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Measurement of *S*-nitrosoalbumin by gas chromatography–mass spectrometry
II. Quantitative determination of *S*-nitrosoalbumin in human plasma using *S*-[^{15}N]nitrosoalbumin as internal standard

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Abstract

A gas chromatographic–mass spectrometric method for the quantitative determination of *S*-nitrosoalbumin (SNALB) in human plasma is described. The method is based on selective extraction of SNALB and its ^{15}N-labeled SNALB analog (S^{15}NALB) used as internal standard on HiTrapBlue Sepharose affinity columns, Hg^{2+}-catalysed conversion of the *S*-nitroso groups to nitrite and [^{15}N]nitrite, respectively, followed by their derivatization to the pentafluorobenzyl derivatives and quantification by GC–MS. Mean recovery of SNALB and S^{15}NALB from plasma was 45%. Mean precision and accuracy within the range 0–10 μ*M* was 95% and 99%, respectively. The limit of quantitation was determined as 100 n*M* at a precision of 93.8% and an accuracy of 94.8%. Considerable improvement of method sensitivity is possible by eliminating nitrite present in the elution buffer. The limit of detection was 0.2 n*M* corresponding to 67 amol of S^{15}NALB. In 0.4-ml aliquots of plasma samples from healthy humans, endogenous SNALB was determined at concentrations of 181±150 n*M* (mean±SD, n=23). External addition of SNALB to these plasma samples at 2 μ*M* and 5 μ*M* serving as quality control samples resulted in quantitative recovery of SNALB. Our results show that SNALB occurs in human plasma at concentrations at least one-order of magnitude smaller than those reported in the literature from measurements using chemiluminescence.

Keywords: *S*-Nitrosoalbumin

1. Introduction

Albumin is the most abundant high-molecular-mass (HMM) reduced thiol in human plasma that contains a single sulfhydryl group located at cysteine-34 [1]. The concentration of this HMM reduced thiol in human plasma is of the order of 300 μ*M* [1]. Reaction of nitric oxide (·NO), a putative endothelium-derived relaxing factor (EDRF) [2,3], with reduced thiols leads in part to nitrosylation of the sulhydryl-group yielding *S*-nitroso compounds [4]. It has been reported that the extremely short-lived ·NO, a half-life of 0.1 s has been described for authentic ·NO in vivo [5], circulates in human

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plasma primarily in the form of *S*-nitroso proteins, *S*-nitrosoalbumin (SNALB) being the most abundant of them [6]. For SNALB plasma concentrations of 7±5 μ*M* in five healthy volunteers have been reported [6]. SNALB is considerably more stable than ·NO but still exerts EDRF-like vasodilatory and antiaggregatory properties in vitro and vivo [6–9]. Therefore, SNALB could serve as a physiological reservoir not only for endogenously produced ·NO [6] but also for potential ·NO-releasing (pro)drugs such as [1-hydroxy-2-oxo-3-(*N*-methyl-3-aminopropyl)- 3 -methyl- 3 -aminopropyl]- 3 -methyl- 1 -triazene (NOC7) [10], glyceryl trinitrate, sodium nitroprusside, sydnonimines and *S*-nitroso compounds such as *S*-nitroso-*N*-acetyl-DL-penicillamine. By this mechanism ·NO-like functions could be regulated in mammals [6]. Thus, the conversion of administered ·NO-releasing (pro)drugs into SNALB could prolong duration of pharmacodynamic effects such as blood pressure due to increases in the half-life of these drugs.

Because of this potential significance we focussed our interest on a method allowing accurate quantitative determination of SNALB in human plasma. In a preceding publication [11], we described the preparation, purification, isolation, characterization and the metabolism of ^{15}N-labeled SNALB (S^{15}NALB) in human blood by gas chromatography–mass spectrometry (GC–MS). The principle of this method illustrated in Fig. 1 is based on specific cleavage of the *S*-nitroso group by Hg^{2+} to nitrite as first shown by Saville [12], conversion of nitrite to the pentafluorobenzyl (PFB) derivative and its analysis by GC–MS as described by us previously for nitrite and nitrate in human plasma and urine [13]. By means of this method we were able to measure S^{15}NALB added to human blood at concentrations within the range 10–25 μ*M*, and we showed that S^{15}NALB is very stable in human blood (half-life of 5.5 h) [11]. In the present work, we describe a fully validated GC–MS method for the selective, accurate and sensitive quantitative determination of endogenous SNALB in human plasma samples. Our results demonstrate that SNALB is physiologically present in human plasma but its concentration is at least one-order of magnitude smaller than that reported by Stamler et al. using chemiluminescence [6].

2. Experimental

2.1. Chemicals and reagents

Sodium [^{15}N]nitrite (98% at ^{15}N) was purchased from Cambridge Isotope Labs. (Andover, MA, USA). Butylnitrite, *N*-acetylcysteine (NAC) and 2,3,4,5,6-PFB bromide were obtained from Aldrich (Steinheim, Germany). Glutathione, L-cysteine and human serum albumin (HSA) were purchased from Sigma (Munich, Germany). Collagen was purchased from Hormonchemie (Munich, Germany). HiTrap-Blue Sepharose affinity columns (1-ml and 5-ml cartridges) were obtained from Pharmacia Biotech (Freiburg, Germany). Centrisart I ultrafiltration cartridges (pore size 4 μm, molecular mass cut-off 20 000) were obtained from Sartorius (Göttingen, Germany).

