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Journal of Chromatography B, 742 (2000) 99–108

JOURNAL OF CHROMATOGRAPHY B

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# Electrospray ionization mass spectrometry of low-molecular-mass *S*-nitroso compounds and their thiols

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Received 15 December 1999; received in revised form 10 February 2000; accepted 10 February 2000

**Abstract**

Low-molecular-mass *S*-nitroso compounds (R–S–N=O) are potent vasodilators and inhibitors of platelet aggregation. This work describes the electrospray ionization mass spectrometric (ESI-MS) analysis of physiological and synthetic low-molecular-mass *S*-nitroso compounds and their thiols including *S*-nitrosoglutathione, *S*-nitrosocysteine, glutathione and cysteine. Mass spectra of the unlabeled and *S*-$^{15}$N-labeled low-molecular-mass *S*-nitroso compounds investigated are characterized by abundant cations due to $[M+H]^+$, $[M+Na]^+$, $[(M+H)-NO]^+$, $[2M+H]^+$, and $[(2M+H)-2NO]^+$. Mass spectra of low-molecular-mass thiols are characterized by abundant cations due to $[M+H]^+$, $[M+Na]^+$ and $[2M+H]^+$. Using off-line electrospray ionization tandem mass spectrometry we unequivocally identified *S*-[$^{15}$N]nitrosoglutathione in human red blood cells formed after their incubation with *S*-[$^{15}$N]nitrosocysteine. These results suggest that ESI-MS in combination with an appropriate liquid chromatographic system should be a useful analytical approach for the on-line quantitative determination of low-molecular-mass *S*-nitroso compounds in biological fluids in the presence of their thiols and nitrite. Considerations were made about on-line ESI-MS and quantitative measurements. © 2000 Elsevier Science B.V. All rights reserved.

*Keywords:* *S*-Nitroso compounds; Thiols

## 1. Introduction

*S*-Nitroso compounds, with the general formula R–S–N=O, are potent vasodilators and inhibitors of platelet aggregation acting both via nitric oxide (NO) release and by themselves (reviewed in [1]). *S*-Nitrosocysteine (SNC) and *S*-nitrosoglutathione (GSNO) are physiologically occurring low-molecular-mass (LMM) *S*-nitroso compounds [2–4]. SNC has been suggested to be a putative endothelium-derived relaxing factor [5]. GSNO has been detected in human bronchial lavage [3]. Also, GSNO has been shown to be formed in vitro in incubation mixtures of NO synthases containing the reduced form of glutathione (GSH) [6,7]. The pathways of formation, action, and metabolism of endogenous *S*-nitroso compounds are currently under intensive scientific investigation. Exogenous LMM *S*-nitroso compounds such as *S*-nitroso-*N*-acetylpenicillamine

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PII: S0378-4347(00)00141-9

(SNAP) have been used as model *S*-nitroso compounds to find out the mechanisms by which *S*-nitroso compounds develop their biological actions [8].

Several analytical methods have been described for the detection of LMM *S*-nitroso compounds [1]. High-performance liquid chromatography (HPLC) with UV absorbance detection at 333 nm [9] is currently the sole method that measures LMM *S*-nitroso compounds in their intact form. The majority of other methods are based on the measurement of NO or nitrite released by chemicals from the *S*-nitroso group of LMM *S*-nitroso compounds [1]. Gas chromatography–mass spectrometry (GC–MS) has been shown to be suitable for the analysis of *S*-nitroso-*N*-acetylcysteine (SNAC) as its pentafluorobenzyl derivative [10]. However, GC–MS is not extendable to other more polar and non-volatile LMM *S*-nitroso compounds such as GSNO and SNC except they are separated by HPLC and converted to nitrite which is finally derivatized to its pentafluorobenzyl derivative [11]. HPLC in combination with fast atom bombardment (FAB) mass spectrometry of the *N*-9-fluorenylmethoxycarbonyl (FMOC) derivative of GSNO has been used to identify and quantify GSNO in rat cerebellum [4].

In recent years ESI has been developed to become a powerful ionization technique in MS, i.e. ESI-MS, for the analysis of low- and high-molecular-mass compounds, in particular amino acids, peptides and proteins [12] including *S*-nitrosohaemoglobin [13]. Recently, Balazy et al. have applied off-line ESI-MS to identify the products of the reaction between peroxynitrite and glutathione [14]. The physicochemical properties of LMM *S*-nitroso compounds dictate the use of ESI-MS for their analysis. Therefore, we investigated the ESI-MS behavior of some physiological and non-physiological LMM *S*-nitroso compounds. Our results suggest that ESI-MS is applicable to this class of compounds. By means of off-line ESI-MS we unequivocally showed that *S*-[$^{15}$N]nitrosocysteine (S$^{15}$NC) is converted in the cytosol of washed human red blood cells by intracellular GSH to *S*-[$^{15}$N]nitrosoglutathione (GS$^{15}$NO). Our results indicate that one of the most important considerations in developing on-line ESI-MS with liquid chromatographic systems will be the pH of the mobile phase and the buffer capacity of the matrix to be analyzed.

