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Artifactual-free analysis of *S*-nitrosoglutathione and *S*-nitroglutathione by neutral-pH, anion-pairing, high-performance liquid chromatography Study on peroxynitrite-mediated *S*-nitration of glutathione to *S*-nitroglutathione under physiological conditions

Dimitrios Tsikas*, Kristina Denker, Jürgen C. Frölich

Institute of Clinical Pharmacology, Hannover Medical School, Carl-Neuberg-Str. 1, D-30623 Hannover, Germany

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Abstract

The endogenous potent vasodilators and inhibitors of platelet aggregation *S*-nitrosoglutathione (GSNO) and *S*-nitroglutathione ($GSNO_2$) are frequently analyzed by high-performance liquid chromatography (HPLC) using mobile phases of acidic pH. These systems are associated with problems stemming from rapid and considerable artifactual formation of GSNO from glutathione (GSH) and ubiquitous nitrite. We describe a novel ion-pairing HPLC method with UV absorbance detection at 334 nm for the highly specific and interference-free analysis of GSNO and $GSNO_2$ in the presence of high GSH and nitrite concentrations. Complete avoidance of artifactual formation of GSNO was accomplished by using the anion-pairing agent tetrabutylammoniumhydrogen sulphate in the mobile phase that enables analysis of GSNO at neutral pH, at which GSH and nitrite do not react to form GSNO. This HPLC system was used to study formation of $GSNO_2$ from GSH and peroxynitrite under physiological conditions. We found by this HPLC system that peroxynitrite (0–300 μM) reacts with GSH (0–5 m*M*) to form $GSNO_2$ at a mean yield of 2%. Analysis of the same samples by a cation-pairing HPLC system with acidic mobile phase (pH 2.0) revealed, however, GSNO plus $GSNO_2$ formation of the order of 20% due to on column reaction of GSH with peroxynitrite-derived nitrite to form GSNO. Ammonium sulfamate is frequently used to remove nitrite from thiol-containing solutions under acidic conditions. By means of the anion-pairing HPLC system it is demonstrated that nitrite removal by this method is incomplete even when ammonium sulfamate is used at high concentrations. These findings underscore the absolute requirement of neutral pH conditions for the analysis of GSNO. The novel anion-pairing HPLC method should be useful to provide reliable data on formation, reaction and metabolism of GSNO and $GSNO_2$ in biological fluids using various detectors including mass spectrometers.

Keywords: Nitrosoglutathione; Nitroglutathione; Glutathione; Peroxynitrite; Organosulfur compounds

1. Introduction

Nitric oxide synthases (NOSs, EC 1.14.13.39) are a family of enzymes which catalyzes the oxidation of L-arginine to nitric oxide (·NO) and citrulline [1].

*Corresponding author. Tel.: +49-511-532-3959; fax: +49-511-532-2750.

E-mail address: tsikas.dimitros@mh-hannover.de (D. Tsikas).

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·NO plays a critical role in signal transduction pathways in the cardiovascular and nervous systems and is a key component of the cytostatic/cytotoxic function of the immune system [2,3]. The amino acid cysteine and cysteine-containing peptides and proteins such as glutathione, albumin and haemoglobin are targets for ·NO and its various derivatives including NO_2, N_2O_3 and peroxynitrite ($ONOO^-$). Peroxynitrite is a potent oxidant that can be formed in biological systems from the reaction of ·NO with superoxide ($O_2^{\cdot -}$) [4]. Since both ·NO and superoxide may be produced simultaneously by NOSs [5], the chemistry of the reaction of the cysteinyl sulfhydryl group with ·NO and ·NO-derived species is very complex [6], and assignment of the respective reaction products is very difficult.

S-Nitroso (RSNO) and *S*-nitro ($RSNO_2$) compounds are two potential classes of compounds that can be formed from the reaction of the cysteinyl sulfhydryl group with ·NO and peroxynitrite. These compounds exhibit ·NO-like biological actions such as vasodilation and inhibition of platelet aggregation (reviewed in [7]). *S*-Nitrosocysteine (SNOC) and *S*-nitrosoglutathione (GSNO) have been reported to be physiological low-molecular-mass (LMM) *S*-nitroso compounds [8–10]. SNOC has been suggested to be a putative endothelium-derived relaxing factor [11]. GSNO has been detected in human bronchial lavage [9]. Also, GSNO has been reported to be formed in vitro in incubation mixtures of NOSs in the presence of reduced glutathione (GSH) [12,13]. However, in all these studies no unequivocal evidence has been reported for the identity of these compounds as SNOC or GSNO.

