy volunteer. Here only the FL signal is shown. Mobile phase B, column 1, and injection volume of 100 μl were used.

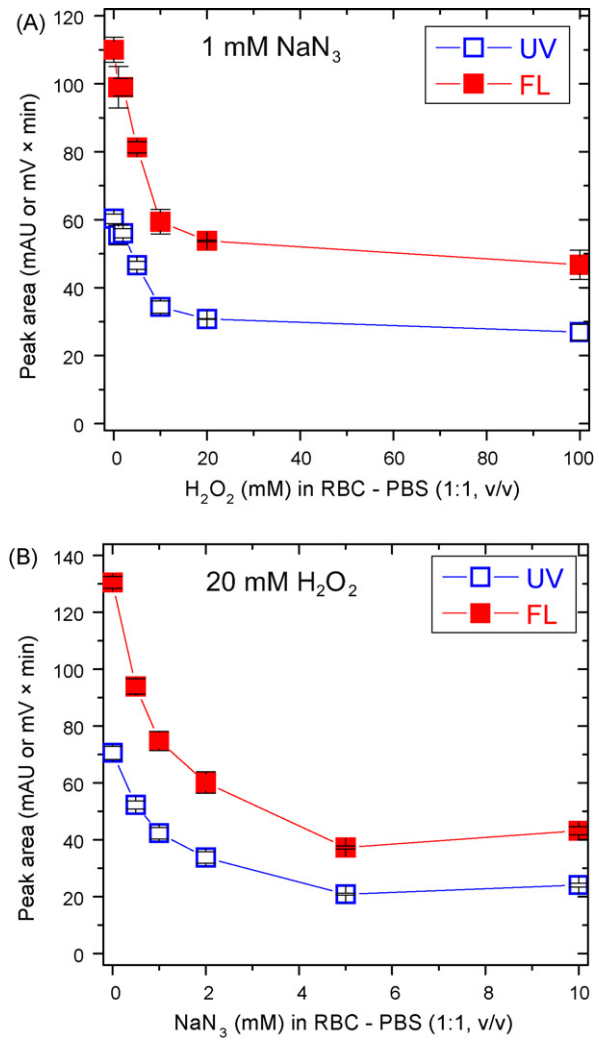


Fig. 10. Effects of hydrogen peroxide (H_2O_2) and sodium azide (NaN_3) on GSH in intact human RBC resuspended in PBS. RBC were isolated by centrifugation from venous blood of a healthy volunteer, the plasma was removed and replaced by the same volume of PBS. (A) First, "blood" was incubated for 2 min with NaN_3 at a final concentration of 1 mM, and then H_2O_2 was added to reach the indicated final concentrations. After 2 min of incubation the samples were put into an ice bath. (B) "Blood" samples were spiked with NaN_3 at the final concentrations indicated and incubated for 2 min. Then, H_2O_2 was added to the "blood" at a final concentration of 20 mM. After 2 min of incubation the samples were put in an ice bath. tGSH was measured in ultrafiltrates of lysed RBC. Mobile phase B, column 1, and injection volume 100 μl were used. Data are shown as mean ± SD from two independent measurements for each concentration examined. Concentration refers to the total volume.

values of the regression equations observed indicate that under the experimental conditions chosen NEM effectively eliminates GSH originally present in the UF (i.e., GSH_{GSH}), and more importantly NEM does not remove GSSG-derived GSH (i.e., GSH_{GSSG}). Obviously, GSH_{GSSG} is condensed with OPA to form the GSH-OPA derivative (i.e., GSH_{GSSG}-OPA) immediately after GSH formation. Most likely, exceeding NACET reacts quantitatively with NEM thus preventing consumption of GSSG-derived GSH (see Eqs. (4)–(8); abbreviations: ex, excess; NACET-S-S-NACET, NACET disulfide).

$$GSH_{GSH} + NEM_{ex} \rightarrow GSH_{GSH}\text{-NEM} + NEM \quad (4)$$

$$NACET + NEM \rightarrow NACET\text{-NEM} \quad (5)$$

$$GSSG + NACET_{ex} \rightarrow GSH_{GSSG} + NACET + NACET\text{-S-S-NACET} \quad (6)$$

$$GSH_{GSSG} + OPA_{ex} \rightarrow GSH_{GSSG}\text{-OPA} + OPA \quad (7)$$

$$(GSH_{GSH}+GSSG) + NEM_{ex} + (NACET_{ex}–NACET\text{-S-S-}NACET–NACET) + (OPA_{ex}–OPA) \rightarrow GSH_{GSH}\text{-NEM} + NACET\text{-NEM} + GSH_{GSSG}\text{-OPA} \quad (8)$$

That *N*-acetyl-cysteine (NAC), i.e., the free acid of NACET, raises GSH levels in different cells in vivo has been reported [28,29]. However, this effect is due to elevated synthesis of GSH from NAC-derived L-cysteine. Grinberg et al. found out that *N*-acetyl-cysteine amide has the ability to generate GSH from GSSG [30]. Presumably, NACET and NAC act on disulfides such as GSSG by the same mechanism, which starts with the nucleophilic attack of the SH group of NACET on the S-S-bond of GSSG, thereby producing GSH and the mixed disulfide GS-S-NACET according to Eq. (9). Due to the excess of NACET over GSSG and GS-S-NACET, exceeding NACET most likely attacks the S-S-bond of GS-S-NACET to form a second GSH molecule and symmetric NACET disulfide, i.e., NACET-S-S-NACET, according to Eq. (10), with the overall reaction being described by Eq. (11). Our results suggest that NACET possesses a much stronger nucleophilic SH group than GSH, which, in synergy with the molar excess of NACET over GSSG, finally leads to quantitative conversion, i.e., reduction of GSSG to GSH. Fig. 7 shows the kinetics of the reaction of NACET with GSSG as studied by measuring GSH formed by an independent previously reported HPLC method based on the use of monobromobimane (mBrB) as the derivatization reagent [7]. In this experiment, GSH recovery from GSSG is incomplete presumably because NACET was used at a considerably lower concentration as compared to the conditions prevailing in the analysis of GSSG in UF of lysed RBC (i.e., 1 mM versus 10 mM; see below).