2.2. Synthesis and standardization of S-nitrosoalbumin standards

SNALB and S^{15}NALB standards were prepared and standardized as described previously [11]. Briefly, 3-ml aliquots of freshly obtained human plasma

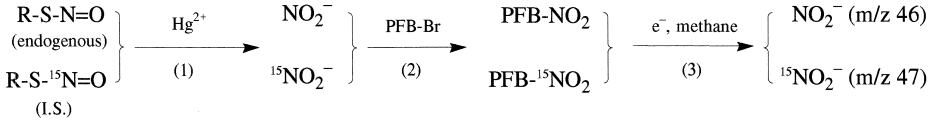

Fig. 1. Simplified schematic drawing of the principle of the GC–MS method for the analysis of *S*-nitrosoalbumin (RSNO, R = albumin-Cys-34) using ^{15}N-labeled *S*-nitrosoalbumin (RS^{15}NO, R = albumin-Cys-34) as internal standard (I.S.). Endogenous plasma RSNO and externally added RS^{15}NO extracted from plasma are converted by Hg^{2+} to NO_2^- and $^{15}NO_2^-$, respectively (1). NO_2^- and $^{15}NO_2^-$ are further derivatized by pentafluorobenzyl bromide (PFB-Br) to the corresponding PFB derivatives (2). In the final step of the method, PFB-NO_2 and PFB-$^{15}NO_2$ are separated by capillary GC, ionized by negative-ion chemical ionization to NO_2^- and $^{15}NO_2^-$, respectively, which are separated by MS according to their mass-to-charge ratios (m/z) and detected by the electron multiplier.

were 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 follows: the clear supernatant was applied to a 5-ml HiTrap-Blue Sepharose affinity column preconditioned with 10 ml of buffer A (50 m*M* KH_2PO_4, pH 7.0). 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). The eluate was ultrafiltered by centrifugation (1800 *g*, 15 min) until a protein fraction volume of about 3 ml was obtained. Concentrations of *S*-nitroso proteins in the final SNALB and S^{15}NALB preparations were determined by GC–MS using [^{15}N]nitrite and nitrite as internal standards, respectively. SNALB and S^{15}NALB preparations were stored at 4°C until use.

2.3. Procedure for the quantification of SNALB in human plasma

Plasma was obtained from normal healthy volunteers who were not taking any drugs or had taken drugs within the preceding two weeks, from patients with chronic renal failure and various liver diseases. Blood was withdrawn from antecubital veins using 5-ml monovettes containing citrate as anticoagulating agent and put immediately on ice. Plasma was recovered by centrifugation at 4°C (1800 *g*, 10 min). Plasma samples from healthy humans were analyzed immediately as described below. Plasma samples from patients were stored at −80°C until use. As a rule, to 400-μl aliquots of plasma samples S^{15}NALB was added to achieve a final concentration of 1 μ*M*. Extraction of proteins from plasma samples was performed as described above with exception of the use of 1-ml HiTrapBlue Sepharose affinity columns preconditioned with 2 ml of buffer A. Cartridges were washed with 4 ml of buffer A, and proteins were eluted from the columns by using 2-ml aliquots of buffer B. Eluates were ultrafiltered by centrifugation for 40 min at 4°C and 1800 *g* in order to obtain a protein fraction of about 400 μl. Two 100-μl aliquots of these fractions were treated with $HgCl_2$ (1 m*M*) and analyzed for nitrite by GC–MS after derivatisation as described [13] with the exception that reaction products were extracted with 300-μl aliquots of toluene. Two 100-μl aliquots of these protein fractions were analyzed for nitrite by GC–MS using [^{15}N]nitrite (1 μ*M*) as the internal standard (no addition of $HgCl_2$). Fig. 2 shows schematically the whole procedure used for quantification of SNALB in human plasma.

2.4. Recovery experiments

Absolute recovery rates of human plasma albumin and S^{15}NALB (spiked at 5 μ*M*) from 400-μl aliquots of plasma samples from healthy humans (n=20) after the HiTrapBlue Sepharose column extraction step was determined by measuring protein

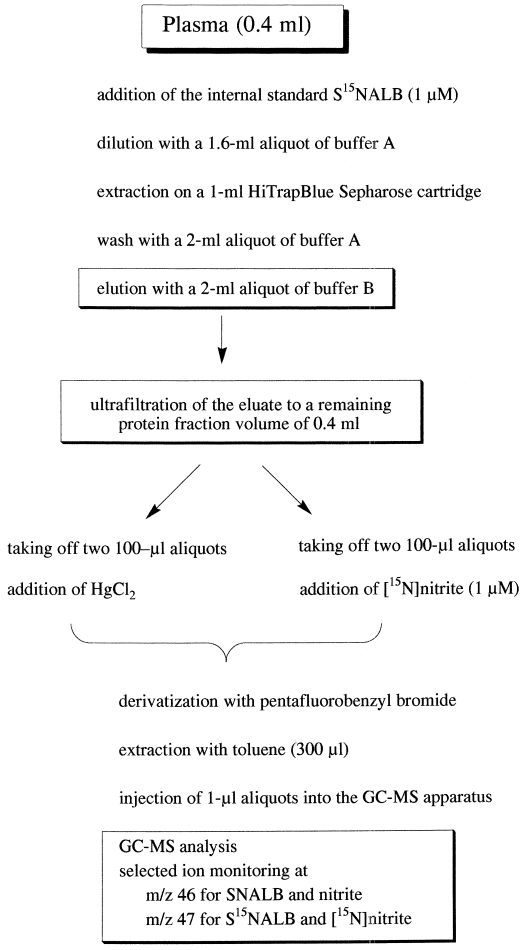

Fig. 2. Summary of the extraction and derivatization steps for the quantification of SNALB in human plasma by GC–MS.

concentrations in human plasma and the eluate (buffer B) by the method of Bradford [14] and by determining S^{15}NALB concentrations using nitrite (at 5 μ*M*) as the internal standard. Within the S^{15}NALB concentration range of 0–10 μ*M*, [^{15}N]nitrite (*y*) was recovered quantitatively from S^{15}NALB (*x*) by using $HgCl_2$ ($y=0.03+1.04x$).

2.5. Precision and accuracy of the method

Freshly obtained plasma from a healthy volunteer was spiked with 1 μ*M* of S^{15}NALB and with 0, 2 and 10 μ*M* of SNALB. Plasma samples were divided into 400-μl aliquots and stored at 4°C until further use. Five 400-μl aliquots of these solutions were analyzed in parallel within the first day to determine method intra-assay precision. On successive five days 400-μl aliquots were analyzed to determine method inter-assay precision.