It is generally believed that GSNO is formed from the reaction of GSH with peroxynitrite [12–16]. However, it has been recently shown by electrospray ionization mass spectrometry (ESI–MS) that the reaction product between peroxynitrite and glutathione is *S*-nitroglutathione ($GSNO_2$) but not *S*-nitrosoglutathione (GSNO) [17]. GSNO and $GSNO_2$ have been reported to possess almost identical physicochemical properties and to co-elute in various high-performance liquid chromatographic (HPLC) systems [17]. It can be expected that *S*-nitroso and *S*-nitro derivatives of other thiols such as cysteine would also co-elute in all HPLC systems. These findings underscore the pressing need of analytical approaches allowing discrimination between *S*-nitroso and *S*-nitro compounds. These observations make mandatory to re-investigate formation, reaction and metabolism of *S*-nitroso and *S*-nitro compounds using specific and artifactual-free analytical methods. ESI–MS in combination with HPLC seems to be the most promising technology in this area of research [17,18]. Alternatively, GC–MS can be used to discriminate indirectly between *S*-nitroso and *S*-nitro compounds [19].

The most challenging analytical task in the field of *S*-nitroso and *S*-nitro compounds research is the development of specific and interference-free HPLC methods. Today, HPLC analysis of intact *S*-nitroso and *S*-nitro compounds is only possible with mobile phases of low pH ranging between 2 and 4 [11–13,17,20,21]. In thiol- and nitrite-rich matrices artifactual formation of considerable amounts of *S*-nitroso compounds during sample preparation under acidic conditions and analysis by these HPLC methods may occur. Indeed, Kluge et al. have reported considerable artifactual formation of GSNO from GSH and nitrite present in rat cerebral extracts despite the use of ammonium sulfamate to remove nitrite under acidic conditions [10]. As *S*-nitroso compounds occur in biological fluids and tissues at very low concentrations [7], artifactual formation of these compounds from nitrite and thiols even of low extent may reach or exceed endogenous levels, and must, therefore, be completely avoided.

The aim of the present study was, therefore, to develop a HPLC method that would enable specific and artifactual-free analysis of *S*-nitroso and *S*-nitro compounds. In previous work we showed that anion-pairing HPLC is useful for the analysis of GSH conjugates of aromatic compounds with electrophilic centers [22]. GSNO and $GSNO_2$ can be regarded as the GSH conjugates of the electrophilic species ^+NO and $^+NO_2$, respectively. Since GSNO and $GSNO_2$ are simply negatively charged within a large pH-range (Fig. 1), we tested the applicability of anion-pairing HPLC for GSNO and $GSNO_2$ analysis. Indeed, we developed a simple and rapid anion-pairing HPLC which uses a mobile phase of pH 7.0 and enables artifactual-free analysis of GSNO. The usefulness of this method for the analysis of GSNO and $GSNO_2$ was demonstrated by studying $GSNO_2$ formation from the reaction between GSH and

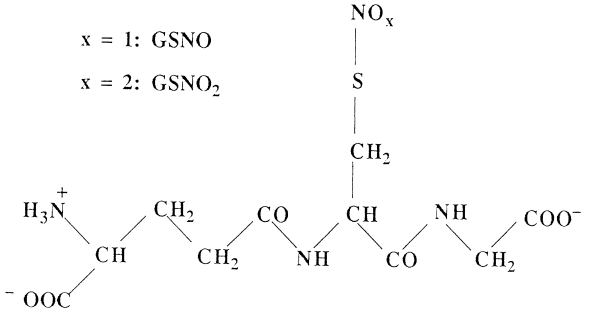

Fig. 1. Chemical structures of GSNO and $GSNO_2$ at neutral pH.

peroxynitrite under physiological pH conditions. This method was compared with a previously reported cation-pairing HPLC method that uses an acidic mobile phase [20].

2. Experimental

2.1. Materials

The sodium salts of [^{15}N]nitrite and [^{15}N]nitrate (both 98.5% at ^{15}N) were purchased from Cambridge Isotope Labs (Andover, MA, USA). The sodium salts of nitrite and nitrate were bought from Riedel-de Haen (Seelze, Germany). 2,3,4,5,6-Pentafluorobenzyl (PFB) bromide, 1-octanesulfonic acid (sodium salt) and nitronium tetrafluoroborate were purchased from Aldrich (Steinheim, Germany). Glutathione in the reduced form, i.e., GSH, ammonium formate and ammonium sulfamate were obtained from SIGMA (Deisenhofen, Germany). Tetramethylammonium peroxynitrite was obtained from Alexis Deutschland (Grünberg, Germany) as a 13.1 m*M* solution in 10 m*M* KOH and stored aliquoted at −80°C. This preparation was declared to contain less than 0.2% nitrite with respect to peroxynitrite. Dilutions of the stock solutions were performed in ice-cold 10 m*M* KOH, stored on ice in the dark during the experiments and used once without re-refregerating. Tetrabutylammoniumhydrogen sulfate (TBAHS) and all other chemicals were bought from Merck (Darmstadt, Germany).