$$GSSG + NACET \rightarrow GSH + GS\text{-S-}NACET \quad (9)$$

$$GS\text{-S-}NACET + NACET \rightarrow GSH + NACET\text{-S-S-}NACET \quad (10)$$

$$GSSG + 2NACET \rightarrow 2GSH + NACET\text{-S-S-}NACET \quad (11)$$

3.3. Method validation for GSH in RBC

The method was validated by using a pooled UF generated from human RBC lysed with distilled water (1:1, v/v). The results from intra-assay validation are shown in Fig. 8. The peak area of the GSH-OPA derivative increased linearly with the concentration of GSH added to the UF as observed by using both detectors. The intra-assay precision (RSD) ranged between 1.3% and 10.6% from the UV signal and between 0.6% and 10.5% from the FL signal. The results from the inter-assay validation experiments performed by analyzing the same pre-prepared UF pool on 5 consecutive days are summarized in Table 1. Derivatized samples were analyzed by HPLC using mobile phase A within two runs. For the first time, analysis was performed separately overnight for each day investigated by using column 1 and by injecting 200 μl aliquots of the derivatization mixture. The second analysis was performed on the shorter HPLC column 2 by injecting 10 μl aliquots of the remaining volume of all samples which had been collected and stored at 4 °C for up to 14 days. The data of Fig. 8 and Table 1 indicate that GSH added to UF of lysed RBC can be precisely determined by the method. Because derivatization under the same conditions of 1 mM GSH standard used as the quality control sample gave peak areas of 91.5 ± 0.4 mV × min from the FL signal and 48 ± 0.1 mAU × min from the UV signal (mean ± SD, n = 10), Fig. 8 and Table 1 also indicate that the method is accurate too across the investigated concentration range of GSH.

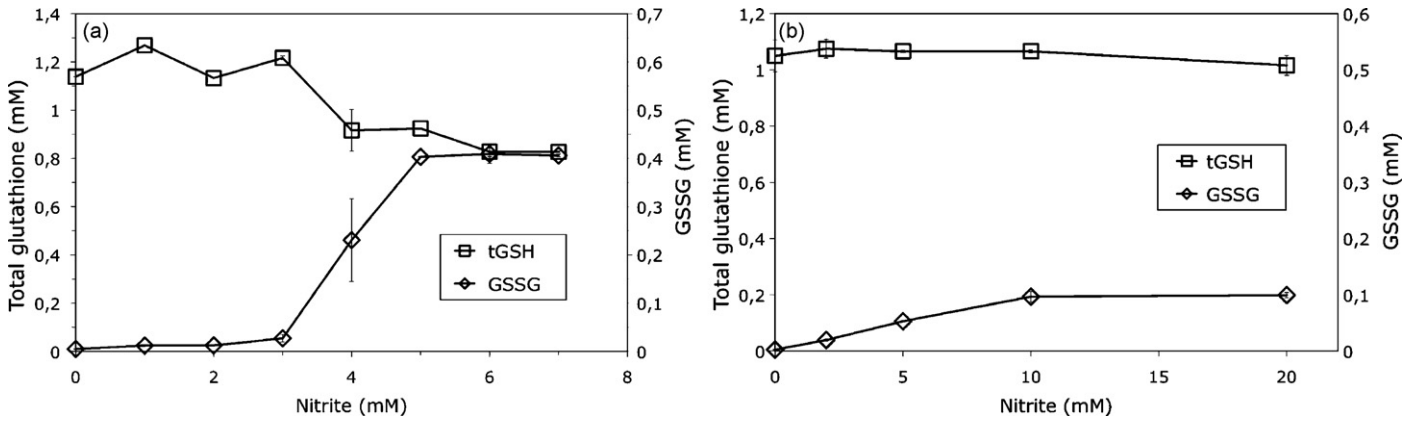


Fig. 11. Effect of nitrite on GSSG formation in lysed (a) and intact RBC (b). (a) Lysed RBC (500 μl aliquots) were incubated at 37 °C in a water bath for 30 min with nitrite at final concentrations of 0–7 mM. Reaction was stopped by putting samples in an ice bath followed by ultrafiltration. (b) Isolated intact erythrocytes combined with the same volume of PBS (500 μl aliquots for each nitrite concentration) were incubated in a water bath at 37 °C for 30 min with nitrite at final concentrations of 0–20 mM. After incubation PBS was removed by centrifugation, erythrocytes were lysed, and aliquots from ultrafiltrates were derivatized and analyzed for GSSG and total glutathione (tGSH). Mobile phase B, column 1, and injection volume of 100 μl were used. Data are shown as mean ± SD from two independent incubations.

