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Measurement of *S*-nitrosoalbumin by gas chromatography–mass spectrometry
I. Preparation, purification, isolation, characterization and metabolism of *S*-[^{15}N]nitrosoalbumin in human blood in vitro

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Abstract

S-Nitrosoalbumin (SNALB) and *S*-[^{15}N]nitrosoalbumin (S[^{15}N]ALB) were prepared by various methods, purified and isolated by a novel selective extraction procedure using HiTrapBlue Sepharose affinity columns and characterized by various techniques including SDS-PAGE electrophoresis, UV–Vis spectroscopy and gas chromatography–mass spectrometry (GC–MS). *S*-Nitrosylation of albumin in freshly obtained human plasma by unlabeled and ^{15}N-labeled butylnitrite at neutral pH revealed the purest preparations. For GC–MS analysis, SNALB and S[^{15}N]ALB were treated with $HgCl_2$ to obtain nitrite and [^{15}N]nitrite, respectively, which were then analysed as their pentafluorobenzyl derivatives. S[^{15}N]ALB preparations were standardized by GC–MS using nitrite as internal standard. S[^{15}N]ALB was prepared and isolated at concentrations of 188±43 μM (mean±SD, $n=8$) at a final yield of about 45%, an isotopic purity of 98%, and SDS-PAGE electrophoretic purity of 90%. ^{15}N-Labeled SNALB was used to study its metabolism in human blood. The half-life of S[^{15}N]ALB (25 μM) in human heparinized blood in vitro was determined by GC–MS as 5.5 h. The GC–MS method described here could be useful for the quantitative determination of SNALB in human plasma using S[^{15}N]ALB as an internal standard.

Keywords: *S*-Nitrosoalbumin

1. Introduction

Nitric oxide (NO) is a putative endothelium-derived relaxing factor (EDRF) [1,2]. It has been reported that endogenously produced NO circulates in human plasma primarily as an *S*-nitroso adduct of serum albumin, i.e. SNALB [3]. EDRF and NO are considerably less stable than SNALB, which nevertheless potently exerts EDRF-like vasodilatory and antiaggregatory properties in vitro and vivo [3–6]. SNALB has therefore been suggested to be a physiological reservoir for NO with which vascular tone can be regulated in mammals [3]. Since the discovery of SNALB in human plasma by Stamler and co-workers in 1992, no reports from other investigators appeared in the literature either on the existence of endogenous SNALB in human plasma nor on its formation in animal models. Also, no

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reliable analytical techniques permitting selective and accurate quantitative determination of SNALB in human plasma or serum have been reported so far. Due to the potential importance of SNALB as a reservoir for endogenous NO [3], and possibly for NO-releasing drugs [7], and as regulator for NO-dependent functions, reliable analytical approaches are necessary and indispensable permitting accurate quantitative measurement of SNALB in human plasma.

The possibility of specific cleavage of *S*-nitroso groups by Hg^{2+} to nitrite [8] and its accurate measurement as its pentafluorobenzyl derivative by gas chromatography–mass spectrometry (GC–MS) [9,10], prompted us to develop a GC–MS method for the quantitative determination of SNALB in human plasma. In this method ^{15}N-labeled SNALB (S[^{15}N]ALB) is used as internal standard. In principle, preparation of SNALB starting from commercially available human or bovine serum albumin (HSA) or plasma albumin can be accomplished by *S*-nitrosylation using nitrite in acidic solution [4,10], aqueous NO solutions [4], butylnitrite [11], or by *S*-transnitrosylation using low molecular weight *S*-nitroso compounds [12,13]. In the present work we describe the preparation of SNALB and S[^{15}N]ALB by using unlabeled and ^{15}N-labeled nitrite and butylnitrite and human plasma, their selective extraction from reaction mixtures by HiTrapBlue Sepharose affinity columns, and their characterization by various analytical techniques and by a bioassay. We demonstrate in this article the careful synthesis and utility of S[^{15}N]ALB and SNALB as internal standards for quantitative measurements in human plasma by GC–MS. The metabolism of S[^{15}N]ALB in human blood in vitro was investigated by this method.

2. Experimental

2.1. Chemicals and reagents

Sodium [^{15}N]nitrite (declared as 98%+ at ^{15}N) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Butylnitrite, 2,3,4,5,6-pentafluorobenzyl (PFB) bromide, 2-mercaptoethanol and *N*-acetyl-cysteine (NAC) were obtained from Aldrich (Steinheim, Germany). Human serum albumin and dithiothreitol (DTT) were purchased from Sigma (Munich, Germany). 1-Butanol, EDTA and $NaBH_4$ were bought from Merck (Darmstadt, Germany). Sulfuric acid was purchased from J.T. Baker (Deventer, The Netherlands). HiTrapBlue Sepharose affinity columns (1-ml and 5-ml cartridges) and Sephadex PD-10 cartridges were obtained from Pharmacia Biotech (Freiburg, Germany). [^{15}N]-Labelled butylnitrite was synthesized as follows: 650 mg (9.4 mmol) of [^{15}N]nitrite were diluted in 10 ml of distilled water and cooled to 0°C. To this solution a 0°C-cold mixture of 1.4 ml concentrated sulfuric acid, 1 ml distilled water and 800 μl (8.8 mmol) 1-butanol were added under stirring. The resulting suspension was stirred for 60 min at 0°C and then centrifuged. The organic phase was taken up by a Pasteur glass pipette, washed two times by shaking with 1-ml aliquots of a mixture containing 25 wt.% NaCl and 2 wt.% $NaHCO_3$ and dried over anhydrous Na_2SO_4. GC–MS analysis revealed ^{15}N-labeled butylnitrite with an isotopic purity of 98% at ^{15}N. Centrisart I® ultrafiltration cartridges (pore size 4 μm, cut-off 20 kDa) were obtained from Sartorius (Göttingen, Germany).

2.2. Synthesis of S-nitroso-albumin standards

A 3-ml aliquot of freshly obtained plasma from a healthy volunteer was treated with aqueous solutions of nitrite or [^{15}N]nitrite at final concentrations each of 600 μ*M* and acidified with 200-μl aliquots of 5 *M* HCl to pH 2. The mixture was allowed to stand at room temperature for 2 h. After dilution with a 7-ml aliquot of buffer A (50 m*M* KH_2PO_4, pH 7.0), and pH adjustment to 7.0 by 5 *M* NaOH, the turbid mixture was centrifuged (1800 *g*, 5 min). The clear supernatant was applied to a 5-ml HiTrapBlue Sepharose affinity column preconditioned with 10 ml of buffer A. The column was washed with 20 ml of buffer A, and proteins were eluted with 10 ml of buffer B (50 m*M* KH_2PO_4, 1.5 *M* KCl, pH 7.0).

A 3-ml aliquot of freshly obtained human plasma was treated with butylnitrite or butyl[^{15}N]nitrite at final concentrations of 3.8 m*M*, and the plasma was allowed to stand at room temperature for 1 h. Proteins were extracted from plasma on a 5-ml

HiTrapBlue Sepharose affinity cartridge as described above but without further dilution.