## 2. Experimental

### *2.1. Chemicals*

L-Cysteine and 1-octanesulfonic acid (sodium salt) were purchased from Aldrich (Steinheim, Germany). *N*-Acetyl-L-cysteine, reduced glutathione (GSH) and cysteinylglycine (CysGly) were obtained from Sigma (Munich, Germany). The sodium salt of nitrite, *o*-phosphoric acid and acetonitrile of gradient grade were obtained from Merck (Darmstadt, Germany). The sodium salt of $^{15}$N-labeled nitrite (98 atom% at $^{15}$N) was purchased from MSD Isotopes Merck Frosst Canada (Montreal, Canada). *S*-Nitroso-*N*-acetyl-D,L-penicillamime (SNAP) was obtained from Biomol (Hamburg, Germany). Stock solutions (4.8 m*M*) of unlabeled and $^{15}$N-labeled *S*-nitroso compounds were prepared by mixing equal volumes of 10 m*M* solutions of nitrite or [$^{15}$N]nitrite and of the reduced thiols in distilled water or unbuffered physiological saline, acidification with a 1/10th volume of 5 *M* HCl and incubation for 5 min at room temperature [9]. *S*-[$^{15}$N]Nitroso-*N*-[$^{2}$H$_3$]acetyl-L-cysteine ([$^{2}$H$_3$,$^{15}$N]SNAC) was synthesized as described [10]. These stock solutions of *S*-nitroso compounds were found not to contain nitrite by an assay based on the Griess reaction [9] nor reduced thiols by the method of Ellman [15]. UV–Vis spectra of these *S*-nitroso compounds showed characteristic maximum absorption at 333 nm with molar absorptivity coefficients (in $M^{-1}$ $cm^{-1}$) of 777 for GSNO, 769 for SNAC, 644 for SNC, and 858 for SNAP.

### *2.2. S-Transnitrosylation in washed human red blood cells*

A 3-ml aliquot of blood (EDTA as anticoagulant) from a healthy volunteer was centrifuged at 4°C (800 *g*, 5 min) and the plasma was removed. The cellular content of the red blood cells preparation was not determined. In the following procedures a physiological saline was used unbuffered and without addition

of other components. The remaining erythrocyte fraction was washed with 5-ml aliquots of physiological saline and centrifuged at 4°C (800 *g*, 5 min). This procedure was repeated three times. The erythrocyte fraction (about 1.5 ml) from the last wash procedure was incubated with a solution of $S^{15}NC$ in physiological saline (1.5 ml, 800 μ*M*, pH 7) to obtain a final volume equivalent to the original blood volume. After incubation for 3 min, erythrocytes were washed five times with physiological saline. The erythrocyte fraction from the last centrifugation was frozen at −80°C for 30 min and then resuspended in distilled water (1:1, v/v). After centrifugation at 10°C (800 *g*, 40 min), a 1-ml aliquot of the supernatant was taken and ultrafiltered at 4°C (1200 *g*, 20 min) using Centrisart I cartridges (cut-off 20 kDa, Sartorius, Göttingen, Germany). A 200-μl aliquot of the ultrafiltrate obtained (pH 7) was analyzed by a previously described, slightly modified HPLC system [9]. Briefly, the stationary phase consisted of a column (250×4.6 mm I.D.) packed with 100-5$C_{18}$ Nucleosil, 5 μm particles size, from Macherey-Nagel (Düren, Germany). The mobile phase was 10 m*M* each of $NaH_2PO_4$ and 1-octanesulfonic acid in acetonitrile–water (9:91, v/v), the pH of which was adjusted to 2.0 by addition of *o*-phosphoric acid. Isocratic runs were carried out at a flow-rate of 1 ml/min. The effluent was monitored at 333 nm. This HPLC system (referred to as HPLC system A) enables baseline separation of SNAC, SNC, GSNO and SNAP [11]. In the present study, nitrite, GSH and GSNO eluted at 2.9, 7.8 and 15.1 min, respectively. The limit of detection of this HPLC system was 0.5 μ*M* for GSNO. For structure elucidation, a 200-μl aliquot of the HPLC fraction eluted at 15.1 min (approximate concentration of 12 μ*M* based on the analysis of a 12.5-μ*M* solution of GSNO in physiological saline; see below) was subjected to solid-phase extraction on a microcon-SCX (100 PK) cartridge from Amicon (Beverly, MA, USA) which was preconditioned with methanol (200 μl) followed with distilled water (500 μl). The cartridge was washed with HCl (10 m*M*, 500 μl) and elution was performed with a 60-μl aliquot of a 1 *M* ammonium acetate buffer (pH 7.0). All steps of this procedure were performed by centrifugation at 800 *g*. Rechromatography by HPLC of a 20-μl aliquot of the eluate showed a single peak with the retention time of synthetic GSNO (UV absorbance detection at 333 nm). A 12.5-μ*M* solution of synthetic GSNO in physiological saline was processed similarly.