3.4. Biomedical applications of the method

3.4.1. Basal concentrations of GSH and GSSG in RBC of healthy humans

This HPLC method was applied to measure total glutathione (use of NACET) and GSSG (use of NEM and NACET) in RBC of twelve healthy volunteers. The concentration of total glutathione (tGSH) and GSSG in these subjects was determined to be 2.35 ± 0.35 mM and 11.4 ± 3.2 μM, respectively. Thus, the mean concentration of GSH in the RBC of our volunteers is calculated to be 2.34 mM. These findings suggest that the molar ratio of GSH to GSSG is of the order of 200:1 in the subjects investigated in the present study. Also, the GSSG:NACET molar ratio in the derivatized samples is calculated to be about 1:1000 and should guarantee complete reduction of GSSG to GSH (see also Fig. 7). Interestingly, Rossi et al. have measured by the mBrB method erythrocytic GSSG concentrations in the range 4–12 μM [7], which are very close to those measured in the present study. By contrast, the GSSG concentrations of 190 ± 20 μM measured by Cereser et al. using HPLC and OPA derivatization [31] are considerably higher. On the other hand, the erythrocytic GSH concentrations reported by Cereser et al. of 2.23 ± 0.15 mM are comparable with our data. It is worth mentioning that Cereser et al. [31] did not measure directly GSSG, but by subtracting the GSH concentration from the total glutathione concentration. Indirect measurement of GSSG in RBC and insufficient avoidance of artificial GSSG formation during sample treatment may be important contributors to falsely high GSSG concentrations in RBC. Typical HPLC chromatograms from the analysis of tGSH and GSSG in a RBC sample of a healthy volunteer are shown in Fig. 9.

3.4.2. Effects of hydrogen peroxide on GSH in RBC

Hydrogen peroxide (H_2O_2) is a potent thiol oxidant. Oxidation of GSH to GSSG has been shown to occur in erythrocytes upon addition of H_2O_2 to intact and lysed erythrocytes [32]. We applied the present HPLC method to study the effects of exogenous H_2O_2 on erythrocytic GSH. Because endogenously produced H_2O_2 is effectively eliminated by catalase in RBC, we used in these experiments the unspecific catalase inhibitor sodium azide (NaN_3) [2] to prevent catalase activity towards externally added H_2O_2. In order to overcome reduction of H_2O_2 by plasma constituents we replaced plasma by the same volume of PBS. The results of these experiments are shown in Fig. 10. Using both detectors we found similar results which indicate that externally added H_2O_2 causes disappearance of GSH + GSSG from the cytoplasm in a concentration-dependent manner in the range of 0–20 mM H_2O_2 (Fig. 10A). Glutathione disappearance increased with increasing NaN_3 concentration, whereas maximum effect was observed for 5 mM of NaN_3 (Fig. 10B). It is worth mentioning that the effect of H_2O_2 on glutathione was more pronounced in lysed erythrocytes (data not shown), suggesting that the erythrocytic cell membrane represents a considerable barrier and/or a sink for H_2O_2. It is also worth mentioning that, in the absence of NaN_3, the effect of H_2O_2 on glutathione in intact RBC was negligible and temporary (data not shown), suggesting that RBC enzymes may avoid GSH oxidation and/or fully regenerate GSH. The results of Fig. 10 suggest that the clear decrease of tGSH caused by H_2O_2 is associated with formation of glutathione mixed disulfides with hemoglobin and other RBC proteins which are not present in the UF analyzed. These findings are supported by a recent study by Aldini et al. who showed abundant formation of *S*-glutathionylated hemoglobin in similar experiments [33].

3.4.3. Effects of nitrite on GSH in RBC

Preliminary investigations showed that incubation of intact or lysed RBC with physiologically and pathologically relevant con-

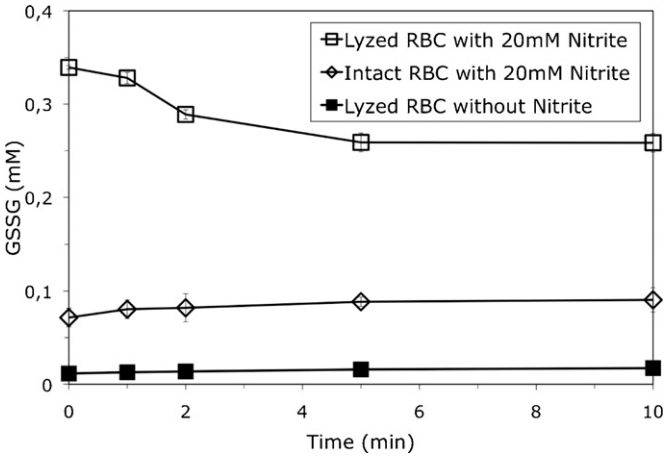


Fig. 12. Effect of nitrite (20 mM) on GSSG formation in lysed and intact RBC upon incubation time (0–10 min) in a water bath at 37 °C. The data from a control experiment with intact RBC in the absence of nitrite were almost identical with those shown for lysed RBC; these data are not shown here for simplicity. Time zero-points refer to the samples which were analyzed immediately after addition of nitrite or of the same volume of a nitrite-free sample. Mobile phase B, column 1, and injection volume of 100 μl were used. Data are mean ± SD from two independent measurements for each time point examined.