Stock solutions (4.8 m*M*) of GSNO were prepared by mixing equal volumes of 10 m*M* solutions of nitrite and GSH in deoxygenated distilled water, acidification with a 1/10th volume of 5 *M* HCl and incubation for 5 min at room temperature [20]. These stock solutions were found not to contain nitrite by an assay based on the Griess reaction [20] nor reduced thiols by the method of Ellman [23]. The UV–Vis spectrum of GSNO showed characteristic maximum absorption at 334 nm with a molar absorptivity coefficient of 780 M^{-1} cm^{-1}. The structure of GSNO prepared by this procedure was elucidated by flow injection analysis ESI–MS as described previously [18]. Intense ions were observed at m/z 307 ($[(M+H)-NO]^+$, 35%), 337 ($[M+H]^+$, 100%), 359 ($[M+Na]^+$, 45%), 613 $[(2M+H)-2NO]^+$, 15%), 673 $[2M+H]^+$, 30%) and 695 $[2M+Na]^+$, 15%). $GSNO_2$ was synthesized from GSH and nitronium tetrafluoroborate as described [17]. A 200-μl aliquot of the reaction mixture was injected into the HPLC system A1 (see below), the fraction with the retention time of GSNO was collected on ice and analyzed immediately by UV–Vis spectroscopy, GC–MS (see below) and ESI–tandem MS. UV–Vis spectra of $GSNO_2$ and GSNO were virtually identical, showing maximum absorption at 334 nm for both compounds (data not shown). Similar UV–Vis spectra of $GSNO_2$ and GSNO have been reported by Balazy et al. [17]. $GSNO_2$ concentrations were determined by using a molar absorptivity coefficient of 980 M^{-1} cm^{-1} [17]. Unlike GSNO [18], collision-activated dissociation (collision energy of 25 eV) of m/z 353 ($[M+H]^+$) of $GSNO_2$ did not result in significant fragmentation. This finding agrees with that reported by Balazy et al. [17].

2.2. High-performance liquid chromatography

HPLC analyses were performed on two Pharmacia LKB pumps model 2150 (Freiburg, Germany) coupled with a variable-wavelength UV–Vis LKB detector model 2151 (Bromma, Sweden) or a variable-wavelength UV–Vis LDC spectromonitor model 1204D (LDC Analytical, Gelnhausen, Germany) both connected with a Shimadzu integrator model C-R3A (Kyoto, Japan). The chromatographic columns (250×4.6 mm I.D.) used were packed with Nucleosil 100-5C_{18}, 5 μm particle size, from Macherey–Nagel (Düren, Germany). Three HPLC systems were used. The mobile phase of HPLC system

A1 consisted of 10 m*M* of K_2HPO_4, 10 m*M* TBAHS and 1 m*M* EDTA in acetonitrile–water (5:95, v/v) the pH of which was adjusted to 7.0 by addition of 6.5 *M* NaOH. EDTA was used to stabilize GSNO and $GSNO_2$. The mobile phase of HPLC system A2 was the same as in HPLC system A1 with the exception of ammonium formate (10 m*M*) which was used instead of K_2HPO_4. The mobile phase of HPLC system B consisted of 10 m*M* of NaH_2PO_4 and 1-octanesulfonic acid each in acetonitrile–water (15:85, v/v) the pH of which was adjusted to 2.0 by addition of ortho-phosphoric acid. This HPLC system has been previously described in detail [20]. Isocratic runs were carried out at a flow-rate of 1.0 ml/min, and the effluent was monitored at 334 nm in all HPLC systems. The concentrations of $GSNO_2$ in the respective experiments were determined by using standard curves of GSNO (0–100 μ*M*) in Tris buffer (0.2 *M*, pH 7.4) and considering the 1.2-fold higher molar absorptivity at 334 nm of $GSNO_2$ compared with GSNO [17]. Linear regression analysis between peak area or peak height and GSNO concentration revealed high regression coefficients with all HPLC systems (r^2=0.991) (see below).

2.3. Reactions of peroxynitrite in Tris buffer in the absence and in the presence of glutathione

Solutions of GSH (0–5 m*M*) in 0.2 *M* Tris buffer, pH 7.4, were treated at room temperature with ice-cold solutions of peroxynitrite (1 or 2 m*M*) in 10 m*M* KOH, and 200-μl aliquots thereof were injected into the HPLC systems A1 and B after short vortexing. In all investigations addition of alkaline peroxynitrite solutions did not change the pH value of the buffered GSH solutions.