### *2.3. Studies on artifactual formation of GSNO during HPLC analysis*

HPLC analysis of GSNO in the present study was performed using mobile phases of acidic pH, i.e. 2.0. Under acidic pH conditions formation of *S*-nitroso compounds from reduced thiols and nitrite is favoured. Thus, *S*-nitrosylation of thiols by nitrite under acidic conditions is utilized to synthesize *S*-nitroso compounds [9]. To study possible artifactual formation of GSNO from GSH and nitrite during HPLC analysis the following experiments were performed. In the first experiment the dependence of GSNO formation from GSH and nitrite upon the pH of the aqueous solution was investigated. In the second experiment the effect of the buffer capacity on the HPLC column GSNO formation was investigated.

A freshly prepared mixture of 1 m*M* GSH and 10 μ*M* nitrite in 0.2 *M* Tris buffer, pH 7.4, was immediately injected onto the HPLC column. These concentrations of GSH and nitrite in the buffer correspond to the concentrations of GSH and GSNO measured in lysed erythrocytes in this and in previous studies under similar experimental conditions [11,16]. We also investigated the formation of GSNO from GSH and nitrite in aqueous solution as a function of the pH value and incubation time. In 0.2 *M* Tris buffer, pH 7.4, a solution of GSH (1000 μ*M*) and nitrite (100 μ*M*) was prepared and constantly stirred at room temperature. Under stirring the pH of this solution was changed successively to 6.0, 4.0, 3.0 and 2.0 by using a 5 *M* HCl solution after an incubation time of at least 30 min. This incubation time should ensure maximum formation of GSNO at the respective pH value. Aliquots (200-μl) were taken and analyzed by HPLC system A.

In the second experiment the influence of the concentration of Tris buffer (0, 25, 50, 100, 200 m*M*) on GSNO formation from GSH (1000 or 3000 μ*M*) and nitrite (100 μ*M*) during HPLC analysis was investigated. Immediately after preparation of

the respective solution, a 100-μl aliquot was injected onto the HPLC column (250×4.6 mm I.D., packed with 100-5$C_{18}$ Nucleosil) which was connected in addition to a precolumn (4×3 mm I.D., packed with ODS, 5-μm particle size) from Phenomenex (Hösbach, Germany). The mobile phase was 10 m*M* each of $NaH_2PO_4$ and 1-octanesulfonic acid in acetonitrile–water (15:85, v/v), pH 2.0. Isocratic runs were carried out at a flow-rate of 1 ml/min. The retention times of nitrite (detection at 333 nm), GSH (detection at 205 nm) and GSNO (detection at 333 nm) in this system were 4.3, 5.8 and 10.8 min, respectively. This HPLC system is referred to as HPLC system B.

GSNO formed in these experiments was quantified by using calibration curves of GSNO in 0.2 *M* Tris buffer, pH 7.4, which were generated by both HPLC systems. Linear regression analysis between peak area and GSNO concentration revealed regression coefficients ($r^2$) of 0.9884 and 0.9910, respectively.

### *2.4. Off-line electrospray ionization mass spectrometry*

ESI-MS and ESI-tandem MS were carried out on an API III+ triple stage quadrupole mass spectrometer (PE-Sciex, Langen, Germany) equipped with the articulated ion spray ionization source. The instrument was calibrated daily with polypropylene glycol standard as recommended by the manufacturer. The mass spectrometer was operated at unit resolution across its entire mass range. Stock solutions of LMM *S*-nitroso compounds and the extracts from HPLC eluates were diluted with 2 vol% formic acid in acetonitrile (1:1, v/v) and analyzed directly. Mass spectra were generated in the positive-ion mode. Samples were infused via a 100-μl Hamilton syringe with a Harvard syringe pump through a 50-μm inner diameter fused-silica capillary directly into the ion spray at 5 μl/min. An orifice voltage of 40 – 50 V was used. The capillary interface tube temperature was kept at 55°C. At least ten scans were taken and summarized. Tandem quadrupole mass spectrometry was performed under identical ionization conditions. ESI-MS analysis of extracts of HPLC eluates was performed by subjecting the parent ions $[M+H]^+$ at *m/z* 337 for GSNO and *m/z* 338 for $GS^{15}NO$ to collision-activated dissociation (CAD) using argon as the collision gas. Collision energy and collision gas thickness were 25 eV and about $300 \cdot 10^{12}$ molecules/$cm^2$. Scans (5 s per scan) were performed at an ionization voltage of 5000 V over a mass range of 50–1000 a.m.u. with a step size of 0.5 a.m.u. About 10 scans were summarized. Data were analyzed by MACSPEC (PE-Sciex).