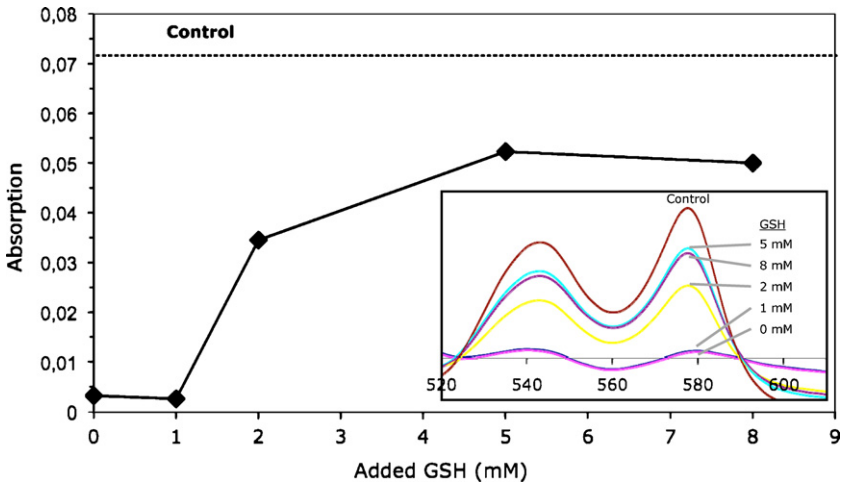


Fig. 13. Effect of exogenous GSH on nitrite-induced methemoglobin formation in lysed human RBC. To 225 μl aliquots of lysed RBC pre-incubated for 1 min at 37 °C in a water bath, 25 μl aliquots of various GSH dilutions were added to reach final GSH concentrations of 0 mM, 2 mM, 5 mM and 8 mM. Then, all samples were treated with 20 μl aliquots of a nitrite stock solution to achieve a final added nitrite concentration of 8 mM and were incubated for further 5 min in the water bath. One sample of lysed RBC, which was not treated with GSH or nitrite and served as a control, was incubated in the water bath in parallel. Reaction was stopped by putting the samples in an ice bath. Samples were diluted with PBS (1:1000, v/v) and spectra were generated between 330 nm and 620 nm (1 cm cuvettes, 50 nm/s, step width 2 nm) on a spectrophotometer model Specord 50 from AnalytikJena (Jena, Germany). *y*-Axis: absorbance at 578 nm. Insertion: spectra within the wavelength window of 520–600 nm. Dotted line indicates the absorbance value of the control sample (no externally added nitrite and GSH).

centrations of nitrite (range 0–10 μM) did not cause changes in erythrocytic glutathione concentrations that could be detected with the present HPLC method. Therefore, the data presented below have been obtained with very high nitrite concentrations. Fig. 11A shows that lysed RBC incubated with nitrite in the range 0–3 mM caused formation of GSSG which amounted to about 25 μM with 3 mM nitrite. Interestingly, at 4 mM nitrite GSSG formation was about 10 times higher than at 3 mM nitrite. Maximum GSSG formation (about 400 μM) in lysed RBC was obtained at 5 mM nitrite. In contrast to lysed RBC, nitrite-induced GSSG formation was much less pronounced in intact erythrocytes, with the maximum GSSG formation being only about 100 μM at 10 mM and 20 mM nitrite (Fig. 11B). The fall in tGSH concentration seen for nitrite concentration ≥4 mM is likely to be due to formation of mixed disulfides with proteins, notably hemoglobin, which have not been analyzed in this study. It is worth mentioning that we noticed a nitrite concentration-dependent change in the color of lysed RBC from light red to dark red indicating methemoglobin formation.

Fig. 12 shows the extent and time course of the concentration of GSSG formed upon incubation of intact and lysed RBC with 20 mM nitrite. Very low extent GSSG formation was noted in intact RBC, which was almost identical in the absence and in the presence of externally added nitrite. Without nitrite addition, GSSG formation in lysed RBC was very low and did not increase considerably upon incubation time. Interestingly, formation of GSSG in lysed erythrocytes was maximum (about 340 μM) immediately after nitrite addition and decreased to about 260 μM after 10 min of incubation. In these experiments total glutathione concentration did not change (data not shown).

It is worth mentioning from the analytical point of view that nitrite in cell-free samples had no effect on GSH and GSSG concentration when measured by the present method (data not shown), so interference by nitrite in the OPA derivatization procedures of our method is very unlikely.

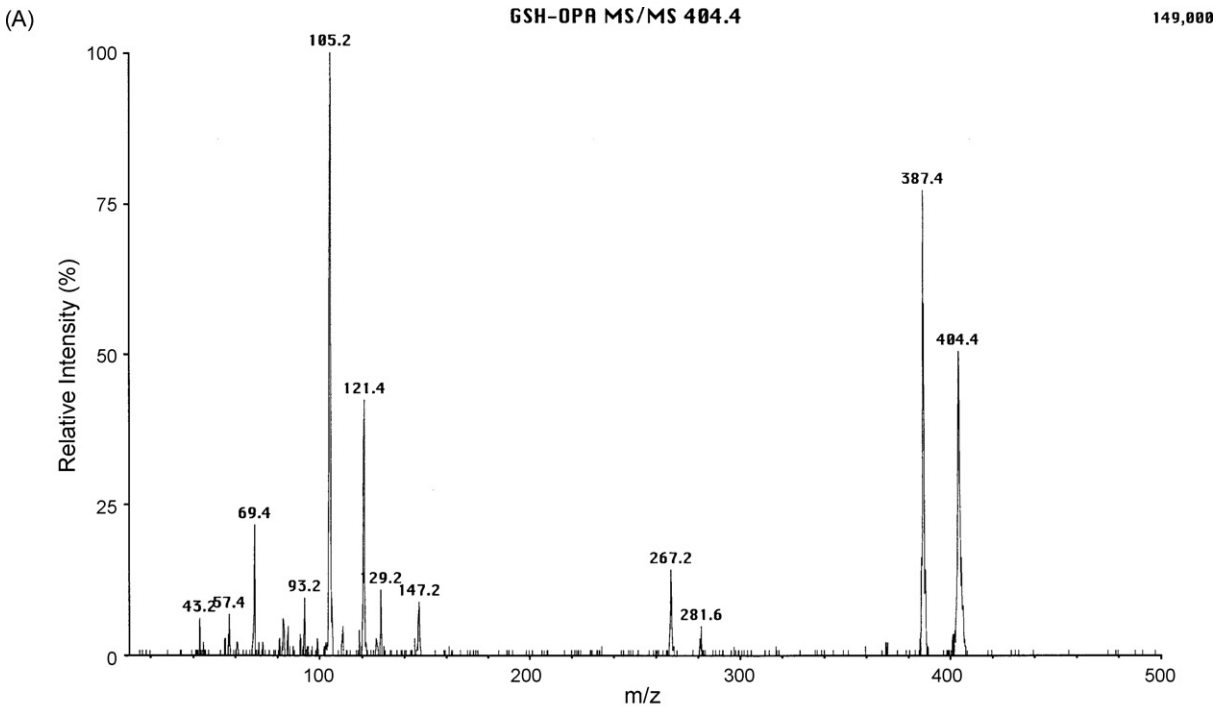
3.4.4. Effects of exogenous GSH on nitrite-induced methemoglobin in lysed RBC