Eluates from both routes of synthesis were ultrafiltered by centrifugation (1800 *g*, 15 min) until protein fraction volumes of about 3 ml were obtained. These SNALB and S[^{15}N]ALB preparations were stored at 4°C until use in the absence or presence of 1 m*M* EDTA.

2.3. GC–MS analysis

Concentration of *S*-nitroso-proteins in the final SNALB and S[^{15}N]ALB preparations were determined by GC–MS as described for nitrite [10] before and after conversion of varying concentrations of the *S*-nitroso-proteins by 1 m*M* $HgCl_2$ to nitrite and [^{15}N]nitrite, respectively, and of fixed concentrations (i.e. each 10 μ*M*) of [^{15}N]nitrite for SNALB and [^{14}N]nitrite for S[^{15}N]ALB used as internal standards. Derivatization of nitrite and [^{15}N]nitrite to their PFB derivatives was performed as described previously [9] with the exception that a 10-μl aliquot of PFB bromide was used because of the higher content of the samples of chloride which also reacts with PFB bromide. Alternatively, a mixture of $CuSO_4$ and cysteine were used for conversion of SNALB and S[^{15}N]ALB to nitrite and [^{15}N]nitrite, respectively.

Mass spectrometry 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&W Scientific (Rancho Cordova, CA) was used. Helium (70 kPa) and methane (200 Pa) were used as the carrier and the reagent gas, respectively, for NICI. Other GC–MS conditions were as described [9]. The most intense mass fragments in the GC–MS NICI mass spectra of PFB–NO_2 and PFB–$^{15}NO_2$ were *m*/*z* 46 (nitrite) and *m*/*z* 47 ([^{15}N]nitrite), respectively (Fig. 1). Less intense ions were observed at *m*/*z* 226 (PFB–NO_2) and *m*/*z* 227 (PFB–$^{15}NO_2$) corresponding both to $[M-1]^-$. The peak area ratio of *m*/*z* 47 to *m*/*z* 46 from selected ion monitoring (SIM) of 1 nmol of PFB–NO_2 was determined as 0.00456±0.00013 (mean±SD, $n=5$) at a RSD of 2.9%. The peak area ratio of *m*/*z* 46 to *m*/*z* 47 from SIM of 1 nmol of PFB–$^{15}NO_2$ was measured as 0.02868±0.00026 (mean±SD, $n=5$) at a RSD of 0.9%. The higher value of the peak area ratio of *m*/*z* 46 to *m*/*z* 47 from PFB–$^{15}NO_2$ compared with that of *m*/*z* 46 to *m*/*z* 47 from PFB–NO_2 is due to the presence of [^{14}N]nitrite in the preparation of the internal standard sodium [^{15}N]nitrite and in the reagents used. The peak area ratio of *m*/*z* 46 to *m*/*z* 47 from SIM of a mixture containing each 0.5 nmol of PFB–NO_2 and PFB–$^{15}NO_2$ was determined as 1.0310±0.0025 (mean±SD, $n=5$) at a RSD of 0.2%. These data indicate no cross-talk between ion channels *m*/*z* 46 and *m*/*z* 47.

2.4. Metabolism of S[^{15}N]ALB in human blood in vitro

S[^{15}N]ALB was added to human venous blood (polypropylene tube, heparin as the anticoagulating agent) to obtain a final concentration of about 25 μ*M* in plasma, and allowed to stand at room temperature for 12 h. Immediately after addition (i.e. time zero), and after 0.5, 1, 2, 4.5, 8.5 and 12 h, aliquots were taken, plasma was generated by centrifugation and SNALB was added to the plasma samples each at a concentration of 30 μ*M*. Samples were processed and analysed by GC–MS in duplicate as described above. S[^{15}N]ALB concentrations (in μ*M*) were calculated by multiplying the respective peak area ratios of *m*/*z* 47 to *m*/*z* 46 observed by the concentration of SNALB (30 μ*M*) used as the internal standard.

3. Results

3.1. Preparation, isolation and characterization of SNALB and S[^{15}N]ALB

Using equimolar concentrations of nitrous acid (i.e. nitrite in acidic solution) and commercially available HSA we found that only about 2% of the protein was susceptible for *S*-nitrosylation despite a decrease in the concentration of nitrous acid of about 20 and 60% within 1 and 40 min, respectively. This finding fully agrees with the concentration of the sulhydryl groups in the HSA preparation which amounted to only 2.2% as measured by the method

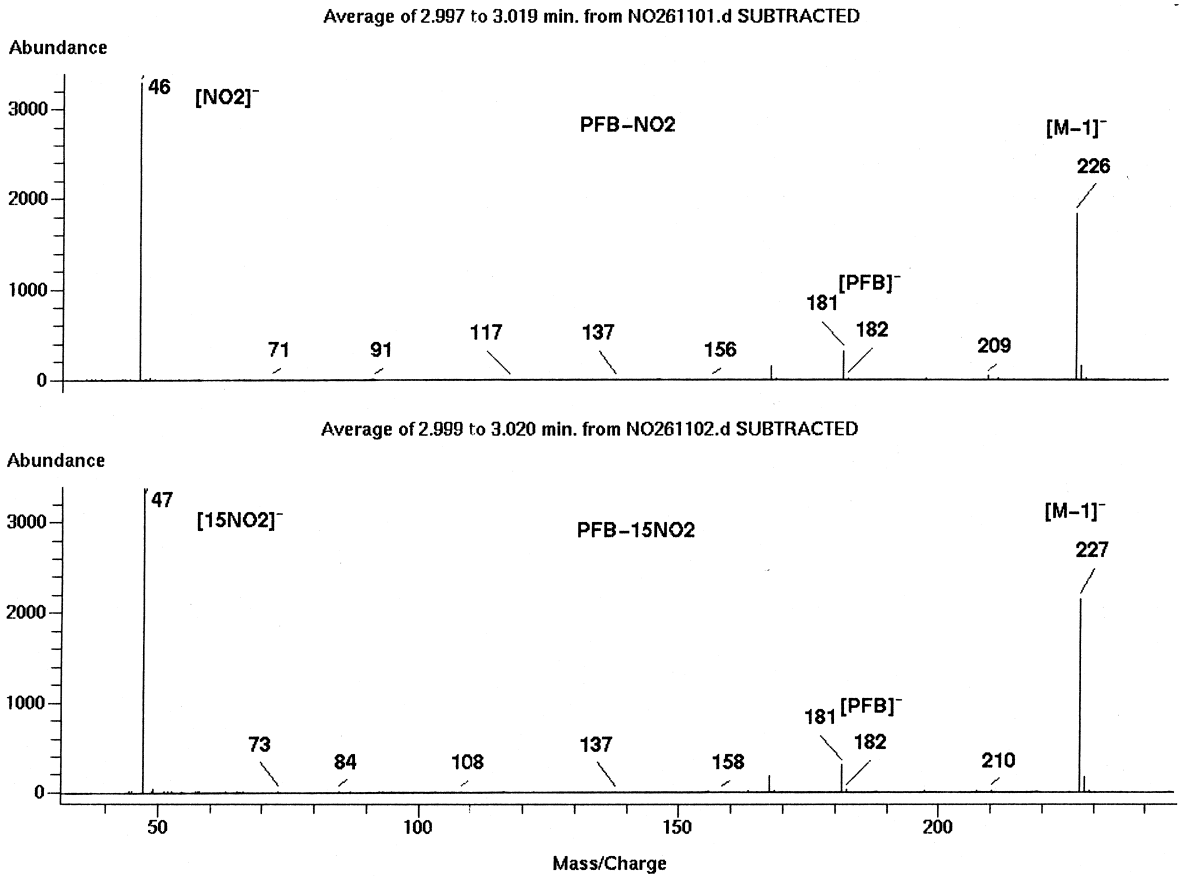

Fig. 1. GC–MS NICI mass spectra of PFB–NO_2 (upper panel) and PFB–$^{15}NO_2$ (lower panel).