## 3. Results and discussion

### *3.1. ESI-MS*

The most intense cations present in the ESI mass spectra of the investigated LMM *S*-nitroso compounds and some thiols are summarized in Tables 1 and 2, respectively. The ESI mass spectra of unlabeled and $^{15}N$-labeled GSNO, i.e. $GS^{15}NO$, are

Table 1
Electrospray ionization mass spectra[a] of unlabeled and $^{15}N$-labeled low-molecular-mass *S*-nitroso compounds

| Ion assigment | Mass-to-charge ratio (intensity, %) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | GSNO | $GS^{15}NO$ | SNC | $S^{15}NC$ | SNAC | $[^2H_3, ^{15}N]$SNAC | SNAP | CysGlySNO |
| $[(M+H)-NO]^+$ | 307 (30) | N.A. | 121 (20) | N.A | 163 (10) | N.A. | 191 (100) | 178 (70) |
| $[(M+H)-^{15}NO]^+$ | N.A. | 307 (40) | N.A. | 122 (15) | N.A. | 166 (10) | N.A. | N.A. |
| $[M+H]^+$ | 337 (100) | 338 (100) | 151 (76) | 152 (85) | 193 (100) | 197 (100) | 221 (60) | 208 (90) |
| $[M+Na]^+$ | 359 (40) | 360 (45) | N.D. | N.D. | 215 (10) | 219 (10) | N.D. | 230 (30) |
| $[(M+H)+CH_3CN]^+$ | N.D. | N.D. | 192 (23) | 193 (45) | 234 (45) | 238 (30) | 262 (15) | 249 (10) |
| $[(2M+H)-2NO]+$ | 613 (18) | N.A. | N.D. | N.A. | 325 (10) | N.A. | 381 (80) | 355 (30) |
| $[(2M+H)-2^{15}NO]^+$ | N.A. | 613 (18) | N.A. | N.D. | N.A. | 331 (20) | N.A. | N.A. |
| $[2M+H]^+$ | 673 (30) | 675 (35) | N.D. | N.D. | 385 (45) | 393 (50) | 441 (95) | N.D. |
| $[2M+Na]^+$ | 695 (15) | 697 (16) | N.D. | N.D. | 407 (10) | 415 (10) | 463 (5) | N.D. |

[a] Characteristic, assigned intense mass fragments are given. N.D., not detected; N.A., not applicable.

Table 2
Electrospray ionization mass spectra[a] of low-molecular-mass thiols

| Ion assignment | Mass-to-charge ratio (intensity, %) | | | |
|---|---|---|---|---|
| | GSH | Cys | NAC | CysGly |
| $[M+H]^+$ | 308 (100) | 122 (100) | 164 (80) | 179 (30) |
| $[M+Na]^+$ | 329 (10) | 144 (30) | 186 (70) | 201 (30) |
| $[(M+H)+CH_3CN]^+$ | N.D. | 163 (35) | 205 (10) | 220 (5) |
| $[2M+H]^+$ | 615 (50) | 243 (50) | 327 (100) | 357 (100) |
| $[2M+Na]^+$ | 636 (5) | 264 (16) | 349 (85) | 379 (15) |

[a] Characteristic, assigned intense mass fragments are given. N.D., not detected.

shown in Fig. 1. The ESI mass spectrum of GSH is shown in Fig. 2. Characteristic cations were observed at $m/z$ ratios corresponding to the protonated monomers $[M+H]^+$ and dimers $[2M+H]^+$ of the molecules. Loss from the monomers and dimers of the *S*-nitroso compounds of one and two molecules of unlabeled or $^{15}$N-labeled NO from the *S*-nitroso groups resulted in the cations due to $[(M+H)-NO]^+$ or $[(M+H)-{}^{15}NO]^+$ and $[(2M+H)-2NO]^+$ or $[(2M+H)-2{}^{15}NO]^+$, respectively. Ions corresponding to adducts of the molecules with $Na^+$ or acetonitrile, i.e. $[M+Na]^+$ or $[(M+H)+CH_3CN]^+$, respectively, were also observed. Under the conditions applied abundant ions due to $[2M+H]^+$ and $[2M+Na]^+$ were observed from all thiols investigated including the *N*-acetylated cysteine, i.e. NAC. On the other hand, $[2M+H]^+$ and $[2M+Na]^+$ ions were not observed from SNC and the *S*-nitroso derivative of CysGly, i.e. CysGlySNO. Evidence for the formation of $[2M+H]^+$ from CysGlySNO is the appearance of the ion at $m/z$ 355 ($[(2M+H)-2NO]^+$.

The ESI mass spectra of the *S*-nitroso compounds clearly differed from those of the corresponding reduced thiols (Tables 1 and 2). As exemplified for GSH in Fig. 2, the ESI mass spectrum of GSH shows intense cations at $m/z$ 308 and 615 due to the protonated monomer $[M+H]^+$ and dimer $[2M+H]^+$ of GSH, respectively. The structures of the dimers were not investigated. Neither did we investigate the conditions under which only the monomers are formed. Formation of a dimer of GSH by ESI-MS analysis of aqueous solutions of GSH in the negative-ion mode has been recently reported by Deutsch et al. [17]. In the ESI mass spectra of GSNO we did not observe a cation at $m/z$ 353 that would be obtained from $GSNO_2$, i.e. the reaction product of GSH and peroxynitrite [14]. These results indicate that the method used for the synthesis of LMM *S*-nitroso compounds, i.e. the nitrosylation of reduced thiols with nitrite in hydrochloric acid aqueous solution [9], results exclusively in quantitative formation of *S*-nitroso compounds.