The effects of GSH externally added to lysed human RBC on the nitrite-induced formation of methemoglobin as measured by spectrophotometry are shown in Fig. 13. Formation of methemoglobin is indicated by a decrease of the absorbance between 520 nm and 600 nm, e.g., at the maximum wavelength of 578 nm. In the absence of externally added GSH, methemoglobin formation by nitrite (8 mM) is maximal. Fig. 13 shows that GSH prevents nitrite-induced methemoglobin formation in a concentration-dependent presumably sigmoid manner. It is worth mentioning that the methemoglobinemia-protecting effect of externally added GSH is almost absent, when GSH is added after nitrite addition to lysed RBC (data not shown).

4. Discussion

4.1. Mass spectrometric characterization of the GSH-OPA derivative

Twenty years ago, Neuschwander-Tetri and Roll [3] discovered that the reaction of GSH with OPA under slightly alkaline conditions leads to the formation of a very stable, highly fluorescent tricyclic isoindole derivative (Fig. 1; GSH-OPA). Neuschwander-Tetri and Roll reported that liquid secondary-ion mass spectrometric analysis of synthetic GSH-OPA derivative provided a cation at *m/z* 404 which had been assigned to $[M-1]^+$ corresponding to a molecular weight of 405 ($C_{18}H_{19}N_3O_6S$) [3]. Also, these authors reported that high resolution electron ionization mass spectrometry analysis of the methyl esters of the GSH-OPA derivative was confirmative of these findings [3]. We have performed flow injection +ESI-MS analysis of the GSH-OPA derivative isolated by HPLC and observed a cation at *m/z* 404 $[M-1]^+$ and an additional intense ion at *m/z* 387 [23]. We are assuming that the ion at *m/z* 387 in the +ESI-MS spectrum of the GSH-OPA derivative results from loss of ammonia (NH_3, 17 Da) from $[M-1]^+$ rather than from loss of water (H_2O, 18 Da) from $[M]^+$. +ESI-MS/MS analysis of the ion *m/z* 404 yielded the product ion mass spectrum shown in Fig. 14A. The proposed mechanism for the collision-induced dissociation (CID) of *m/z* 404 is shown in (Fig. 14B). Our data from MS and MS/MS analyses of the GSH-OPA derivative support the structure proposed by Neuschwander-Tetri and Roll [3]. Furthermore, our results suggest putative structures for the cation $[M-1]^+$ of the GSH-OPA derivative with *m/z* of 404 and for the most intense product ions at *m/z* 387 and *m/z* 105 (Fig. 14B).