A number of 400-μl aliquots of a freshly obtained plasma sample from a healthy volunteer were spiked with 1 μ*M* of S^{15}NALB and varying concentrations of SNALB (0, 1, 2, 4, 6, 8, 10 μ*M*) and treated as described above. Two 100-μl aliquots of the eluates were taken and derivatized and analyzed by GC–MS. The remaining 1.8-ml portions were ultrafiltered to remaining protein fraction volumes of about 400 μl. Four 100-μl aliquots of these solutions were derivatized and analyzed by GC–MS.

2.6. Limits of detection (LOD) and quantitation (LOQ)

The LOD was determined as follows: solutions of varying concentrations of S^{15}NALB (0, 0.1, 0.2, 0.4, 0.6, 0.8, 1, 2, 4, 6, 8, 10, 20, 40, 80 and 100 n*M*) were prepared in buffer B. One hundred-μl aliquots of these solutions were derivatized and analyzed by GC–MS in triplicate without previous extraction on HiTrapBlue Sepharose cartridges.

The LOQ was determined as follows: for each concentration, a 1.5-ml aliquot of a freshly obtained plasma sample from a healthy volunteer was spiked with 0.85 μ*M* of S^{15}NALB and with varying concentrations of SNALB (0, 0.1, 0.2, 0.4, 0.7, 1.0 μ*M*). Three 0.4-ml aliquots of each sample were extracted on 1-ml HiTrapBlue Sepharose cartridges and the eluates were ultrafiltered to remaining protein fraction volumes of about 400 μl. Four 100-μl aliquots of these solutions were derivatized and analyzed by GC–MS.

2.7. Identification of SNALB by platelet aggregation measurements and as SNAC

Identification of SNALB in human plasma was also performed by the following experiment. Freshly obtained human plasma was divided into two 3-ml aliquots. One sample, (sample A) was extracted on a 5-ml HiTrapBlue Sepharose cartridge without addition of SNALB. The second sample (sample B) was spiked with 4.4 μ*M* of SNALB and extracted on a second 5-ml HiTrapBlue Sepharose cartridge. Eluates of both samples were concentrated by ultrafiltration to remaining protein fraction volumes each of 3 ml (*conc sample A* and *conc sample B*). Using [^{15}N]nitrite as internal standard SNALB concentrations were determined by GC–MS in these fractions. Each 10-μl aliquots of *conc sample A* and *conc sample B* were used to test inhibitory potency on collagen-induced (1 μg/ml) aggregation in 250-μl suspensions of washed platelets in the absence and in the presence of L-cysteine (400 μ*M*). Also, each 50-μl aliquots of these samples were diluted each with 450 μl of buffer A and treated each with 100 μl of a 200 m*M* solution of NAC in water. Ten min after incubation at room temperature, samples were ultrafiltered and 200-μl aliquots of the ultrafiltrates were analyzed for *S*-nitroso-*N*-acetylcysteine (SNAC) by high-performance liquid chromatography (HPLC) as described elsewhere [15].

2.8. GC–MS analysis

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 Optima (15 m×0.25 mm I.D., 0.25 μm film thickness) from Macherey & Nagel (Düren, Germany) was used. Helium (30 kPa) and methane (200 Pa) were used as the carrier and the reagent gas, respectively, for negative-ion chemical ionization (NICI). Other GC–MS conditions were as described [13]. Selected ion monitoring (SIM) was performed at *m/z* 46 for nitrite and

SNALB, and at m/z 47 for [^{15}N]nitrite and S^{15}NALB.

3. Results

3.1. Recovery

Recovery rates from plasma using HiTrapBlue Sepharose affinity columns were determined as (mean±SD) 45±10% by measuring protein concentrations and 46±8% by measuring S^{15}NALB by GC–MS. These data indicate that SNALB and albumin behave identically with respect to extraction on HiTrapBlue Sepharose affinity columns.

3.2. Standard curves and calculation of SNALB concentrations in plasma

Four hundred-μl aliquots of plasma samples were spiked with various amounts of SNALB resulting in final added concentrations of 0, 1, 2, 4, 6, 8 and 10 μM and with a fixed amount of S^{15}NALB (5 μM). Samples were extracted on HiTrapBlue Sepharose cartridges. Two 100-μl aliquots of the eluates (E) were derivatized and analyzed by GC–MS. The remaining 1.8-ml aliquots were ultrafiltered to a remaining protein fraction (U) of about 0.4 ml. Each two 100-μl aliquots of these samples were derivatized and analyzed by GC–MS. The corresponding mean RSD values were 5.6% and 6.3%. Plotting of the mean peak area ratios of m/z 46 to m/z 47 (y) measured vs. the SNALB concentration added (x) resulted in straight lines with the regression equations: $y_E = 0.464 + 0.184x$ ($r = 0.997$) in the eluate fractions and $y_U = 0.289 + 0.186x$ ($r = 0.999$) in the ultrafiltered protein fractions. The slopes of these regressions are almost identical. On the other hand, ultrafiltration decreases the y-axis intercept. The y-axis intercepts correspond to the sum of endogenous plasma SNALB and nitrite present in the respective fractions which is mainly derived from buffer B. In untreated buffer B we measured nitrite at concentrations of 290±60 nM (mean±SD, $n = 50$).

These results show that SNALB-unrelated nitrite will contribute to the peak area ratio R of m/z 46 to m/z 47 in accordance to Eq. (1):

$$R = (\text{nitrite}_E + \text{SNALB}_E)/\text{S}^{15}\text{NALB}_E \quad (1)$$

where nitrite$_E$, SNALB$_E$ and S^{15}NALB$_E$ are the amounts of nitrite, SNALB and S^{15}NALB in the eluate, respectively.