The daughter ion mass spectra obtained from CAD of the parent ions $[M+H]^+$ of synthetic GSNO ($m/z$ 337) and $GS^{15}NO$ ($m/z$ 338) formed in the erythrocytes following incubation with $S^{15}NC$ are shown in Fig. 3. The formation of the product ion at $m/z$ 307 from the parent ion at $m/z$ 338 provides clear evidence for the presence of a *S*-[$^{15}$N]nitroso group in the molecule of this compound. CAD of $m/z$ 337 of GSNO to form the product ion at $m/z$ 307 has also been reported recently by Balazy et al. [14]. The parent ions at $m/z$ 337 and 338 subjected to CAD and the daughter ions at $m/z$ 307 are not present in the mass spectrum of GSH (Fig. 2). These observations are unequivocal evidence for the formation of $GS^{15}NO$ in human erythrocytes incubated with $S^{15}NC$.

### *3.2. Artifactual formation of GSNO from GSH and nitrite*

Injection of a solution of 1 m*M* GSH and 10 μ*M* nitrite in 0.2 *M* Tris buffer, pH 7.4, into HPLC system A did not result in formation of detectable amounts of GSNO. Fig. 4 shows that at pH 7.4 no detectable concentrations of GSNO were formed from GSH (1000 μ*M*) and nitrite (100 μ*M*) in 0.2 *M* Tris buffer. This result indicates that no GSNO is formed from GSH and nitrite during HPLC analysis using HPLC system A. At lower pH values of the

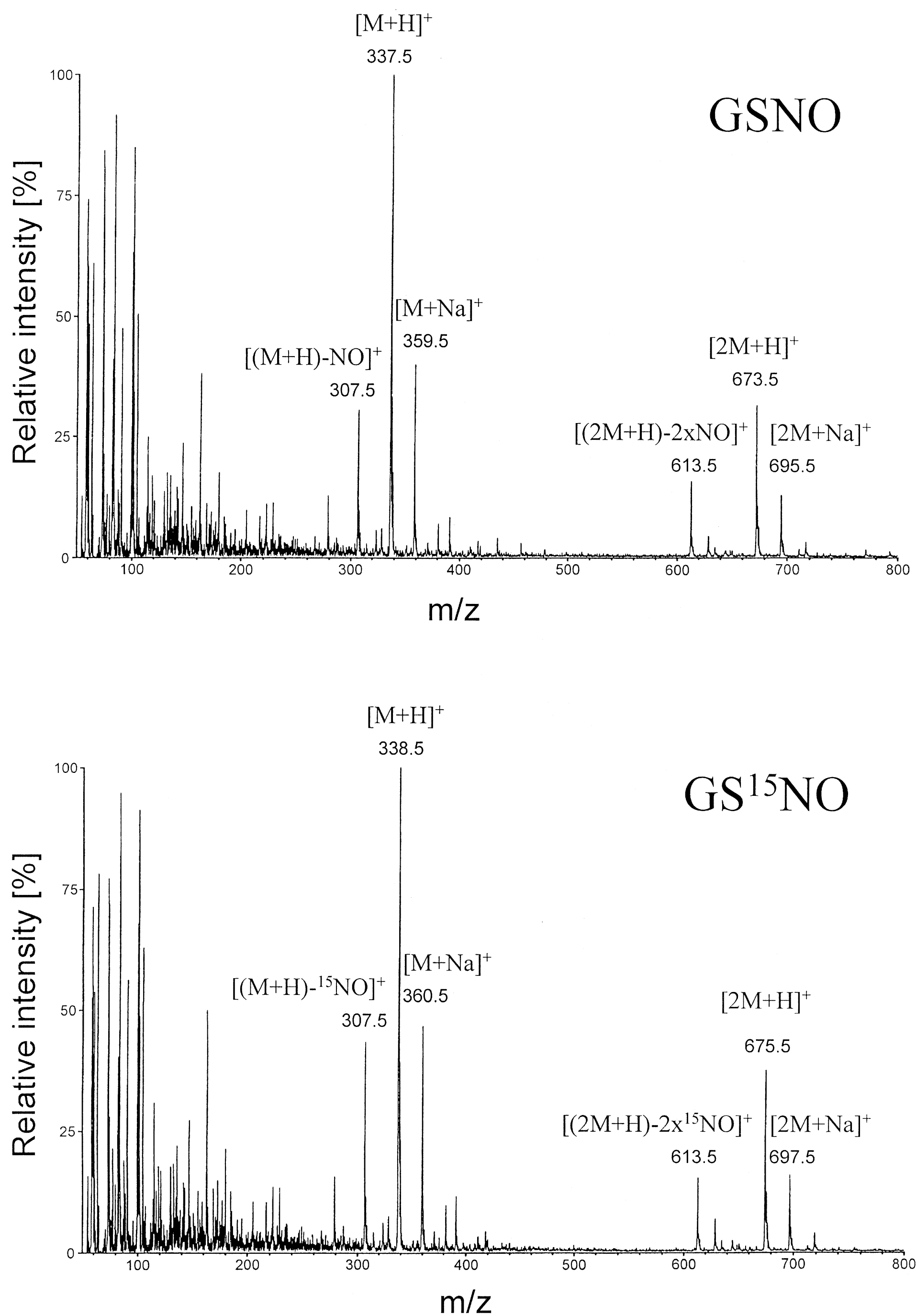


Fig. 1. ESI mass spectra of synthetic unlabeled *S*-nitrosoglutathione (upper panel) and $^{15}$N-labeled *S*-nitrosoglutathione (lower panel).