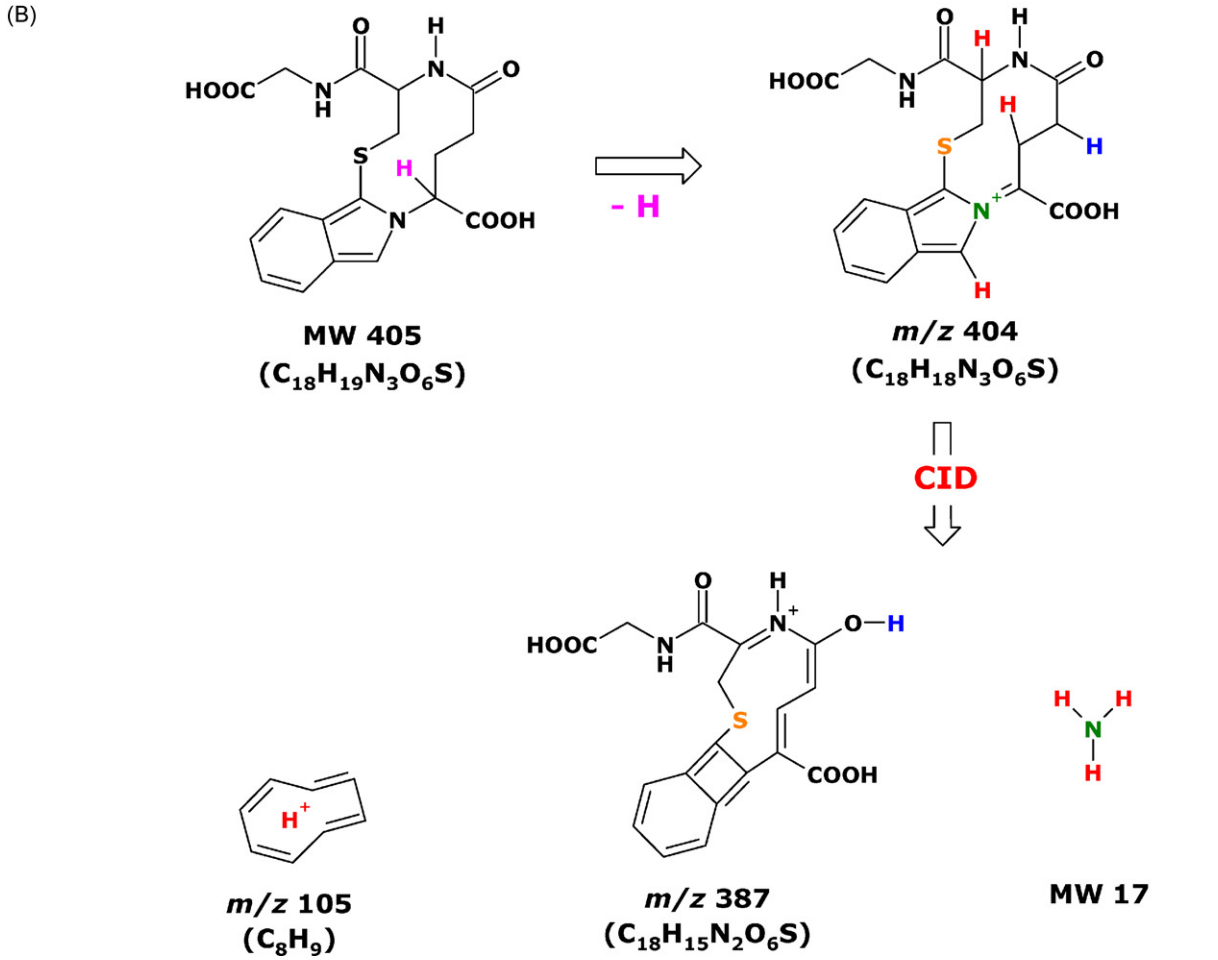


Fig. 14. (A) Electrospray ionization product ion mass spectrum of the GSH-OPA derivative in the positive ion mode. The ion with *m/z* 404 was subjected to collision induced dissociation (CID). (B) Proposed structures for the GSH-OPA derivative, for the ion with *m/z* 404 and the product ions with *m/z* 387 and *m/z* 105.

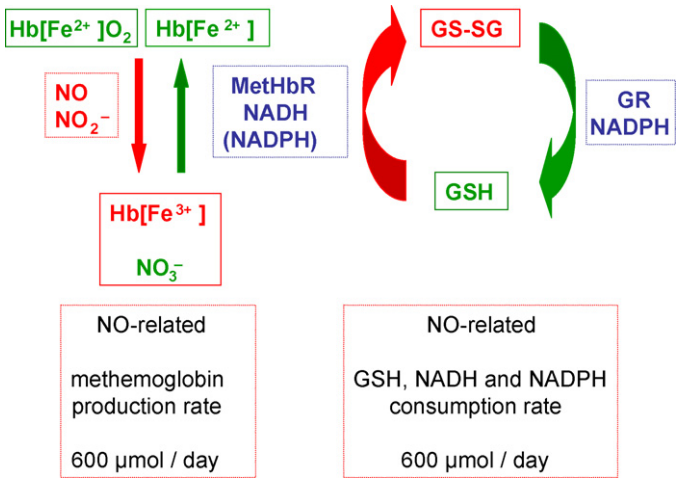


Fig. 15. Proposed mechanism for the involvement of GSH in the reduction of methemoglobin formed during NO and nitrite oxidation to nitrate by oxyhemoglobin. MetHbR, methemoglobin reductase; GR, glutathione reductase.

4.2. Utility of NACET in the analysis of GSH and GSSG in RBC as GSH-OPA derivatives

In consideration of the high specificity of the reaction of GSH with OPA to form the GSH-OPA derivative and the high stability of this derivative in the derivatization mixture, we wanted to apply pre-column OPA derivatization for measuring erythrocytic GSH and GSSG (after reduction to GSH) by the method originally reported by Neuschwander-Tetri and Roll [3]. Unexpectedly, we always observed two HPLC peaks when we analyzed erythrocytic GSH, of which the earlier eluting peak co-eluted with the GSH-OPA derivative prepared with GSH dissolved in pure water or in PBS. Interestingly, treatment of UF samples with NACET prior to OPA derivatization apparently avoided formation of the later eluting peak. Alternatively, NACET could have converted the later eluting compound into the GSH-OPA derivative. Although not definitely delineated, our study shows that GSH present in UF of lysed RBC behaves differently from GSH in aqueous buffered solutions with regard to its reaction with OPA and/or chromatography. Non-consideration of this finding would inevitably lead to erroneous, falsely too low concentrations for erythrocytic GSH with this method. In this work we point out to this potential pitfall and provide a simple and effective solution to overcome this analytical problem, i.e., by adding NACET to the UF prior to OPA derivatization. In consideration of the negligible GSSG concentration in RBC, the method reported here provides a useful analytical means to accurately and precisely measure GSH in RBC. Moreover, this work shows that the method is also useful for the measurement of GSSG in RBC in the presence of an almost 200 molar GSH excess which is alkylated with NEM, unlike GSSG-derived GSH. The GSH and GSSG concentrations measured in RBC of healthy volunteers by the present method are in line with those obtained from use of reliable and artefact-free analytical methods [7]. In the present study we did not investigate whether commonly used disulfide-bond reducing agents such as DTT [3], 2-mercaptoethanol [23] or dithionite [34] are useful in the method reported here.