Considering a mean recovery rate of 50% and a dilution factor of 5 [i.e., volume of plasma (0.4 ml) divided by volume of eluate (2 ml)], the concentrations of endogenous SNALB and of the internal standard S^{15}NALB in the eluate fractions will be each about 1/10th of those in plasma. Thus, for low endogenous SNALB concentrations, nitrite will greatly contribute to the peak area ratio m/z 46 to m/z 47. In order to calculate the concentration of SNALB in plasma from the ratio R measured in the eluate and the known concentration of the internal standard S^{15}NALB added to plasma Eq. (3) was developed from Eq. (1) via Eq. (2):

$$R = [\text{SNALB}]_P/[\text{S}^{15}\text{NALB}]_P + 1/A \times V_E/V_P \times ([\text{nitrite}]_E/[\text{S}^{15}\text{NALB}]_P) \quad (2)$$

$$[\text{SNALB}]_P = [\text{S}^{15}\text{NALB}]_P \times R - 1/A \times V_E/V_P \times [\text{nitrite}]_E \quad (3)$$

where A is the recovery rate (an A value of 0.5 corresponds to 50%); V_E and V_P are the volumes of the eluate and plasma, respectively; $[\text{SNALB}]_P$ and $[\text{S}^{15}\text{NALB}]_P$ are the concentrations of SNALB and S^{15}NALB in plasma; $[\text{nitrite}]_E$ is the concentration of nitrite in the eluate.

The validity of Eqs. (2) and (3) was checked as follows. A 2-ml aliquot of buffer A containing 4.4 μM of S^{15}NALB and 4.3 μM of SNALB was extracted on a 5-ml HiTrapBlue Sepharose cartridge and proteins were eluted with 10 ml of buffer B. Ratio R was determined in the remaining protein fraction after ultrafiltration to various V_E volumes. Plotting of the ratio R measured (y) vs. the V_E (x) resulted in a straight line with the regression equation $y = 1.09 + 0.388x$, $r = 0.982$. This result indicates that the term $\{1/A \times 1/V_P \times ([\text{nitrite}]_E/[\text{S}^{15}\text{NALB}]_P)\}$ of Eq. (2) does not change upon ultrafiltration. The y-axis intercept of the straight line agrees well with the theoretical ratio of $[\text{SNALB}]_P/[\text{S}^{15}\text{NALB}]_P$ which amounts to 4.3:4.4 = 0.977. Also, from the slope of the regression equation the nitrite concentration in the eluate, i.e., $[\text{nitrite}]_E$, is calculated

to 339 n*M* which is close to the measured nitrite concentration of 306±20 n*M* in this experiment. These results indicate that the concentration of SNALB in plasma can be accurately calculated by using Eq. (3) from the measured ratio *R*, the known concentration of the internal standard S^{15}NALB (in n*M*) added to the plasma, the known volume ratio V_E/V_P and the nitrite concentration which also must be determined in eluate fractions.

3.3. Study on interferences

3.3.1. Low-molecular-mass S-nitroso compounds and nitrite

Low-molecular-mass (LMM) *S*-nitroso compounds have been reported to occur physiologically in human plasma at concentrations of the order of 200 n*M* [6]. Because treatment of such compounds with $HgCl_2$ would also form nitrite [15], we investigated the potential interference by *S*-nitrosocysteine (SNC) and *S*-nitrosoglutathione (GSNO), the most favored structures for endogenous LMM *S*-nitroso compounds. Since nitrite is also physiologically present in human plasma at concentrations above those of LMM *S*-nitroso compounds [15], we also investigated a potential interference by nitrite. SNC, GSNO and nitrite solutions in buffer A (2 ml, each 24 μ*M*) were separately subjected to extraction on 1-ml HiTrapBlue Sepharose affinity columns as described above for SNALB. Each 1-ml aliquot of the eluate (2 ml) was analyzed for SNC, GSNO and nitrite by a modification [15] of the method of Saville [12]. We found in the eluates 0.4±0.2 μ*M* of SNC (i.e., 1.7% of total), 0.3±0.1 μ*M* of GSNO (i.e., 1.2% of total) and 0.2±0.1 μ*M* of nitrite (i.e., 0.8% of total). These results suggest that physiological concentrations of these compounds will not interfere with the measurement of SNALB.

3.3.2. Human albumin

Using the extraction procedure described here, unnitrosylated human albumin is co-extracted to a comparable degree with SNALB. In the eluate albumin is present at a high molar excess over SNALB. In order to investigate a potential interference of albumin in the determination of SNALB, the following experiment was carried out: In buffer B, solutions of commercially available HSA were prepared at concentrations of 0 and 380 μ*M*. To aliquots of these solutions SNALB was added at final concentrations of 0, 2.8, 5.6, 8, 11 and 12.8 μ*M*. Using S^{15}NALB (6.5 μ*M*) as internal standard samples were analyzed by GC–MS. Linear regression analysis between the peak area ratio of *m*/*z* 46 to *m*/*z* 47 measured (*y*) and SNALB amounts added (*x*) resulted in parallel straight lines with the following regression equations: $y=0.167+0.140x$ ($r=0.997$) and $y=0.201+0.137x$ ($r=0.995$), respectively. The slight increase in the *y*-axis intercept of the straight line from samples containing HSA could be due to nitrite present in the HSA preparation.

3.3.3. Effect of haemoglobin

Using the extraction procedure described here for SNALB we found that haemoglobin (0.5 mg/ml in buffer A) is retained on the HiTrapBlue Sepharose affinity cartridges and is eluted from them at a mean recovery rate of 43% which is very close to that of albumin. Haemoglobin concentrations were determined by the method of Bradford [14] by using a calibration curve generated in buffer A. The results suggest that plasma should be carefully prepared in order to avoid haemolysis and thus interference by *S*-nitrosohaemoglobin which has been reported to occur physiologically in human blood [16]. The concentration ratio of haemoglobin in plasma and blood is of the order of $1:10^5$. It seems therefore to be highly unlikely that plasma *S*-nitrosohaemoglobin would significantly contribute to SNALB.

3.3.4. Effect of anticoagulants

Blood was taken from a healthy donor by using monovettes that contained heparin, citrate or EDTA as an anticoagulant, and plasma was generated by centrifugation. Two 400-μl aliquots of each plasma were spiked with S^{15}NALB (5 μ*M*) and extracted using 1-ml HiTrapBlue Sepharose affinity columns. GC–MS analyses of the eluates resulted in the following SNALB concentrations (mean±SD): 110±30 n*M* for heparin, 120±20 n*M* for citrate and 120±15 n*M* for EDTA. These results indicate that the antiacoagulants tested have no significant effect on the measurement of SNALB by the method.