Decomposition of peroxynitrite in 0.2 *M* Tris buffer, pH 7.4, was investigated by adding various concentrations of peroxynitrite to the buffer and incubating for 60 min to guarantee complete degradation of peroxynitrite. Each three 100-μl aliquots of these solutions in 1.5–ml glass vials were spiked with a mixture of [^{15}N]nitrite and [^{15}N]nitrate (each at 20 μ*M*), treated with acetone (400 μl) and PFB bromide (10 μl) and samples were heated at 50°C for 60 min to form the PFB derivatives of nitrite and nitrate [19]. After acetone evaporation under nitrogen and extraction with toluene (1 ml) the organic phase was transferred into 1.5–ml glass vials. Aliquots (0.5 μl) were injected in the splitless mode into the GC–MS system and analyzed for nitrite and nitrate as described below.

2.4. Gas chromatography–mass spectrometry

GC–MS was carried out on a Hewlett-Packard MS Engine 5989A connected directly to a gas chromatograph 5890 series II (Waldbronn, Germany). A fused-silica capillary column DB-5 MS (30 m×0.25 mm I.D., 0.25-μm film thickness) from J and W Scientific (Rancho Cordova, CA, USA) was used. Helium (70 kPa) and methane (200 Pa) were used as the carrier and the reagent gas, respectively, for negative-ion chemical ionization. The interface, injector, ion source and quadrupole were kept at 280, 200, 225, and 120°C, respectively. The column was held at 70°C for 1 min and then increased to 280°C at a rate of 30°C/min. Selected ion monitoring (SIM) of the ions at *m/z* 46 ($[^{14}NO_2]^-$) for [^{14}N]nitrite, *m/z* 47 ($[^{15}NO_2]^-$) for [^{15}N]nitrite, *m/z* 62 ($[^{14}NO_3]^-$) for [^{14}N]nitrate and *m/z* 63 ($[^{15}NO_3]^-$) for [^{15}N]nitrate was carried out. Linear relationships between the peak area ratio of *m/z* 46 to 47 (*y*1) or *m/z* 62 to 63 (*y*2) and the concentration of nitrite (*x*1) or nitrate (*x*2) in the range 0–100 μ*M* in 0.2 *M* Tris buffer, pH 7.4, were observed: $y1=0.112+0.05x1$ ($r^2=1.000$) and $y2=0.206+0.04x2$ ($r^2=0.998$). The concentration of the internal standards [^{15}N]nitrite and [^{15}N]nitrate was 20 μ*M* each.

3. Results

3.1. High-performance liquid chromatography

Fig. 2 shows chromatograms from the HPLC analysis of Tris buffer alone (A), of synthetic GSNO (B) and $GSNO_2$ (D) in Tris buffer, of a reaction mixture of GSH and peroxynitrite in Tris buffer using HPLC systems A1 and A2 (E), and of GSNO diluted in the respective mobile phase (C). This figure clearly shows that GSNO and $GSNO_2$ co-elute in both HPLC sytems. The other peaks eluting in front of GSNO and $GSNO_2$ were observed from