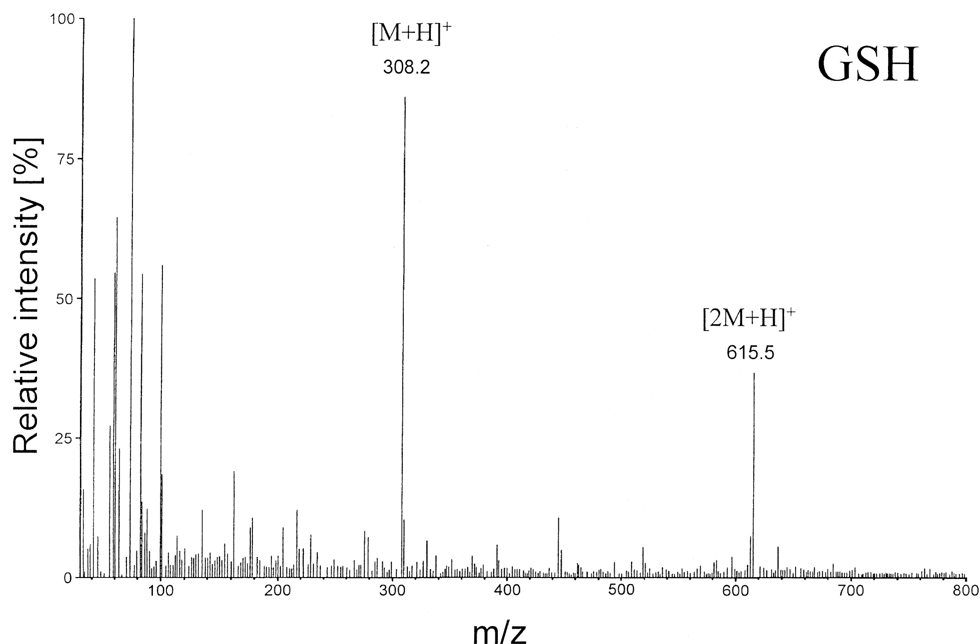


Fig. 2. ESI mass spectrum of reduced glutathione.

buffer, however, GSNO was formed at significant concentrations. Rapid and quantitative formation of GSNO was observed at pH values of 3.0 and 2.0. Formation of GSNO from GSH and nitrite at pH 6.0 and 4.0 was less marked and preceded considerably slower. Similar results have been also obtained for cysteine and *N*-acetylcysteine [18]. On the other hand, Fig. 5 shows that using HPLC system B a low but clear formation of GSNO was observed from injection of solutions of GSH and nitrite in 0.2 *M* Tris buffer (pH 7.4). Furthermore, this figure shows that GSNO formation increased when the concentration of Tris decreased. A possible explanation for these findings could be on column reaction of GSH with nitrite, i.e. nitrous acid, to form GSNO within the first minutes of analysis, i.e. prior complete chromatographic separation of nitrite and GSH. Since in HPLC system B the retention times of nitrite (4.3 min) and GSH (5.8 min) are less distinct than in HPLC system A (2.9 and 7.8 min), on column formation of GSNO using HPLC system B greatly predominates over GSNO formation using HPLC system A. Fig. 5 clearly shows that artifactual formation of GSNO from HPLC analysis of matrices that contain GSH and nitrite will greatly depend upon their buffer capacity and GSH content when mobile phases of low pH will be used. In the presence of high GSH concentrations as their occur in the cytosol of many cells up to 50% of nitrite present in the matrix to be analyzed could be converted to GSNO only during HPLC analysis using mobile phase of pH 2.0.

In the experiment of the present study with human erythrocytes cytosolic $GS^{15}NO$ was determined as 12 μ*M* by HPLC system A. In the cytosol of untreated human erythrocytes we could not detect any endogenous GSNO by HPLC. The concentration of $GS^{15}NO$, [$^{15}$N]nitrate and [$^{15}$N]nitrite in the cytosol of human erythrocytes in similar experiments of previous studies [11,16] were determined as 15 μ*M*, 40 μ*M*, and 1 μ*M*, respectively, by GC–MS [9,11]. The mean cytosolic GSH concentration was determined as 2.3 m*M* by HPLC [19]. Using HPLC system A, analysis of ultrafiltrate samples from lysed erythrocytes without and with external addition of nitrite to obtain an added concentration of 10 μ*M* did not result in on column formation of GSNO most likely due to the sufficient buffer capacity of the red blood cells cytosol. Therefore, $GS^{15}NO$ quantitated by HPLC and structurally elucidated by ESI-MS