3.3.5. Effect of the time point of spiking S^{15}NALB to plasma and blood

Using EDTA monovettes blood was taken from two healthy volunteers (one female and one male).

Two 3-ml aliquots of each plasma sample were spiked with S^{15}NALB at a final concentration of 4.2 μ*M*. Immediately thereafter, blood from these volunteers was collected directly by dropping into the EDTA monovettes to which S^{15}NALB had been added previously. The final concentration of S^{15}NALB in the resulting plasma samples was calculated to be about 4 μ*M*. Two 3-ml aliquots of each plasma sample were subjected to extraction on 5-ml HiTrapBlue Sepharose cartridges. GC–MS analysis of the samples resulted in the following peak area ratios of *m*/*z* 46 over *m*/*z* 47: 0.588±0.006 and 0.767±0.002 (male), and 0.559±0.026 and 0.715±0.01 (female), for S^{15}NALB added to plasma and blood, respectively. The higher ratios measured in the samples in which S^{15}NALB was added to blood could be due to a lower concentration of S^{15}NALB in the resulting plasma samples than estimated, i.e., 4 μ*M*. These results indicate that endogenous SNALB remains unchanged until plasma generation, and suggest that the time-point of addition of S^{15}NALB to plasma is not crucial. This is supported by the finding that S^{15}NALB has a half-life of 5.5 h in human blood in vitro [11].

3.4. Precision and accuracy of the method

The concentration of SNALB in plasma samples was determined as [mean±SD, (RSD, %)] 0.43±0.04 μ*M* (8.6%), 2.46±0.12 μ*M* (4.7%) and 10.3±0.3 μ*M* (3.3%) for unspiked plasma and plasma spiked with 2 μ*M* and 10 μ*M* of SNALB, respectively, when samples were analyzed within a day. When the corresponding samples were analyzed on five successive days the concentration of SNALB was determined as 0.43±0.04 μ*M* (9.3%), 2.4±0.16 μ*M* (6.6%) and 10.3±0.3 μ*M* (2.9%), respectively.

Data on accuracy of the method for ultrafiltered and non-ultrafiltered samples are presented in Table 1. According to these data, mean accuracy was 102% for non-ultrafiltered and 99% for ultrafiltered samples, and the corresponding mean RSD values were 7.8% and 5.2% in the concentration range investigated.

3.5. LOD and LOQ

Plotting of the peak area ratio of *m*/*z* 47 to *m*/*z* 46 (*y*) from GC–MS analysis of the LOD samples vs. the S^{15}NALB concentrations (in n*M*) (*x*) within the whole concentration range tested revealed a straight line with the regression equation: $y = 0.0054 + 0.0017x$ ($r = 0.993$). The mean ratios of *m*/*z* 47 to *m*/*z* 46 were determined as 0.0052 (RSD of 2.7%) for 0.0 n*M* of S^{15}NALB and 0.0058 (RSD 5.7%) for 0.2 n*M* of S^{15}NALB. The signal-to-noise ratio for 0.2 n*M* of S^{15}NALB was determined as 27.7:1±1.5 (RSD of 5.5%); the amount injected was calculated as 67 amol.

Plotting of SNALB concentrations measured (*y*) vs. the SNALB added (*x*) from GC–MS analysis of the LOQ samples resulted in a straight line with the regression equation: $y = 98 + 1.073x$, $r = 0.990$. The concentration of endogenous SNALB in the plasma used was determined as 98 n*M* at an RSD of 7.7%. The lowest concentration of externally added SNALB of 100 n*M* was determined at a mean

Table 1
Accuracy of the method[a]

SNALB added (μ*M*)	SNALB found (μ*M*) [mean±SD, (RSD, %)]		Accuracy (%)	
	Without ultrafiltration	With ultrafiltration	Without ultrafiltration	With ultrafiltration
0.0	0.111±0.018 (16)	0.146±0.017 (11.6)	n.a.	n.a.
1.0	1.235±0.148 (12)	1.155±0.064 (5.5)	111	101
2.0	2.090±0.226 (10.8)	2.110±0.127 (6.0)	99	98
4.0	4.195±0.233 (5.6)	4.090±0.198 (4.8)	102	99
6.0	6.130±0.156 (2.5)	6.025±0.199 (2.9)	100	98
8.0	8.050±0.255 (3.2)	8.030±0.255 (3.2)	99	99
10.0	10.185±0.488 (4.8)	10.045±0.262 (2.6)	101	99
Mean RSD/mean accuracy (%)	7.8	5.2	102	99

[a] The concentration of the internal standard S^{15}NALB was 1 μ*M*. The concentration of blank nitrite in buffer B was 290±60 n*M* (mean±SD, $n = 50$). Eq. (3) was used for calculation of SNALB concentrations.

accuracy of 105% and a mean imprecision (RSD) of 6.2%. SNALB added at 200 n*M* was determined at an accuracy of 92.3% and an imprecision (RSD) of 11.7%.

3.6. Platelet aggregation measurement

Fig. 3A shows original tracings from GC–MS analyses of SNALB in *conc sample A* and *conc sample B* using [^{15}N]nitrite as internal standard. The concentration of SNALB in these samples was determined as 0.36±0.01 μ*M* and 11.3±0.03 μ*M*, respectively. Representative tracings from aggregation measurements are shown in Fig. 3B. The results from aggregation measurements shown in Fig. 3C confirm the results from GC–MS measurements of physiological SNALB in plasma of healthy humans indicating that SNALB is present in plasma of healthy humans at concentrations much lower than those reported by Stamler et al. [6].