4.3. Nitric oxide- and nitrite-associated GSH consumption in human RBC

In the present study, we applied the HPLC method to study potential effects of nitrite on erythrocytic GSH. Our working hypothesis was that GSH may be consumed for the reduction of methemoglobin which is produced from the oxidation of NO (Eq. (1)) and nitrite (Eq. (2)) to nitrate by oxyhemoglobin [24]. Considering a daily NO production rate of about 600 μmol by healthy humans [35], it may be calculated that about 10% of erythrocytic GSH would be required daily for regeneration of methemoglobin formed solely due to NO- and nitrite-oxidation in human RBC. Thus, we expected that measurement of GSSG formed upon incubation of RBC with NO or nitrite (Eq. (3)) would be a reliable approach to test our hypothesis.

Preliminary studies showed that intact RBC possess very effective mechanisms to avoid GSH oxidation and/or regenerate GSH from GSSG. By using relevant concentrations of NO and nitrite (up to 10 μM), we were unable to demonstrate by this HPLC method NO/nitrite-induced formation of GSSG in RBC at concentrations clearly above the basal GSSG concentrations (data not shown). Therefore, demonstration of GSSG formation in RBC required use of extraordinarily high mM-concentrations of nitrite and potent oxidants such as H_2O_2 in combination with the use of the non-specific catalase inhibitor sodium azide [2]. Our results show that nitrite can indeed cause GSSG formation in RBC. With regard to the RBC physiology, these findings should be treated with caution. On the other hand, they might be relevant for cases of intoxication with nitrite or for diseases associated with excessive NO production such as in sepsis [35].

It is well known that NO and nitrite oxidation in RBC is accompanied by oxidation of hemoglobin to methemoglobin [24,36]. Also, it has been reported that methemoglobin is reduced back to hemoglobin, with GSH being oxidized to GSSG [26]. Formation of mixed disulfides of GSH with proteins including hemoglobin (HbSSG) has been observed by other investigators [33,37,38]. Measurement of HbSSG should be possible with the method reported here, for instance by adding NACET to the protein fraction of lysed RBC, subsequent ultrafiltration, and HPLC analysis of the UF, but measurement of HbSSG was beyond the aim of the present study. Nevertheless, our findings suggest considerable formation of HbSSG in lysed RBC incubated with nitrite and hydrogen peroxide. The recent findings by Aldini et al. [33] are supportive of the results of the present study.

The considerable differences between lysed and intact RBC with respect to nitrite-induced GSSG formation are likely to be due to involvement of more effective enzyme systems for conversion of GSSG and mixed glutathione disulfides back to GSH in intact as compared to lysed erythrocytes. Also, because of the instantaneous formation of GSSG in lysed erythrocytes incubated with nitrite, the low-extent formation of GSSG in intact RBC upon incubation with nitrite could be in part due to the limited transport of nitrite across the erythrocyte cell membrane. Indeed, May et al. [39] found that 0.8 mM nitrite are uptaken by RBC with a half-life of 11 min. The time gained by the limitation of nitrite transport through the intact RBC membrane is obviously satisfactory enough to fully regenerate GSSG to GSH within the RBC. In this context, performance of investigations on lysed RBC, for instance in the framework of oxidative stress, seems not to be very meaningful.

It is known for several decades that human RBC possess NADH- and NADPH-dependent methemoglobin reductase activity (NADH-cytochrome b_5 reductase; EC 1.6.2.2.) [40]. Interestingly, Stock and Smith have reported that methemoglobin formation in RBC requires high nitrite concentrations and that methemoglobin reductase activity is virtually abolished in lysed RBC [40]. These findings are supportive of the remarkable differences we found in the present study from the use of intact and lysed RBC regarding GSSG kinetics. As reduction of GSSG to GSH as well as of methemoglobin to hemoglobin in RBC requires NADH and NADPH as cofactors [40] (Fig. 15), and considering a daily NO production rate of about 600 μmol [35], it can be estimated that under basal conditions about 10% of erythrocytic GSH would be required for hemoglobin regeneration. In turn, these processes would be associated with

considerable consumption of the cofactors NADH and NADPH in RBC, which are generated by glycolysis and hexose monophosphate shunt metabolism, respectively [40,41]. To the best of our knowledge, these potential ramifications have not been addressed satisfactorily so far, and are worth of more detailed investigation in the future. Because the mechanisms of nitrite-related GSH oxidation in erythrocytes are not fully elucidated, mechanistic studies and measurement of wider a spectrum of analytes are advisable and necessary.