of Ellman [14]. Reduction of commercially available HSA by DTT [11], $NaBH_4$ [15] or 2-mercaptoethanol [16], and subsequent nitrosylation by unlabeled or ^{15}N-labeled nitrous acid or butylnitrite was found to be very time-consuming, cumbersome and to result in low yields and unstable preparations of S[^{15}N]ALB (not shown). On the other hand, incubation of albumin immediately after its extraction from freshly obtained human plasma with various nitrite concentrations in acidic solution (pH 2) was found to lead to satisfactory *S*-nitrosylation of albumin (Fig. 2). This figure shows that the formation of SNALB parallels the formation of unknown nitrosated species that are in excess over SNALB in the whole concentration range tested. Fig. 2 shows that at initial nitrite concentrations below 60 μ*M*, SNALB formation linearly depends on nitrite concentration and slopes at nitrite concentrations above 60 μ*M*. Thus, under acidic conditions maximum *S*-nitrosylation of albumin by nitrite requires about two molar excess of nitrite over free sulfhydryl groups. Under such conditions significant amounts of unreacted nitrite will also be present in the reaction mixtures.

The reaction of nitrous acid and butylnitrite with plasma proteins was also investigated by UV–Vis spectrophotometry (Fig. 3). Reaction of nitrous acid with plasma resulted in continuous increases in absorbance between 300 and 400 nm (Fig. 3, left panel). By contrast reaction of 1-butylnitrite with plasma led to a continous decrease in absorbance in the same wavelength range. Fig. 4 showing UV–Vis spectra of 1-butylnitrite, nitrite and of *S*-nitroso-*N*-acetyl-cysteine (SNAC) demonstrates that measure-

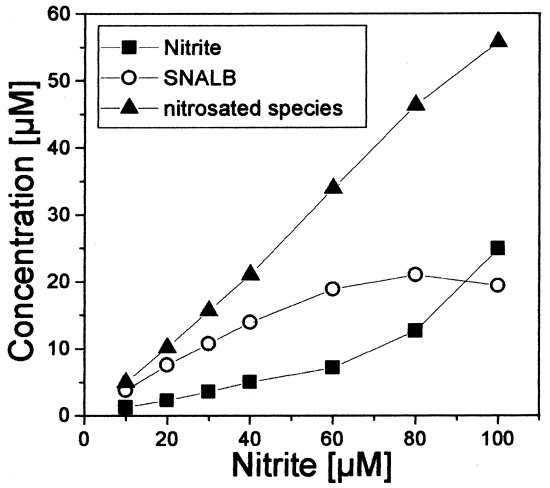

Fig. 2. Concentration dependent formation of SNALB and other nitrosated albumin species on the initial nitrite concentration used. A 120 μ*M* concentration (by Bradford [17]) of albumin extracted from freshly obtained human plasma containing 44 μ*M* sulfhydryl groups (by Ellman [14]) were incubated with the indicated nitrite concentrations, acidified by 5 *M* HCl to pH 2 and allowed to stand at room temperature for 60 min. Nitrite and SNALB concentrations were determined by GC–MS [10]. The concentration of the unknown nitrosated species was calculated by mass balance.

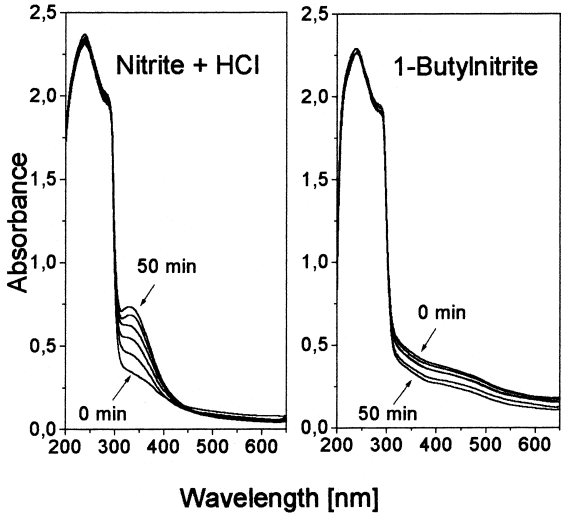

Fig. 3. UV–Vis spectra from incubation mixtures of freshly obtained human plasma (diluted 1:10 with distilled water) and nitrous acid at pH 2 (left panel) or butylnitrite at pH 7.4 (right panel) at room temperature for 50 min. Spectra were generated consecutively every 5 min within 50 min. Initial concentrations were: 76 μ*M* for plasma proteins (by Bradford [17]), 600 μ*M* for nitrous acid and 380 μ*M* for butylnitrite. The reference cuvette contained plasma (diluted 1:10 with distilled water).

ment of *S*-nitroso compounds by spectrophotometry around 340 nm may lead to erroneous results. The decrease of absorbance in the reaction of butylnitrite with plasma (Fig. 3, right panel) is most likely due to the decrease in the concentration of butylnitrite resulting from reaction of 1-butylnitrite and/or from its decomposition. At the end of these experiments, the concentration of *S*-nitroso-proteins were determined by GC–MS as 20 μ*M* (from nitrous acid) and 22 μ*M* (from butylnitrite). On the basis of the molar absorption coefficient of the *S*-nitroso group in *S*-nitroso-cysteinyl derivatives including SNALB of the order of 900 M^{-1} cm^{-1} around 340 nm [11], formation of 20 μ*M* of SNALB would cause an increase of about 0.002 absorption units. However, we obtained increases of about 0.4 absorption units using nitrous acid (Fig. 3, left panel). This finding suggests that the increases in the absorption in the wavelength range 300–400 nm of plasma proteins nitrosated by a 10-fold excess of nitrous acid over sulfhydryl concentration are not caused by *S*-nitrosylation of proteins-associated cysteine but are rather due to nitrosation of aromatic residues of proteins such as tyrosine, phenylalanine and tryptophan. This is supported by the observation, that reaction of plasma proteins with nitrous acid is accompanied with a change of the color of the plasma to green. It is notable that human serum albumin contains a single reduced cysteine but 18 tyrosine, 31 phenylalanine and one tryptophan residues [16]. Preparation of SNALB by nitrous acid is also accompanied by protein precipitation due to denaturation of albumin. These results strongly suggest that investigations on formation and decomposition of SNALB by spectrophotometry by measuring absorption changes around 340 nm for physiological concentrations of SNALB (e.g. below 7 μ*M* [3]) will be impossible (see also Ref. [11]).