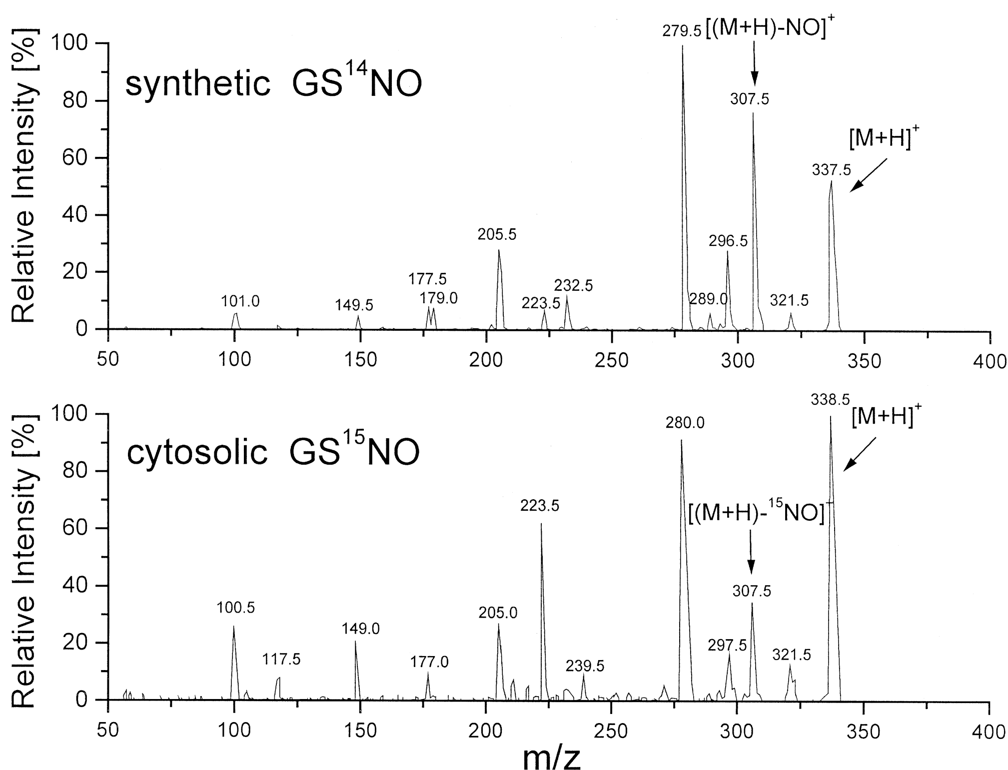


Fig. 3. Tandem mass spectra generated from cytosolic $^{15}$N-labeled *S*-nitrosoglutathione formed after incubation of washed human erythrocytes with $^{15}$N-labeled *S*-nitrosocysteine (lower panel) and from synthetic unlabeled *S*-nitrosoglutathione (upper panel). The parent ions $[M+H]^+$ at *m/z* 337.5 for unlabeled and at *m/z* 338.5 for $^{15}$N-labeled *S*-nitrosoglutathione were subjected to collision-activated dissociation at a collision energy of 25 eV.

must be regarded as the reaction product of externally added $S^{15}NC$ with cytosolic GSH via a *S*-transnitrosylation reaction [11,16]. This is further supported by the facts that the cytosolic $GS^{15}NO$ concentrations are several times higher than that of cytosolic [$^{15}$N]nitrite and that no significant amounts of $S^{15}NC$ were formed although the cytosol contained cysteine at about 100 μ*M* [16].

### 3.3. Considerations about on-line ESI-MS and quantitative measurement

On-line HPLC separation and ESI-MS detection of intact *S*-nitroso compounds has not been described to date. 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 [14]. In the present work we show that off-line ESI-MS is a suitable analytical approach to structurally identify $GS^{15}NO$ formed in the cytosol of human erythrocytes. By using this technique we unequivocally confirmed the conversion of cytosolic GSH of human erythrocytes to $GS^{15}NO$ after their incubation with $S^{15}NC$ previously shown by HPLC and GC–MS [11,16]. Our study indicates that artifactual-free on-line analysis of *S*-nitroso compounds by liquid chromatographic systems and ESI-MS is a great analytical challenge. Several problems have to be overcome and precautions have to be taken especially regarding artifactual formation in thiols- and nitrite-rich matrices during sample treatment and HPLC analysis. This is very important especially in consideration of the extremely low concentration of *S*-nitroso compounds in

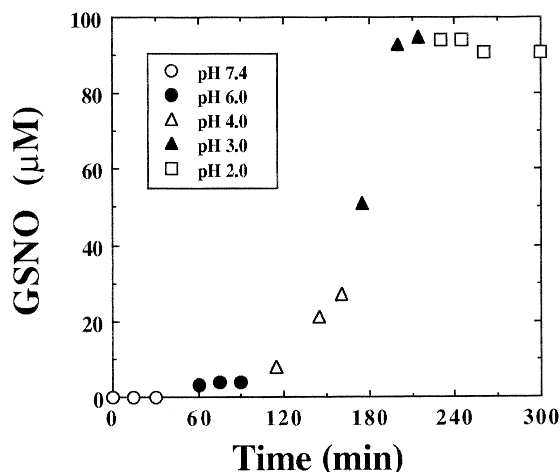


Fig. 4. Formation of GSNO from GSH and nitrite in aqueous buffer as a function of the pH. GSH (1000 μ*M*) and nitrite (100 μ*M*) were incubated in 0.2 *M* Tris buffer, pH 7.4, for up to 45 min. Immediately after pH adjustment and at the indicated time samples were taken and analyzed by HPLC for GSNO. HPLC system A was used.

biological fluids [1,3,20,21] and of the high or very high concentrations of thiols throughout the organism and the ubiquitous nitrite.