This finding was further verified by converting SNALB into SNAC and its analysis by HPLC. Incubation of *conc sample A* with NAC did not result in formation of detectable amounts of SNAC (LOD of 170 n*M*) while incubation of *conc sample B* with NAC did result in formation of SNAC at a concentration of 6.1 μ*M*. From a similar experiment using aqueous solutions of SNALB (0–15 μ*M*) we

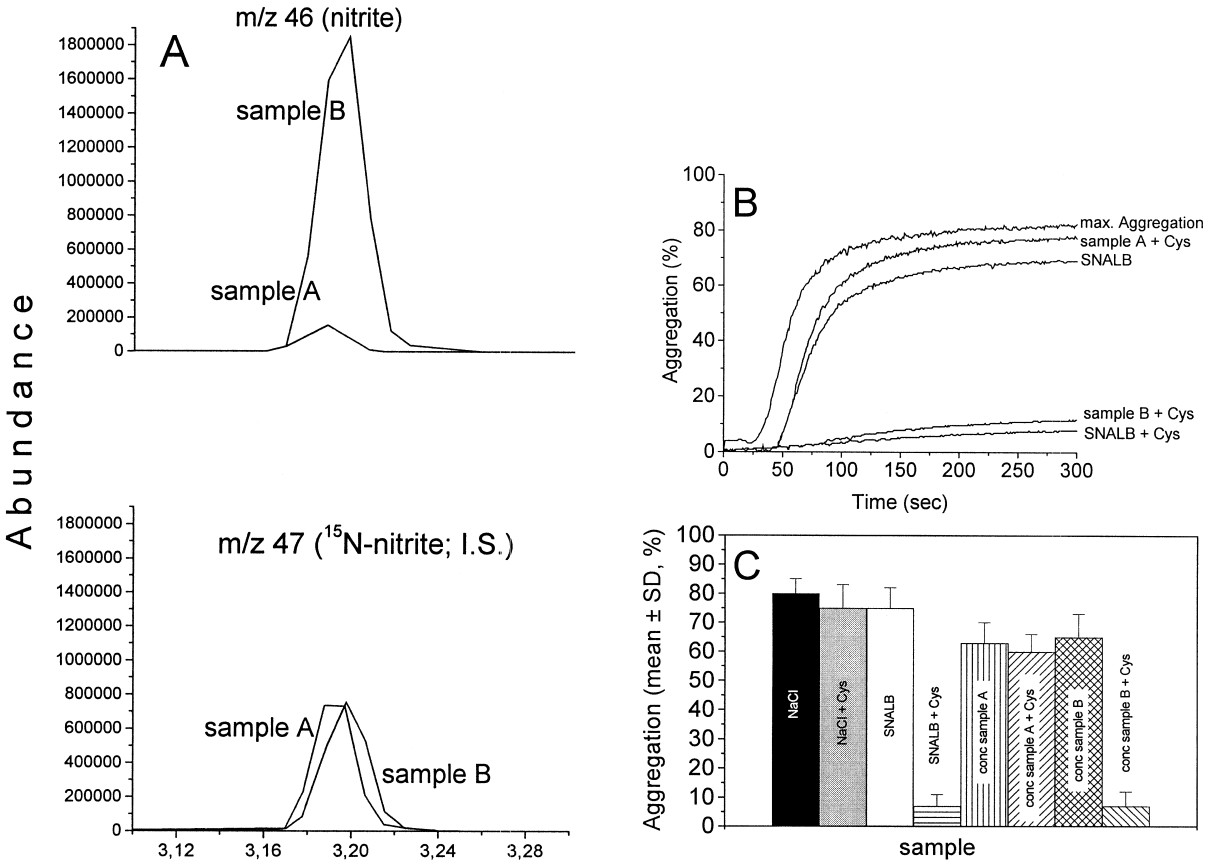

Fig. 3. (A) Partial GC–MS chromatograms from analyses of SNALB in *conc sample A* and *conc sample B* using [^{15}N]nitrite as internal standard. (B) Representative tracings from aggregation measurements in washed human platelet suspensions using a physiological NaCl solutions (used a control), pure SNALB (at 2 μ*M*), *conc sample A* and *conc sample B*. For simplicity, tracings from the use of SNALB, *conc sample A* and *conc sample B* without the use of cysteine are not shown. (C) Extent of aggregation from aggregation measurements in the absence and in the presence of cysteine (400 μ*M*). Cysteine had no effect on platelet aggregation but was used to potentiate the antiaggregatory action of SNALB.

obtained a linear relationship between the SNAC concentration (y) formed from the reaction of NAC with SNALB and the concentration of SNALB (x): $y = 0.513 + 0.833x$.

3.7. SNALB levels in plasma of healthy and ill humans

Table 2 summarizes the SNALB and nitrate levels measured by the method in plasma of healthy humans and in humans suffering from hepatic and renal diseases. For quality control to each of these plasma samples was added SNALB at final concentrations of 2 and 5 μ*M* and analyzed in parallel in duplicate. Mean accuracy and imprecision for quality control samples were 93% and 10.5%, respectively, for samples spiked with 2 μ*M* of SNALB, and 104% and 7.5%, respectively, for samples spiked with 5 μ*M* of SNALB. Fig. 4 shows typical chromatograms from the GC–MS analysis of SNALB in plasma of a healthy human. GC–MS analysis of plasma samples unspiked with S^{15}NALB resulted in nitrite peaks (m/z 46, retention time 3.4 min) of similar abundance to that shown in Fig. 4 and in very low [^{15}N]nitrite peaks (m/z 47, retention time 3.4 min) the abundance of which corresponded to the natural abundance of ^{15}N. With the exception of two humans suffering from an hepatic disease SNALB plasma levels were within the range 0–560 n*M*. No correlation was found between plasma SNALB and nitrate or albumin levels in the groups investigated (not shown). The mean concentrations measured in the present work are more than one-order of magnitude smaller than those reported by Stamler et al. which were reported to be about 7±5 μ*M* in five healthy humans [6]. In none of the plasma samples from healthy humans investigated in our study SNALB concentrations were detected within the range reported by Stamler et al. [6]. Our results are also supported by measurement of nitrite in native plasma samples of four healthy humans in the absence and in the presence of $HgCl_2$. We found very similar nitrite concentrations: 1.38±0.09 μ*M* without $HgCl_2$ and 1.53±0.13 μ*M* with $HgCl_2$. In the corresponding ultrafiltrate samples nitrite was determined as 1.35±0.22 μ*M* and 1.33±0.12 μ*M*, respectively. Similar results have also been reported for the sum of nitrite plus *S*-nitroso compounds in human plasma [17]. These findings suggest that SNALB, and possibly other LMW *S*-nitroso compounds, are present in human plasma at concentrations in the low n*M*-range.