To avoid loss of sulfhydryl groups by oxidation during the isolation of plasma proteins by extraction on HiTrapBlue Sepharose columns or Sephadex PD-10 cartridges and to remove excess of nitrous acid or butylnitrite and side-products, we preferably performed *S*-nitrosylation in fresh native plasma and then extracted *S*-nitroso-proteins. SDS-PAGE electrophoresis under non-reducing conditions of aliquots

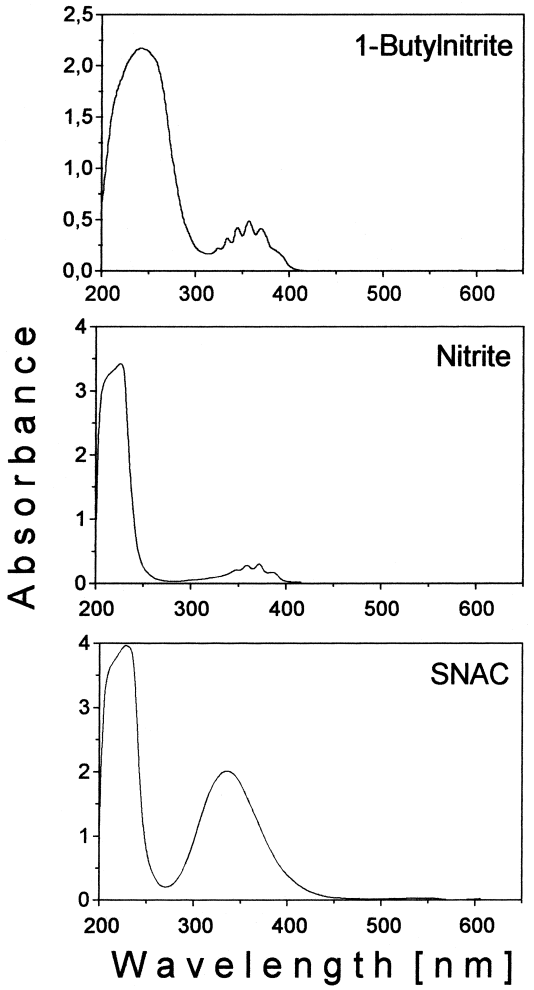

Fig. 4. UV–Vis spectra of 1-butylnitrite (10 m*M*), nitrite (9.5 m*M*) and *S*-nitroso-*N*-acetyl-cysteine (SNAC; 2.4 m*M*) in aqueous solution (pH 7.4). The molar extinction coefficients (in M^{-1} cm^{-1}) were determined as 48 for 1-butylnitrite (at 357 nm), 33 for nitrite (at 358 nm) and 769 for SNAC (at 333 nm).

from extracts of ^{15}N-nitrosylated human plasma proteins by using a two molar excess of [^{15}N]nitrite over sulfhydryl groups in HCl acid solution revealed a purity of about 90% (HiTrapBlue Sepharose cartridge) and 75% (Sephadex PD-10 cartridge) with respect to the compound co-migrating with commercially available HSA (Fig. 5). Thus, the most abundant protein in eluates from the extraction of ^{15}N-nitrosylated plasma proteins both by HiTrapBlue Sepharose and Sephadex PD-10 columns is S[^{15}N]ALB. However, the use of HiTrapBlue Sepharose cartridges results in S[^{15}N]ALB preparations of higher purity than the use of Sephadex PD-10 cartridges. GC–MS analysis of aliquots of $HgCl_2$-treated gel bands with the migration time of HSA and ^{15}N-nitrosylated human plasma albumin gave a peak area ratio of m/z 47 to m/z 46 of 0.006±0.002 and 0.042±0.006 (mean±SD, $n=3$), respectively, indicating formation of S[^{15}N]ALB. The relative low level for the peak area ratio m/z 47 to m/z 46 for the sample containing S[^{15}N]ALB most probably results from the decomposition of the *S*-[^{15}N]nitroso group of S[^{15}N]ALB during the electrophoretic step. Similar results were obtained also for ^{15}N-nitrosylated proteins prepared using ^{15}N-labeled butylnitrite.

^{15}N-Nuclear magnetic resonance spectra (16 384 scans per spectrum) of isolated (by HiTrapBlue Sepharose) S[^{15}N]ALB and synthetic GS[^{15}N]O were generated on a Bruker DMX-600 instrument using a resonance frequency of 150 MHz. At the maximum concentration of S[^{15}N]ALB achievable (about 2 m*M*), no signals could be obtained in the spectrum of S[^{15}N]ALB. Using GS[^{15}N]O at concentrations of 10 m*M* a weak signal was obtained at 751 ppm in reference to a 20 m*M* aqueous [^{15}N]nitrite solution at 587 ppm (Fig. 6). Bonnett et al. [18] have reported a chemical shift at 748.3 ppm for *S*-[^{15}N]nitroso-*N*-acetyl-cysteine. Our findings agree with a previous report by Bonnett et al. [18] indicating that high concentrations of *S*-[^{15}N]nitroso compounds are required to obtain ^{15}N-Nuclear magnetic resonance spectra. On the other hand, our results disagree with the report by Stamler et al. [4] who obtained a strong signal from 2 m*M* of S[^{15}N]ALB prepared by [^{15}N]nitrous acid.

3.2. Standardization of S[^{15}N]ALB

Within a time period of 1 year we prepared S[^{15}N]ALB standards utilizing fresh human plasma three times by using [^{15}N]nitrite in acidic solution and eight times by using butyl[^{15}N]nitrite. Rough estimation of S[^{15}N]ALB concentrations in dilution of its preparations was achieved spectrophotometrically by the modified Saville [8] method using the Griess reaction [10]. Accurate standardization of S[^{15}N]ALB standards was managed by GC–MS