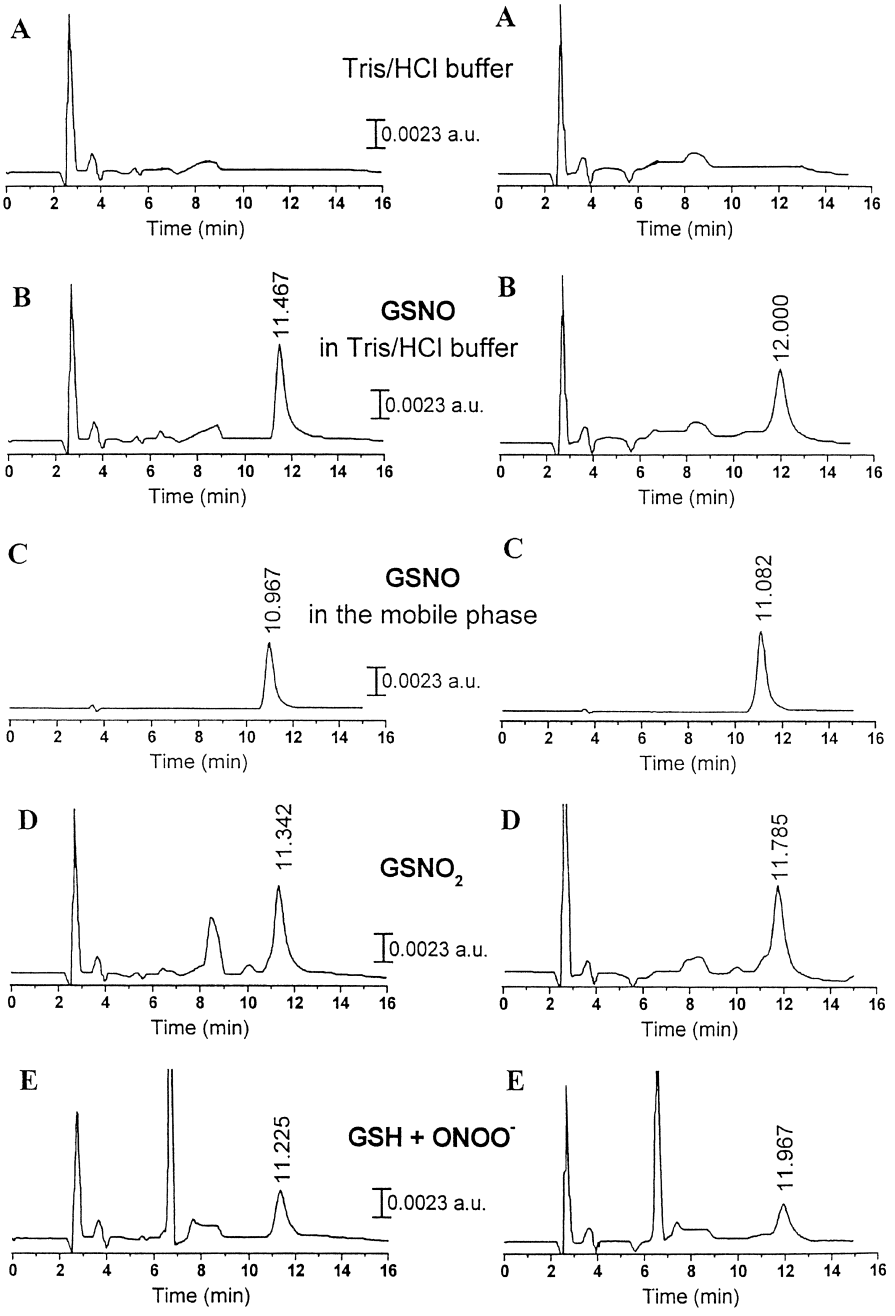

Fig. 2. HPLC chromatograms from the analysis of (A) 0.2 *M* Tris buffer, pH 7.4, of synthetic GSNO diluted (B) in 0.2 *M* Tris buffer, pH 7.4, and (C) in the respective mobile phase, (each 20 μ*M*), (D) of synthetic $GSNO_2$ (24 μ*M*), and (E) of aliquots injected 1 min after addition of peroxynitrite (2 m*M*, 37 μl) to 0.2 *M* Tris buffer, pH 7.4, (213 μl), that contained GSH (5 m*M*); the concentration of $GSNO_2$ formed was calculated to 11 μ*M* (system A1) and 10 μ*M* (system A2). HPLC systems A1 (left panel) and A2 (right panel) were used; the flow-rate was 0.9 ml/min in both systems. The LDC spectromonitor model 1204D was set at 334 nm. Aliquots (200 μl) were injected from each sample. The attenuation of the integrator was 2^8 in all analyses. Inserted vertical bars indicate quantitative absorption in absorbance units (a.u.).

injection of solutions of these compounds in Tris buffer (Fig. 2A, C, and D) but not when solutions in the mobile phase were injected (Fig. 2B). These observations agree with those obtained on other HPLC systems [17]. The retention time of GSNO in HPLC systems A1 and A2 obtained from injections (200 μl) of standard solutions (0–100 μ*M*) in 0.2 *M* Tris buffer, pH 7.4, was determined as (mean±S.D., n=15) 9.73±0.014 min (R.S.D. 0.142%) and 9.72±0.030 min (R.S.D. 0.34%), respectively. In HPLC system B, GSNO and $GSNO_2$ diluted in Tris buffer eluted as double peaks at (mean±S.D., $n=3$) 7.3±0.08 and 10.9±0.12 min. Standard curves were generated for GSNO in Tris buffer by using HPLC systems A1 and A2. Linear regression between peak area (y) and concentration of GSNO (x) in Tris buffer revealed the regression equations $y=3066+10\,700x$ ($r^2=0.995$) for HPLC system A1 and $y=1530+9950x$ ($r^2=0.992$) for HPLC system A2. The lowest GSNO concentration injected, i.e., 1 μ*M*, was detected at a signal-to-noise ratio of 7:1 and a precision (R.S.D.) of 3.8% with HPLC system A1 (LKB detector model 2151). The detection limit for GSNO using HPLC system B was previously determined as 0.5 μ*M* (signal-to-noise ratio of 3:1) [24]. The retention time and the peak area from injection (200 μl) of 5-μ*M* solutions of GSNO in Tris buffer on ten consecutive working days were determined with a precision (R.S.D.) of 0.342% and 1.56%, respectively, using HPLC system A1. The retention times of nitrite and nitrate in this HPLC system (detected at 210 nm, no EDTA in the mobile phase) were 6.8 and 9.1 min, respectively.