4. Discussion

Measurement of nitrite as its PFB derivative by GC–MS using [^{15}N]nitrite as an internal standard is a highly accurate and sensitive analytical approach [13]. The possibility of specific conversion of the *S*-nitroso group to nitrite by Hg^{2+} [12] prompted us to develop a GC–MS method for the quantitative determination of SNALB using S^{15}NALB as internal standard. The present work describes this method and demonstrates its excellent applicability to accurately and precisely quantify SNALB in human plasma.

The physiological occurrence of nitrite and LMM *S*-nitroso compounds in human plasma made imperative the complete separation of these compounds from SNALB prior to addition of the Hg^{2+} reagent. Therefore, the first step of our method involves selective extraction of SNALB from human plasma by means of HiTrapBlue Sepharose affinity cartridges. We have recently shown that HiTrapBlue Sepharose affinity cartridges result in extracts that contain considerably less impurities than those from the use of unselective PD-10 gel filtration cartridges

Table 2
SNALB and nitrate concentrations measured in plasma[a] of healthy and ill humans

	SNALB (n*M*) (mean±SD)	Nitrate (μ*M*)
Healthy volunteers ($n = 23$)	181±150	35.6±15.2
Patients with hepatic diseases ($n = 40$)	161±274	56.5±41.5
Patients with chronic renal failure ($n = 6$)	147±55	68.8±20.1

[a] SNALB was measured in ultrafiltered eluate fractions; nitrate was measured in native plasma.

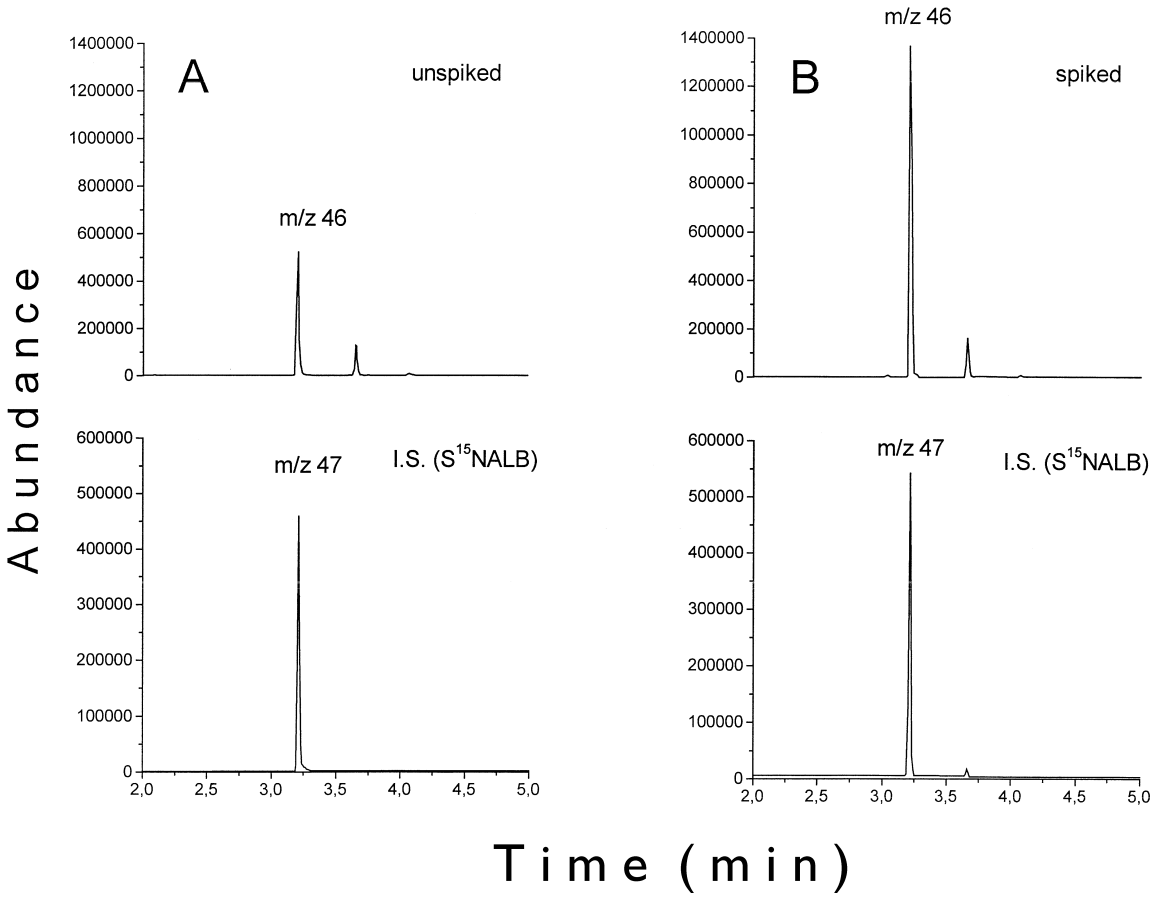

Fig. 4. Partial GC–MS chromatograms from analyses of SNALB in a 0.4-ml aliquot of a plasma sample from a healthy volunteer using S^{15}NALB (1 μ*M*) as internal standard without (A) and after addition of 1 μ*M* of SNALB (B).

[11]. Using 1-ml HiTrapBlue Sepharose affinity columns SNALB was extracted from 0.4-ml aliquots of untreated human plasma at a recovery rate of about 45%. Human albumin was co-extracted at an almost identical recovery rate. However, albumin did not significantly interfere with the measurement of SNALB by this method. Our results suggest that *S*-nitrosohaemoglobin would interfere with the measurement of SNALB. However, this interference can be eliminated by avoiding haemolysis of the blood. Our results also suggest that plasma LMM *S*-nitroso compounds such as *S*-nitrosocysteine and *S*-nitrosoglutathione and plasma nitrite would not interfere at physiological concentrations.