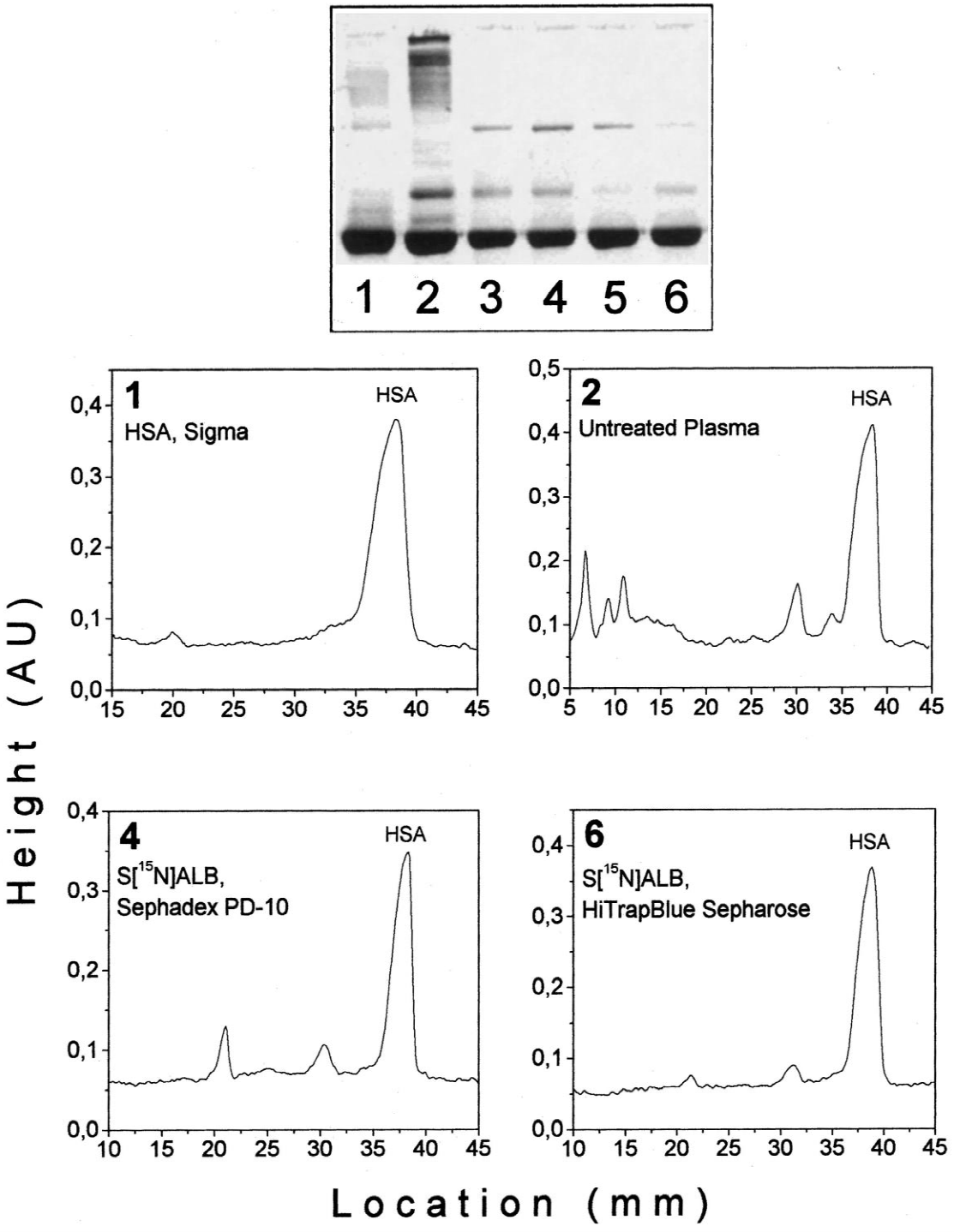

Fig. 5. SDS-PAGE electropherogram and gel scan chromatograms from the analysis of commercially available HSA (lane 1), of freshly obtained human plasma without extraction (lane 2), and of S[^{15}N]ALB preparations after extraction by Sephadex PD-10 (lane 4) and HiTrapBlue Sepharose cartridges (lane 6) of reactions mixtures of fresh human plasma with [^{15}N]nitrite in HCl acidic solution (pH 2). Lanes 3 and 5 correspond to S[^{14}N]ALB preparations extracted by Sephadex PD-10 and HiTrapBlue Sepharose cartridges, respectively.

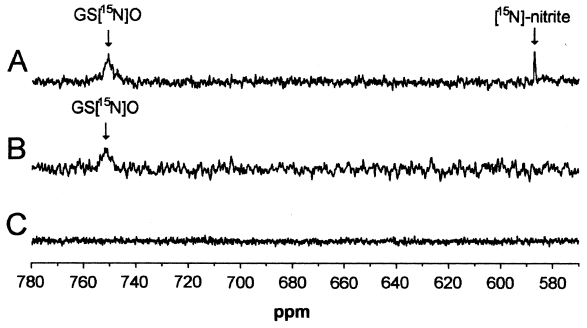

Fig. 6. ^{15}N-Nuclear magnetic resonance spectra generated from 10 m*M* GS[^{15}N]O (A and B) and from 2 m*M* S[^{15}N]ALB (C). S[^{15}N]ALB was prepared in fresh human plasma using [^{15}N]nitrite and HCl acid (pH 2), extracted on HiTrapBlue Sepharose cartridges and concentrated by ultrafiltration (cut-off 20 kDa) to a final concentration of about 2 m*M*. The chemical shift of GS[^{15}N]O and [^{15}N]nitrite were 751 and 587 ppm, respectively. GS[^{15}N]O was prepared by incubation of equal volumes of GSH and [^{15}N]nitrite and acidification by 5 *M* HCl.

analysis of mixtures containing varying concentrations of S[^{15}N]ALB and a fixed known concentration of nitrite used as the internal standard. Typical GC–MS chromatograms from such analyses are shown in Fig. 7. Linear regression analysis between the peak area ratio m/z 47 over m/z 46 and the expected concentration ratio of S[^{15}N]ALB to [^{14}N]nitrite used as the internal standard revealed straight lines (r=0.989). The concentration of S[^{15}N]ALB in its stock solutions was calculated from the slope of the respective regression equations. Using $HgCl_2$ (1 m*M*), the concentration of total [^{15}N]nitrite in the S[^{15}N]ALB preparations isolated (2 ml of buffer B) was determined as (mean±SD) 156±30 μ*M* using [^{15}N]nitrous acid and 188±43 μ*M* using ^{15}N-labeled butylnitrite. Similar results were also obtained by the use of a mixture of $CuSO_4$ and cysteine at final concentrations of 500 n*M* and 50 μ*M*, respectively, instead of 1 m*M* $HgCl_2$. This finding is further strong evidence for the formation of S[^{15}N]ALB. Without external addition of Cu^{2+} ions, formation of NO (y) linearly dependent on the SNALB concentration (x) in the range 0 to 20 μM: $y=-3.6+0.151x$ (r^2=0.990) at 500 μ*M* of cysteine. NO was detected in this experiment by a NO electrode from World Precision Instruments (Berlin, Germany). In