3.2. Decomposition of peroxynitrite in Tris buffer to nitrite and nitrate

GC–MS analysis of solutions of peroxynitritre in Tris buffer showed that they contained nitrite and nitrate. Linear regression analysis between nitrite concentration ($y1$) or nitrate concentration ($y2$) measured and peroxynitrite concentration (x) added resulted in the regression equations $y1=5.2+1.247x$ ($r^2=0.998$) and $y2=10.6+1.095x$ ($r^2=0.977$), respectively. The mean yield of nitrite and nitrate from peroxynitrite (0–100 μ*M*) in Tris buffer were 125 and 110%, respectively. Thus, the total yield of nitrite and nitrate amounted to 235%, i.e., it is 2.3 times greater than the expected yield. Similar results have been reported for the decomposition of peroxynitrite in 0.1 *M* phosphate buffer, pH 7.0, in the absence and presence of carbonate [25]. Uppu et al. have assumed that peroxynitrite decomposed exclusively to nitrate via isomerization, and that the concentration of nitrite in peroxynitrite preparations decomposed in buffers represented the contamination of nitrite in the original preparation [25]. In their study, Uppu et al. have used peroxynitrite which was synthesized by ozonation of an aqueous solution of sodium azide at pH 12. In our study we used a commercial preparation of peroxynitrite as tetramethylammonium salt, which was declared to contain less than 0.2% nitrite with respect to peroxynitrite.

3.3. Peroxynitrite-mediated conversion of GSH to $GSNO_2$

The concentration of $GSNO_2$ formed from the reaction between peroxynitrite and GSH in Tris buffer at pH 7.4 was found to linearly increase with increasing peroxynitrite concentration (Fig. 3). Linear regression analysis between the concentration of $GSNO_2$/GSNO (y) measured and added peroxynitrite concentration (x) resulted in the regression equations $y=1.85+0.176x$ ($r^2=0.987$) for HPLC system B, and $y=-0.288+0.0186x$ ($r^2=0.991$) for HPLC system A1. Thus, by means of HPLC system A1, about 1.9% of peroxynitrite were found to be converted to $GSNO_2$ (Fig. 3A). However, analysis of the same samples by HPLC system B revealed that about 18% of peroxynitrite were converted to $GSNO_2$ (Fig. 3B). Moro et al. have reported a maximal yield of 1–3% of GSNO from GSH (1 m*M*) and peroxynitrite (0–1 m*M*) in phosphate buffer as measured by HPLC [15]. However, these authors did not present conclusive evidence for the formation of GSNO from GSH and peroxynitrite, and, as we now know, they in fact generated $GSNO_2$ [17].

We repeated the above experiment with nitrite (0–100 μ*M*) instead of peroxynitrite. By means of HPLC system A1, no GSNO could be detected above the limit of detection, i.e., 0.5 μ*M* (not shown). By means of HPLC system B, however,

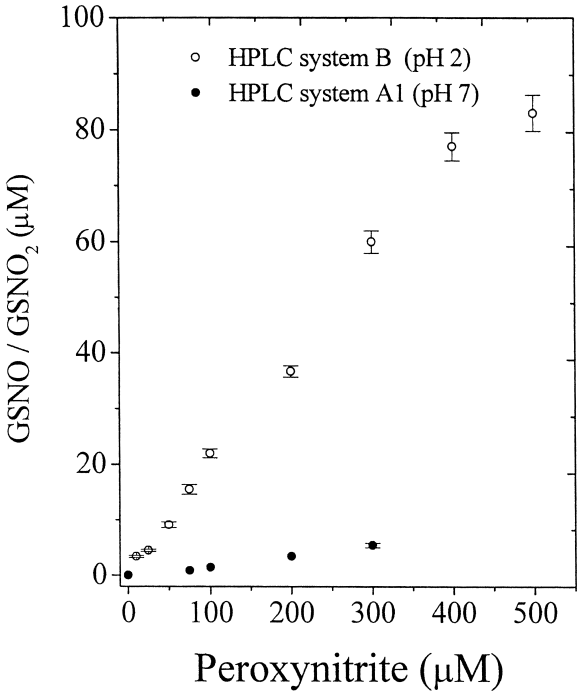

Fig. 3. Peroxynitrite-mediated formation of $GSNO_2$ from GSH (5 m*M*) in 0.2 *M* Tris buffer, pH 7.4, as observed from the use of the novel HPLC system, i.e. HPLC system A1, and of a previously described cation-pairing HPLC system with a mobile phase of pH 2.0, i.e., HPLC system B [20]. Immediately after addition of peroxynitrite at the indicated concentrations to the GSH-containing Tris buffer, each 200-μl aliquots were taken and analyzed by the two HPLC systems in parallel.