In our HPLC systems we used 1-octanesulfonic acid as the cation-pairing agent. Such systems allow separation of various LMM *S*-nitroso compounds from their corresponding thiols and nitrite [9,16]. This is an absolute requirement for artifactual-free analysis of LMM *S*-nitroso compounds. Phosphate and 1-octanesulfonic acid were found to disturb ESI-MS analysis of the *S*-nitroso compounds investigated in this study (not shown). Therefore we solid-phase extracted cytosolic $GS^{15}NO$ prior to ESI-MS analysis. Other HPLC systems do not use ion-pairing agents for some *S*-nitroso compounds [5–7,14]. The suitability of these systems for the separation of other LMM *S*-nitroso compounds, their thiols and nitrite has not been described. Nevertheless, in all these HPLC systems mobile phases of acidic pH (2–4) are used. Artifactual formation of GSNO from GSH and nitrite during HPLC analysis in these HPLC systems cannot completely be excluded. Our present results underline this possibility. This problem can be overcome by the development of HPLC systems that would allow use of mobile phases of neutral pH values. Anion-pairing HPLC could be a proper solution for this problem.

Intact and derivatized GSNO and $GS^{15}NO$ coelute in various HPLC systems [4,9,11,21]. It can be assumed that other *S*-nitroso compounds would also coelute on HPLC and other chromatographic systems with their stable isotope labeled analogs. $^{15}N$-Labelled *S*-nitroso compounds are not ideal internal standards due to the considerable contribution of the $^{13}C$ isotope of the unlabelled material to the $^{15}N$-labelled analog. Multiple labelling of the *S*-nitroso group with $^{15}N$ and $^{18}O$, for example by using commercially available or laboratory-made $^{15}N^{18}O_2^-$, should be an ease procedure in principle applicable for every *S*-nitroso compound. Alternative labelling techniques as applied by us for the preparation of [$^2H_3$,$^{15}N$]SNAC are possible [10] but not applicable for physiological *S*-nitroso compounds such as GSNO and SNC. With respect to the high sensitivity required for quantitative measurements of LMM *S*-nitroso compounds in the low n*M* range further investigations are needed in order to obtain the protonated monomers in high abundance for ESI-MS or ESI-tandem MS analysis. The results by Deutsch et al. [17] suggest that in the negative-ion mode deprotonated monomers and dimers of *S*-nitroso compounds would be obtained. In this case optimi-

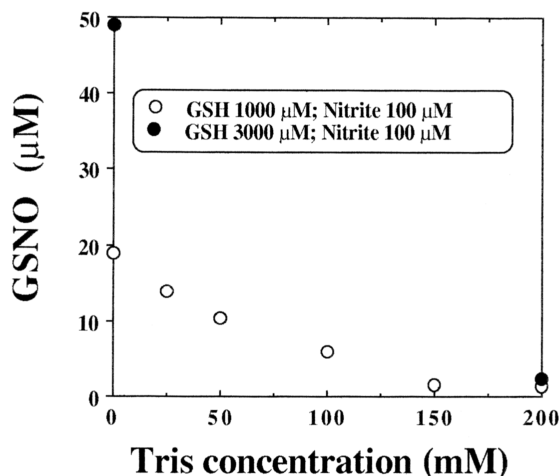


Fig. 5. On column formation of GSNO from GSH (1000 or 3000 μ*M*) and nitrite (100 μ*M*) in aqueous buffer as a function of the concentration of the Tris buffer (0–200 m*M*; pH 7.4) during HPLC analysis using HPLC system B.

zation of the deprotonated monomers would be necessary.

## 4. Conclusions

The analysis of LMM *S*-nitroso compounds in biological fluids is associated with many problems. They originate from the occurrence of these compounds in very low concentrations, their lability in biological fluids, the lack in sensitivity of various detection techniques and from the artifactual formation during sample treatment and analysis. The present work shows the suitability of ESI-MS for the analysis of a series of LMM *S*-nitroso compounds. By means of this technique we could unequivocally show artifactual-free formation of $GS^{15}NO$ in human erythrocytes incubated with $S^{15}NC$. ESI-MS in combination with liquid chromatographic systems such as highly resolving HPLC could be an analytical technique for the direct assessment of intact, non derivatized LMM *S*-nitroso compounds in biological fluids. Further work is needed for the development of such a method. The inherent accuracy and high sensitivity of MS would help to better understand formation, metabolism, occurrence and biological role(s) of physiological LMM *S*-nitroso compounds in humans.

## Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft (Grant TS 60/2-1).

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