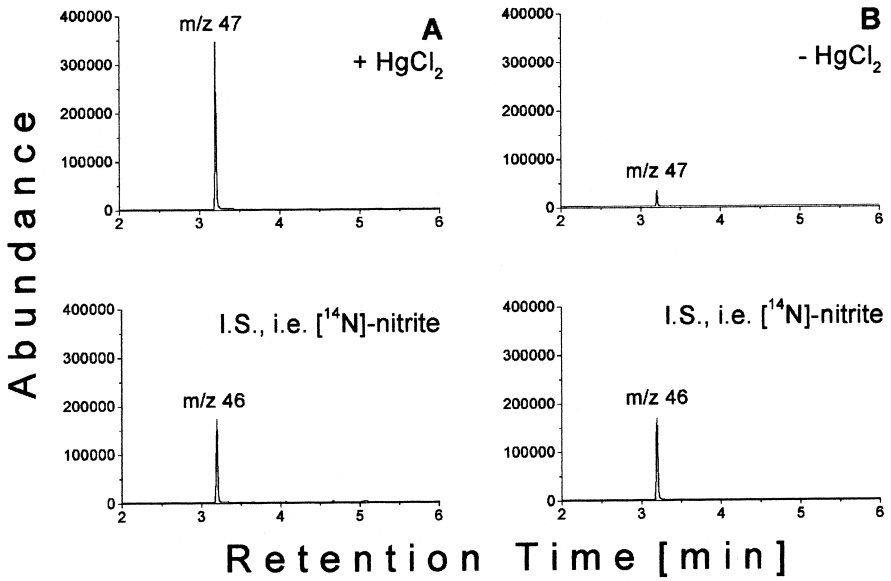

Fig. 7. Partial chromatograms from GC–MS analyses of S[^{15}N]ALB prepared by butyl [^{15}N]nitrite starting with fresh human plasma. (A) A 100-μl aliquot of a S[^{15}N]ALB preparation (163 μ*M*) spiked with [^{14}N]nitrite (80 μ*M*) for use as internal standard was treated with $HgCl_2$ to obtain [^{15}N]nitrite. (B) A second 100-μl aliquot of the same preparation was also spiked with [^{14}N]nitrite (80 μ*M*) but not treated with $HgCl_2$. The samples were derivatized by PFB bromide and analysed by GC–MS in the selected ion monitoring mode by detecting m/z 47 for [^{15}N]nitrite (upper traces) and m/z 46 for [^{14}N]nitrite (lower traces).

the absence of $HgCl_2$ or of the $CuSO_4$/cysteine reagent, the mean [^{15}N]nitrite concentrations in the S[^{15}N]ALB preparation were (mean±SD) 23.5±1.4 and 9.7±5.2 μ*M*, respectively. It is unknown whether the [^{15}N]nitrite portion in the S[^{15}N]ALB preparation completely originates from sources other than *S*-nitroso groups or partly derived from S[^{15}N]ALB due to thermal decomposition of the *S*-[^{15}N]nitroso group during derivatisation by PFB bromide. Because of the considerably lower [^{15}N]nitrite content of the S[^{15}N]ALB preparations obtained from the use of butyl[^{15}N]nitrite, the latter method is more suitable for the preparation of S[^{15}N]ALB for use as an internal standard in quantitative measurements by GC–MS. Considering a mean recovery rate of 45% for the extraction of albumin and SNALB on HiTrapBlue Sepharose columns as found by measuring protein concentrations by the method of Bradford [17], our results suggest that about 300 to 400 μ*M* of albumin, i.e. about 50% of total albumin, in freshly obtained human plasma is susceptible to *S*-nitrosylation. Using both ^{15}N-labeled nitrous acid and butylnitrite, S[^{15}N]ALB preparations were measured to contain about 98% of ^{15}N which is very close to the ^{15}N-content of the sodium [^{15}N]nitrite preparation used.

Standard curves were generated by GC–MS analysis of mixtures containing SNALB (0, 1, 2, 5, 7.5, 10, 15, and 20 μ*M*) and S[^{15}N]ALB at a fixed concentration of 5 μ*M*. Plotting of the mean SNALB/S[^{15}N]ALB, i.e. *m*/*z* 46 to *m*/*z* 47 peak area ratio (*y*) against the concentration of SNALB (*x*) resulted in a straight line with the regression equation $y=0.03+0.202x$ ($r^2=0.998$). This result clearly demonstrates the utility of S[^{15}N]ALB as an internal standard for the quantitative determination of SNALB by GC–MS.

The factors influencing stability of SNALB are still poorly understood. Hg^{2+} at high concentrations catalyses rapidly and quantitatively the conversion of SNALB to nitrite. Cu^{2+} at nanomolar-concentrations in combination with cysteine also converts rapidly and quantitatively SNALB to nitrite by a mechanism involving formation of *S*-nitroso-cysteine and subsequent release of NO (not shown). The decomposition of *S*-nitroso-cysteine to nitrite has been shown to be catalysed by trace amounts of Cu^{2+} ions [11]. In order to avoid Cu^{2+}-catalysed decomposition of SNALB we added the chelating agent EDTA at a concentration of 1 m*M* to a SNALB preparation and stored it at 4°C for about 2 months. The nitrite content of this SNALB preparation increased from 12.5% at day zero to 27, 31 and 32% at days 21, 37 and 53, respectively. Thus, S[^{15}N]ALB may considerably decompose on storage even in the presence of EDTA so that the concentration of S[^{15}N]ALB has to be accurately determined at the time of use in quantitative measurements.

3.3. Metabolism of S[^{15}N]ALB in human blood

Fig. 8 shows that the concentration of S[^{15}N]ALB in human plasma exponentially decreases on incubation time. Plotting of the logarithmus naturalis of the ratio concentration of S[^{15}N]ALB at time zero to the concentration of S[^{15}N]ALB at the respective time points vs the incubation time resulted in a straight line with the regression equation $y=0.1264x$ ($r^2=0.970$). From the slope of this equation a reaction rate constant *k* of 0.1264 h^{-1} and a half-life of 5.5 h were calculated for the first-order decay of S[^{15}N]ALB concentration in human plasma. The

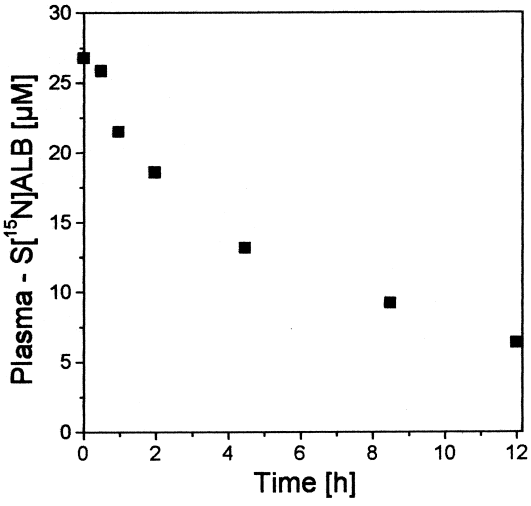

Fig. 8. Concentration–time profile for the decay of the S[^{15}N]ALB concentration upon incubation in human blood for 12 h. The initial concentration of S[^{15}N]ALB was about 25 μ*M*. At the indicated time points blood aliquots were taken and S[^{15}N]ALB concentrations were determined by GC–MS following addition of the internal standard SNALB (30 μ*M*) to the plasma samples.

mean RSD from analyses performed in duplicate was determined as 6.7% (range: 2.1 to 10.5%).