References

[1] P.C. Jocelyn, Biochemistry of the SH Group, Academic Press, 1972.
[2] B. Halliwell, J.M.C. Gutteridge, Free Radicals in Biology and Medicine, third edition, Oxford University Press, 1999.
[3] B.A. Neuschwander-Tetri, F.J. Roll, Anal. Biochem. 179 (1989) 236.
[4] M.A. Mansoor, A.M. Svardal, P.M. Ueland, Anal. Biochem. 200 (1992) 218.
[5] E. Camera, M. Picardo, J. Chromatogr. B 781 (2002) 181.
[6] D. Giustarini, I. Dalle-Donne, R. Colombo, A. Milzani, R. Rossi, Free Radic. Biol. Med. 35 (2003) 1365.
[7] R. Rossi, A. Milzani, I. Dalle-Donne, D. Giustarini, L. Lusini, E. Colombo, P. Di Simplicio, Clin. Chem. 48 (2002) 742.
[8] R. Rossi, I. Dalle-Donne, A. Milzani, D. Giustarini, Clin. Chem. 52 (2006) 1406.
[9] J.S. Bains, C.A. Shaw, Brain Res. Brain Res. Rev. 25 (1997) 335.
[10] A. Jain, J. Martensson, E. Stole, P.A. Auld, A. Meister, Proc. Natl. Acad. Sci. U.S.A. 88 (1991) 1913.
[11] G.A. Keller, R. Barke, J.T. Harty, E. Humphrey, R.L. Simmons, Arch. Surg. 120 (1985) 941.
[12] J. Martensson, A. Jain, E. Stole, W. Frayer, P.A. Auld, A. Meister, Proc. Natl. Acad. Sci. U.S.A. 88 (1991) 9360.
[13] I. Nemeth, D. Boda, Biomed. Biochim. Acta 48 (1989) S53.
[14] O. Paamoni-Keren, T. Silberstein, A. Burg, I. Raz, M. Mazor, O. Saphier, A.Y. Weintraub, Arch. Gynecol. Obstet. 276 (2007) 43.
[15] I. Rahman, W. MacNee, Eur. Respir. J. 16 (2000) 534.
[16] J.B. Schulz, J. Lindenau, J. Seyfried, J. Dichgans, Eur. J. Biochem. 267 (2000) 4904.
[17] M.B. Spina, G. Cohen, Proc. Natl. Acad. Sci. U.S.A. 86 (1989) 1398.
[18] T.E. Meyer, H.Q. Liang, A.R. Buckley, D.J. Buckley, P.W. Gout, E.H. Green, A.M. Bode, Int. J. Cancer 77 (1998) 55.
[19] T. Schnelldorfer, S. Gansauge, F. Gansauge, S. Schlosser, H.G. Beger, A.K. Nussler, Cancer 89 (2000) 1440.
[20] A. Sharma, B. Patrick, J. Li, R. Sharma, P.V. Jeyabal, P.M. Reddy, S. Awasthi, Y.C. Awasthi, Arch. Biochem. Biophys. 452 (2006) 165.
[21] H. Liu, H. Wang, S. Shenvi, T.M. Hagen, R.M. Liu, Ann. N.Y. Acad. Sci. 1019 (2004) 346.
[22] I. Dalle-Donne, R. Rossi, R. Colombo, D. Giustarini, A. Milzani, Clin. Chem. 52 (2006) 601.
[23] D. Tsikas, J. Sandmann, D. Holzberg, P. Pantazis, M. Raida, J.C. Frölich, Anal. Biochem. 273 (1999) 32.
[24] Å. Wennmalm, G. Benthin, A. Edlund, L. Jungersten, N. Kieler-Jensen, S. Lundin, U. Nathorst Westefelt, A.-S. Petersson, F. Waagstein, Circ. Res. 73 (1993) 1121.
[25] J. Takahashi, Comp. Biochem. Physiol. B 76 (1983) 1.
[26] I. Stepuro, N. Chaikovskaya, T. Piletskaya, A. Solodunov, Pol. J. Pharmacol. 46 (1994) 601.
[27] D. Tsikas, S. Dehnert, K. Urban, A. Surdacki, H.H. Meyer, J. Chromatogr. B. 877 (2009) 3442.
[28] E. Skrzydlewska, R. Farbiszewski, Drug Alcohol Depend. 57 (1999) 61.
[29] J. Traber, M. Suter, P. Walter, C. Richter, Biochem. Pharmacol. 43 (1992) 961.
[30] L. Grinberg, E. Fibach, J. Amer, D. Atlas, Free Radic. Biol. Med. 38 (2005) 136.
[31] C. Cereser, J. Guichard, J. Drai, E. Bannier, I. Garcia, S. Boget, P. Parvaz, A. Revol, J. Chromatogr. B 752 (2001) 123.
[32] R. Rossi, A. Milzani, I. Dalle-Donne, F. Giannerini, D. Giustarini, L. Lusini, R. Colombo, P. Di Simplicio, J. Biol. Chem. 276 (2001) 7004.
[33] L. Regazzoni, A. Panusa, K.J. Yeum, M. Carini, G. Aldini, J. Chromatogr. B 877 (2009) 3456.
[34] A.P. Senft, T.P. Dalton, H.G. Shertzer, Anal. Biochem. 280 (2000) 80.
[35] D. Tsikas, F.-M. Gutzki, D.O. Stichtenoth, Eur. J. Clin. Pharmacol. 62 (2006) 51.
[36] M.P. Doyle, J.G. Herman, R.L. Dykstra, Free Radic. Biol. Med. 1 (1985) 145.
[37] J.J. Mieyal, D.W. Starke, S.A. Gravina, C. Dothey, J.S. Chung, Biochemistry 30 (1991) 6088.
[38] B.R. Shivakumar, S.V. Kolluri, V. Ravindranath, J. Pharmacol. Exp. Ther. 274 (1995) 1167.
[39] J.M. May, Z.C. Qu, L. Xia, C.E. Cobb, Am. J. Physiol. Cell Physiol. 279 (2000) C1946.
[40] J.M. Stolk, R.P. Smith, Biochem. Pharmacol. 15 (1966) 343.
[41] J.H. Ströme, L. Eldjarn, Biochem. J. 84 (1962) 406.