The most important interfering species in our method is the ubiquitously occurring nitrite. In dependence on the concentration of nitrite in the SNALB-containing eluate nitrite may considerably contribute to the peak area ratio *R* of *m*/*z* 46 to *m*/*z* 47. Because of incomplete recovery of SNALB and apparent dilution of the plasma by a factor of 5, the concentrations of SNALB and of the internal standard S^{15}NALB in the eluate are only about 1/10th of those in the native plasma. Therefore, the contribution of nitrite to the ratio *R* is greater than that of SNALB when SNALB is present in plasma at concentrations below 1 μ*M*. For this reason, SNALB concentrations in plasma cannot be calculated directly by multiplying the measured ratio *R* with the concentration of the internal standard as it is usually performed in stable isotope dilution techniques. In order to increase the concentration of SNALB in the

eluate we concentrated SNALB and S^{15}NALB by ultrafiltrating the eluate from 2 ml to about 0.4 ml. Since by this procedure nitrite concentration remains almost unchanged, SNALB and S^{15}NALB are enriched in the protein fraction by a factor of 5 and the contribution of SNALB to the ratio *R* becomes greater in comparison with nitrite. The fact that the ratio *R* decreases upon ultrafiltration suggests the presence of considerable amounts of nitrite in the eluate. For calculation of SNALB concentrations in plasma it was therefore necessary to accurately determine nitrite concentration in the eluate.

Also, nitrite is the most important parameter influencing the LOQ and specificity of our method. In the absence of any nitrite our method would allow accurate quantification of SNALB in plasma at concentrations below 1 n*M* without the need of using the time-consuming and costly ultrafiltration step. Therefore, our current efforts are aimed at the finding ways to remove quantitatively nitrite from the elution buffer and other relevant solutions used in our method. These efforts involve the use of ammonium sulfamate which has been shown to convert quantitatively nitrite to nitrogen gas [12]. Using a 200 n*M* solution of [^{15}N]nitrite in buffer B we found by GC–MS that under acidic conditions less than 1% of [^{15}N]nitrite remained unchanged.

The time point of addition of the internal standard to the blood or plasma is not crucial. This is in line with the relatively high stability of SNALB in human blood (half-life of 5.5 h [11]). Also, the nature of the anticoagulant used did not affect at all the quantification of SNALB in plasma. This suggests that the most abundant LMW thiol in plasma, i.e., cysteine, which is present in EDTA plasma at much higher concentrations than in citrate plasma [18,19], does not change significantly SNALB concentration by *S*-transnitrosylation to *S*-nitrosocysteine and subsequent spontaneous degradation to nitrite. This finding agrees well with the observation that the initial rate of the *S*-transnitrosylation between SNALB and plasma levels of cysteine and glutathione is slow [20]. By contrast, it has been recently reported that SNALB is present in plasma at higher concentrations when measured immediately after samples have been taken than when stored for 2–3 weeks [21].

Our results demonstrate that SNALB physiologically occurs in human plasma. However, the concentration of SNALB measured in plasma samples of a large number of healthy humans by our method is at least one-order of magnitude smaller than that reported by using a chemiluminescence assay [6]. Recently, it has been reported that SNALB concentrations in human plasma are ranging within 0.25 and 1 μ*M* [21] which are considerably lower than those firstly reported [6]. Our data showing low SNALB plasma concentrations were verified by platelet aggregation measurements, analysis of *S*-nitroso compounds by HPLC following conversion to SNAC and quantitation of nitrite plus *S*-nitroso compounds in native plasma by GC–MS. In patients suffering from hepatic diseases and chronic renal failure we measured slightly lower mean SNALB but definitely higher nitrate levels in plasma compared with those of the control group. The elevated plasma nitrate levels measured in the patients with chronic renal failure are most likely due to impaired urinary excretion of nitrate (29.1 ± 17.3 vs. 80.1 ± 11.4 μmol nitrate/mmol creatinine in healthy humans, mean±SD). By contrast, plasma nitrate levels in patients with hepatic diseases were found to correlate with disease activity (unpublished observations) but not with SNALB or albumin levels. The influence of thiol status on ·NO synthesis and SNALB plasma levels in health and disease remains to be investigated.

The major advantage of our GC–MS method over chemiluminescence is the use of a reliable internal standard which permits accurate quantification of SNALB in plasma. Other methods for measurement of SNALB in human plasma have not been published so far. It has only been reported that *S*-nitroso compounds are present in human blood at concentrations below 2 μ*M* [22]. Based on our data, the molar excess of albumin over SNALB in human plasma is of the order of 1500. Because of the very low difference in the molecular masses of albumin and SNALB, and because of lack in chromatographic systems completely separating these macromolecules [15,23], MS in combination with liquid chromatography seems to be not useful for the quantification of plasma SNALB in the presence of high excess of albumin [23].

Using the present method we determined the equilibria constants of the *S*-transnitrosylation re-

actions between SNALB and cysteine (SNALB + Cys⇔ALB + SNC) and between SNALB and glutathione (SNALB + GSH⇔ALB + GSNO) as 0.75 and 0.59, respectively. Based on these data, on the concentration of SNALB in plasma of healthy humans measured in this study and on the plasma levels of albumin, cysteine and glutathione of 300 μ*M* [11], 10 μ*M* and 4 μ*M* [18], respectively, it can be calculated that the concentration of SNC and GSNO in human plasma would be about 5 n*M* and 2 n*M*, respectively. By contrast, using the mean concentration of SNALB of 7 μ*M* measured by Stamler et al. [6] the concentrations of SNC and GSNO in human plasma would be estimated as 175 n*M* and 55 n*M*, respectively. Currently, no data are available on the concentration of SNC and GSNO in human plasma.

5. Conclusions

The GC–MS method described in this work is a useful analytical approach to accurately and precisely quantify SNALB in human plasma. By this method we measured SNALB at concentrations ranging between 0 and 1000 n*M* with a mean concentration of about 180 n*M* in plasma of healthy humans. Our GC–MS method should be useful in studies on the significance of SNALB – formed from endogenous ·NO and/or from ·NO-releasing (pro)drugs – for the regulation of ·NO-like biological activities such as vasodilation and antiaggregation in vivo.

Acknowledgements

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