GSNO concentration increased linearly with increasing nitrite concentration (not shown). The mean yield of GSNO from nitrite was determined as 27%. These data suggest that the major part of $GSNO_2$ measured by HPLC system B is in fact GSNO, which was artifactually formed from the reaction between GSH and peroxynitrite-derived nitrite during HPLC analysis.

3.4. Formation of GSNO during elimination of nitrite by ammonium sulfamate

When analyzing *S*-nitroso compounds in biological fluids under acidic aconditions, elimination of thiols and/or nitrite from the matrix is a frequently used method to avoid acid-catalyzed formation of *S*-nitroso compounds from the reaction of thiols with nitrite [10,13]. Ammonium sulfamate is commonly used to eliminate nitrite via its reduction to nitrogen gas [10,13]. This reaction takes place under acidic conditions so that artifactual formation of *S*-nitroso compounds cannot be excluded during this procedure [10]. The possibility to analyze *S*-nitroso compounds by HPLC under neutral pH conditions offers us the opportunity to discriminate between formation of *S*-nitroso compounds during sample treatment and HPLC analysis. Under experimental conditions very similar to those used by Mayer et al. [13] for the elimination of nitrite in 50 m*M* phosphate buffered solutions (pH 7) by ammonium sulfamate, we investigated artifactual formation of GSNO from GSH and nitrite by means of the novel HPLC method (HPLC system A1) by using three different concentrations of ammonium sulfamate and acidification of the buffer to pH 3 [13]. Fig. 4 clearly shows that artifactual formation of GSNO occurs in any case in a time-dependent manner during this procedure. Even the use of ammonium sulfamate at the very high concentration of 10 m*M* cannot completely avoid artifactual formation of GSNO: the maximum extent of artifactual GSNO formation under this

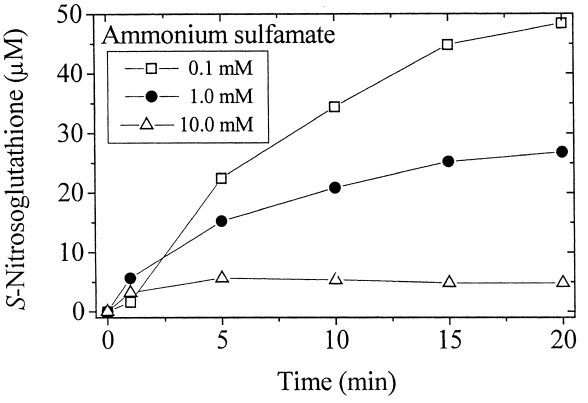

Fig. 4. Time-dependent formation of GSNO from GSH (1 m*M*) and nitrite (100 μ*M*) in 50 ml of 50 m*M* phosphate buffer, pH 7.0, after addition under stirring of ammonium sulfamate to achieve final concentrations of 0.1, 1.0, and 10 m*M*, respectively, and acidification to pH 3.0 by phosphoric acid. This procedure is almost identical to that previously described by Mayer et al. [13]. Samples (200 μl) were analyzed with HPLC system A1 (pH 7.0).

condition amounts to about 5% with respect to nitrite initially present.

4. Discussion

HPLC is frequently used in the field of *S*-nitroso compound research. Artifactual-free analysis of *S*-nitroso compounds by HPLC systems coupled with various detection techniques including UV–Vis absorbance and fluorescence detectors and mass spectrometers is still a great analytical challenge. Several problems have to be overcome and precautions have to be taken especially regarding artifactual formation of these compounds in thiol- and nitrite-rich matrices, both during sample treatment and HPLC analysis. This is very important especially in consideration of the extremely low concentrations of *S*-nitroso compounds in biological fluids [7], of the high concentrations of thiols throughout the organism and the ubiquitous nitrite from endogenous and exogenous sources. Under acidic pH conditions thiols react very rapidly with nitrite to form *S*-nitroso compounds [18]. We showed for GSH that significant artifactual formation of GSNO is possible even at slightly acidic conditions, e.g. at pH 6 [18]. The extent of artifactual on-column formation of *S*-nitroso compounds during HPLC analysis of thiols and nitrite containing matrices mainly depends upon the separation of thiols and nitrite on the column and on their concentrations [18]. Rapid separation of these compounds reveals low on-column formation while slow and poor separation results in considerable formation of the respective *S*-nitroso compounds [18]. Use of neutral or sligthly alkaline conditions during sample preparation and HPLC analysis is the best way to avoid artifactual formation of *S*-nitroso compounds. However, working under these conditions may be associated with decomposition of these compounds due to their lower stability under neutral and alkaline pH conditions. Nevertheless, artifactual formation of *S*-nitroso compounds should be avoided at all costs, because it can cause formation of these compounds at concentrations which may by far exceed their physiological levels, leading thus to momentous misinterpretations. Considering these mandatory requirements we developed an anion-pairing HPLC method for the specific and artifactual-free analysis of GSNO and $GSNO_2$ in matrices that contain high concentrations of GSH and nitrite. The usefulness of the method for artifactual-free analysis of $GSNO_2$ formed from the reaction between GSH and peroxynitrite is demonstrated.