4. Discussion

The lack of suitable analytical methods for the measurement of SNALB in human plasma and other biological fluids has hampered reliable determination of SNALB levels in human plasma and investigations on formation, metabolism and reactions of SNALB at physiologically relevant concentrations. The potential significance of SNALB as a reservoir for endogenous NO as well as for appropriate pharmacologically active NO-containing or NO-releasing drugs including organic nitrates/nitrites and sodium nitroprusside with which vascular tone, platelet aggregation, and possibly other NO-mediated biological activities could be regulated in vivo in humans, makes mandatory the development of specific and accurate analytical approaches for SNALB.

Chemiluminescence is the only method used to measure endogenous SNALB levels in human plasma so far. By this technique mean SNALB levels of about 7 ± 5 μM were measured in the plasma of five healthy subjects [3]. This method has not yet been described in detail. Formation and decomposition of SNALB during *S*-transnitrosylation reactions have been studied almost exclusively by spectrophotometry [11–13]. However, measurements by this approach are indirect because SNALB can not be detected around 340 nm due to a very small molar extinction coefficient [11]. Also, a possible interference by stronger absorbing nitrosated proteins can not be excluded. The greatest drawback of chemiluminescence and spectrophotometric assay methods for quantitative measurement of SNALB is, however, the inability to use an internal standard.

Unlike chemiluminescence and spectrophotometry, MS is the only approach that enables reliable quantitative determination of analytes even in complex matrices by using stable isotope-labeled analogs of the analyte to be measured. Measurement of intact SNALB by MS has not been reported so far. In the presence of great excess of albumin over SNALB, the molar ratio of albumin to SNALB could be in the order of 50:1 to 300:1 [3]. Therefore, and because of the very low difference in the molecular weights of albumin and SNALB, mass spectrometric distinction between SNALB and albumin is difficult or impossible. In the present work, we describe an alternative MS method which is based on selective Hg^{2+}-catalyzed [8] cleavage of the *S*-nitroso group of SNALB to nitrite and its accurate quantification of it by a previously described specific GC–MS method [9,10].

Well-characterized and pure unlabeled SNALB standards for use in chemiluminescence, spectrophotometry and in other MS-unrelated assay methods, and stable isotope-labeled SNALB standards for use in MS methods are necessary to calibrate the systems or for use as internal standards for purpose of quantitation. Also, such SNALB standards are indispensable for biological experiments. Synthesis of unlabeled and stable isotope-labeled, well-characterized *S*-nitroso compounds from low molecular weight thiols is easy to perform [4,11,19–21]. Synthesis of labeled and unlabeled SNALB standards is, however, unequally more difficult (see Fig. 9). The most frequently used method to prepare SNALB involves the use of nitrite in acidic solution, i.e. nitrous acid, and butylnitrite at neutral pH. The sole cysteine residue of albumin possessing a free sulfhydryl group per albumin molecule for *S*-nitrosylation competes with other supernumerary aromatic residues of HSA which are also susceptible for nitrosation (Fig. 9). The results of the present study indicate that butylnitrite is superior to nitrite for many reasons regarding preparation of SNALB standards. The major advantages of the use of butylnitrite over nitrous acid are careful conditions and the preparation of SNALB standards with a relatively low content of nitrosated albumin residues. Reaction of butylnitrite with other residues of albumin such as tyrosine are apparently negligible due to its weak electrophilic potency against aromatic rings of albumin. Satisfactory *S*-nitrosylation of albumin by nitrous acid requires at least a two molar excess over sulfhydryl groups. Under these conditions considerably nitrosation of other residues of albumin occurs yielding green-colored products. Nitrosated albumin interferes in GC–MS analysis as shown in this work and could also interfere in other analytical methods and in biological experiments. Synthesis of SNALB standards by *S*-nitrosylation using low molecular weight *S*-nitroso compounds is

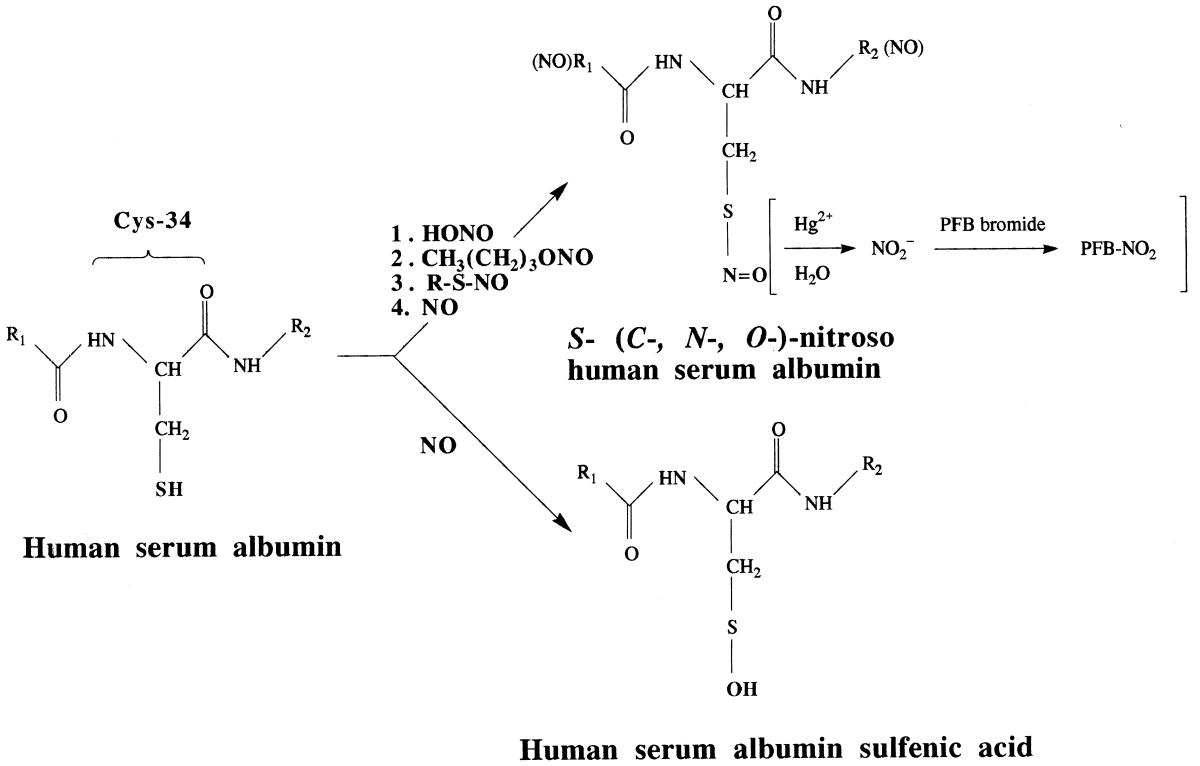

Fig. 9. Schematic drawing of the proposed reactions of human serum albumin (HSA) with nitrous acid, butylnitrite, *S*-nitroso compounds (RSNO) and NO to form nitros(yl)ated HSA and HSA sulfenic acid. Nitrous acid, butylnitrite, *S*-nitroso compounds and NO react with the sulhydryl group of cysteine-34 of HSA to form *S*-nitroso HSA. These agents also react with HSA residues distinct from cysteine-34 (R_1 and R_2) to form *C*-, *N*- and *O*-nitroso HSA (shown in parenthesis). In addition, NO oxidizes HSA to its sulfenic acid [22]. The Hg^{2+}-catalysed conversion of *S*-nitroso HSA to nitrite and its subsequent derivatization by PFB bromide to the PFB–NO_2 for GC–MS analysis is shown in brackets.

possible but requires an additional analytical step [12,13]. SNALB standards can also be prepared by using NO [4]. However, it must be considered that during exposure of albumin to NO considerable oxidation of the free sulfhydryl group of HSA to sulfenic acid may occur (Fig. 9) [22].

It has been shown that reduction of bovine serum albumin by DDT before nitrosylation leads to extensive molecular heterogeneity via oligomerization [23]. It is also possible that such changes may also occur under reducing conditions using $NaBH_4$ and 2-mercaptoethanol. We found that reduction of commercially available HSA by DDT, $NaBH_4$ or 2-mercaptoethanol is time-consuming, cumbersome and is associated with low recovery rates of reduced HSA. Because of these disadvantages preparation of SNALB using freshly obtained human plasma and butylnitrite is highly favored. Pure SNALB standards can be obtained from reaction mixtures by extraction on HiTrapBlue Sepharose columns.

Our results show that the use of HiTrapBlue Sepharose cartridges for extraction of SNALB from plasma and of Hg^{2+} ions to convert SNALB to nitrite are useful procedures in the quantitative measurement of SNALB concentrations in human plasma by GC–MS. This method allowed us to investigate S[^{15}N]ALB metabolism in human heparinized venous blood in a concentration range close to the physiological range reported for SNALB (7 ± 5 μM) [3]. In this work we assessed a half-life of about 5.5 h for S[^{15}N]ALB in human blood in vitro. Stamler et al. have reported a half-life of about 40 min for *S*-nitroso bovine serum albumin in plasma [4]. A method involving addition of

S[^{15}N]ALB to human plasma, extraction of endogenous SNALB and S[^{15}N]ALB from plasma by HiTrapBlue Sepharose cartridges, treatment of the eluate with Hg^{2+} ions to obtain nitrite and [^{15}N]nitrite, respectively, and their GC–MS analysis should be a useful approach to accurately and sensitively quantitate endogenous SNALB levels in plasma at concentrations below those measured in the in vitro experiment in this article, i.e. below 7 μM.

5. Conclusions

The most suitable method to prepare S[^{15}N]ALB in satisfactory yield and stability involves *S*-nitrosylation of albumin in freshly obtained human plasma by using an excess of ^{15}N-labeled butylnitrite, which can easily be prepared by sulfuric acid catalyzed *O*-nitrosylation of 1-butanol. Subsequent extraction of the reaction products on HiTrapBlue Sepharose affinity cartridges reveals S[^{15}N]ALB of high purity. Nitrosation by butylnitrite of albumin functionalities distinct from the sulfhydryl group of albumin and/or thermal decomposition of S[^{15}N]ALB of the order of 5% can not, however, be avoided. S[^{15}N]ALB can be selectively and quantitatively converted to [^{15}N]nitrite by $HgCl_2$. S[^{15}N]ALB may be a useful internal standard for the quantitative determination of endogenous SNALB in human plasma by GC–MS.

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References

[1] R.M.J. Palmer, A.G. Ferrige, S. Moncada, Nature 327 (1987) 524.
[2] M. Feelisch, M. the Poel, R. Zamora, A. Deussen, S. Moncada, Nature 368 (1994) 62.
[3] J.S. Stamler, O. Jaraki, J. Osborne, D.I. Simon, J. Keaney, J. Vita, D. Singel, C.R. Valeri, J. Loscalzo, Proc. Natl. Acad. Sci. U.S.A. 89 (1992) 7674.
[4] J.S. Stamler, D.I. Simon, J.A. Osborne, M.E. Mullins, O. Jaraki, T. Michel, D.J. Singel, J. Loscalzo, Proc. Natl. Acad. Sci. USA 89 (1992) 444.
[5] J.F. Keaney Jr., D.I. Simon, J.S. Stamler, O. Jaraki, J. Scharfstein, J.A. Vita, J. Loscalzo, J. Clin. Invest. 91 (1993) 1582.
[6] J.S. Scharfstein, J.F. Keaney Jr., A. Slivka, G.N. Welch, J.A. Vita, J.S. Stamler, J. Loscalzo, J. Clin. Invest. 94 (1994) 1432.
[7] Y. Minamiyama, S. Takemura, M. Inoue, Biochem. Biophys. Res. Commun. 225 (1996) 112.
[8] B. Saville, Analyst 83 (1958) 670.
[9] D. Tsikas, R.H. Böger, S.M. Bode-Böger, F.-M. Gutzki, J.C. Frölich, J. Chromatogr. B 661 (1994) 185.
[10] D. Tsikas, F.-M. Gutzki, S. Rossa, H. Bauer, Ch. Neumann, K. Dockendorff, J. Sandmann, J.C. Frölich, Anal. Biochem. 244 (1997) 208.
[11] D.J. Meyer, H. Kramer, N. Özer, B. Coles, B. Ketterer, FEBS Lett. 345 (1994) 177.
[12] H. Zhang, G.E. Means, Anal. Biochem. 237 (1996) 141.
[13] M.S. Studebaker, H. Zhang, G.E. Means, Anal. Biochem. 237 (1996) 193.
[14] G.L. Ellman, Arch. Biochem. Biophys. 82 (1959) 70.
[15] M.A. Mansoor, A.M. Svardal, P.M. Ueland, Anal. Biochem. 200 (1992) 218.
[16] T. Peters Jr, Adv. Protein Chem. 37 (1985) 161.
[17] M.M. Bradford, Anal. Biochem. 72 (1976) 248.
[18] R. Bonnett, R. Hollyhead, B.L. Johnson, E.W. Randall, J. Chem. Soc. Perkins Trans. 1 (1975) 2261.
[19] D.R. Arnelle, J.S. Stamler, Arch. Biochem. Biophys. 318 (1995) 279.
[20] D. Tsikas, R.H. Böger, S.M. Bode-Böger, G. Brunner, J.C. Frölich, J. Chromatogr. A 699 (1995) 363.
[21] D. Tsikas, S. Rossa, D.O. Stichtenoth, M. Raida, F.-M. Gutzki, J.C. Frölich, Biochem. Biophys. Res. Commun. 220 (1996) 939.
[22] E.G. DeMaster, B.J. Quast, B. Redfern, H.T. Nagasawa, Biochemistry 34 (1995) 11494.
[23] J.F. Ewing, D.V. Young, D.R. Janero, D.S. Garvey, T.A. Grinell, J. Pharmacol. Exp. Therap. 283 (1997) 947.