By means of the novel HPLC method we have shown that in 0.2 *M* Tris buffer at pH 7.4 only 1.8% of authentic peroxynitrite are converted to $GSNO_2$ from its reaction with GSH. For comparison we performed analyses in parallel with HPLC system B that uses the cation-pairing agent 1-octanesulfonic acid at acidic pH, i.e., pH 2.0. By means of this HPLC system, the apparent yield of $GSNO_2$ from the reaction between peroxynitrite and GSH was about 10 times higher. We found by GC–MS that solutions of decomposed peroxynitrite in 0.2 *M* Tris buffer at pH 7.4 contained nitrite and nitrate at a molar ratio of approximately 1:1. These solutions were also found to contain unreacted GSH. Therefore, the higher GSNO concentrations measured by HPLC system B reflect considerable artifactual formation of GSNO under the acidic conditions of this HPLC system. These and previous [18] data show on-column formation of GSNO from analysis of buffered solutions of GSH and nitrite by the HPLC system B, but non on-column formation of GSNO by the novel HPLC method. Other HPLC systems for some *S*-nitroso compounds do also work with mobile phases of acidic pH [11–13,17,20,21]. The potential for significant artifactual formation of *S*-nitroso compounds by using such HPLC systems is high. Our results suggest that considerable artifactual formation of GSNO from GSH and nitrite during HPLC analysis with HPLC systems that run with acidic mobile phases may occur. The use of the anion-pairing agent TBAHS in the mobile phases of the novel HPLC system enables analysis of GSNO and $GSNO_2$ at neutral pH. This is a great advantage since GSH and other thiols do not react with nitrite to form the corresponding *S*-nitroso compounds at neutral pH [18], even when thiols and nitrite are not separated chromatographically.

An alternative way to prevent artifactual on-column formation of *S*-nitroso compounds is the elimination of nitrite, for instance by ammonium sulfamate [10,13], which, however, may not be complete as shown by Kluge et al. [10], and by us in the present study. Since elimination of nitrite by am-

monium sulfamate requires acidic conditions, *S*-nitroso compounds are formed artifactually during this procedure in a non-negligible extent that primarily depends upon time and ammonium sulfamate concentration.

On-line HPLC separation and ESI–MS detection of intact *S*-nitroso compounds has not been described so far. Balazy et al. have demonstrated the usefulness of off-line ESI–tandem MS for structure elucidation of reaction products of GSH with peroxynitrite after their isolation by HPLC [17]. This technique provided evidence that the product from the reaction between GSH and peroxynitrite is $GSNO_2$ but not GSNO as had been generally expected. We have recently reported that off-line ESI–MS is a useful approach to analyze various LMM *S*-nitroso compounds in the positive-ion mode [18]. Deutsch et al. have shown that ESI–MS applies to the analysis of GSH and its oxidative metabolites in the negative-ion mode, too [26]. Thus, mass spectrometry, e.g., ESI–MS of intact [17,18] and GC–MS of derivatized *S*-nitroso and *S*-nitro compounds [19], is the sole methodology that can discriminate and detect all compounds which are formed in reactions of ·NO and its numerous derivatives including that of peroxynitrite with thiols. Especially ESI–MS appears currently the most favorable methodology to investigate occurrence and mechanisms of formation and reactions of *S*-nitroso and *S*-nitro compounds in biological fluids. The HPLC method described in this work, i.e., the HPLC systems A1 and A2, should now enable on-line ESI–MS detection of GSNO without the danger of artifacts. In most analyses, which were performed in this study, we used phosphate as the buffering agent in the mobile phase. Despite recent advances in ESI–MS instrumentation, especially regarding elimination of buffer constituents, such as phosphate, precipitating salts may cause problems with sensitivity and performance of the mass spectrometry. This potential problem may be presumably overcome by using ammonium formate instead of phosphate in the mobile phase. In previous work we found that the cation-pairing agent 1-octanesulfonic acid disturbed off-line ESI–MS analysis of GSNO [18]. Whether the anion-pairing agent tetrabutylammonium hydrogensulfate or other tetrabutylammonium salts may also cause problems in the ESI–MS analysis of GSNO and $GSNO_2$, remains to be investigated. In the present work we focussed our interest on GSNO and $GSNO_2$ as the derivatives of the most abundant thiol in various cells. Extension of this anion-pairing HPLC system to other *S*-nitroso and *S*-nitro compounds that are negatively charged at neutral or slightly alkaline pH should be possible.

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