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Journal of Chromatography B, 772 (2002) 335–346

JOURNAL OF CHROMATOGRAPHY B

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Measurement of *S*-nitrosoalbumin by gas chromatography–mass spectrometry
III. Quantitative determination in human plasma after specific conversion of the *S*-nitroso group to nitrite by cysteine and Cu^{2+} via intermediate formation of *S*-nitrosocysteine and nitric oxide

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Received 16 January 2002; accepted 19 February 2002

Abstract

Highly contradictory data exist on the normal plasma basal levels in humans of *S*-nitrosoproteins, in particular of *S*-nitrosoalbumin (SNALB), the most abundant nitric oxide (·NO) transport form in the human circulation with a range of three orders of magnitude (i.e., 10 n*M*–10 μ*M*). In previous work we reported on a GC–MS method for the quantitative determination of SNALB in human plasma. This 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, $HgCl_2$-catalysed conversion of the *S*-nitroso groups to nitrite and [^{15}N]nitrite, respectively, their derivatization to the pentafluorobenzyl derivatives and quantification by GC–MS. By this method we had measured SNALB basal plasma levels of 181 n*M* in healthy humans. It is generally accepted that $HgCl_2$-catalysed conversion of *S*-nitroso groups into nitrite is specific. In consideration of the highly divergent SNALB plasma levels in humans reported so far, we were interested in an additional method that would allow specific conversion of *S*-nitroso groups into nitrite. We found that treatment with cysteine plus $CuSO_4$ is as effective and specific as treatment with $HgCl_2$. The principle of the cysteine/$CuSO_4$ procedure is based on the transfer of the *S*-nitroso group from SNALB to cysteine yielding *S*-nitrosocysteine, and its subsequent highly Cu^{2+}-sensitive conversion into nitrite via intermediate ·NO formation. Similar SNALB concentrations in the plasma of 10 healthy humans were measured by GC–MS using $HgCl_2$ (156±64 n*M*) and cysteine/$CuSO_4$ (205±96 n*M*). Our results strongly suggest that SNALB is an endogenous constituent in human plasma and that its concentration is of the order of 150–200 n*M* under physiological conditions.

Keywords: *S*-Nitrosoalbumin; Cysteine; Copper

1. Introduction

In 1992, it was reported that nitric oxide (·NO), whose half-life in vivo is 0.1 s [1], circulates in human plasma primarily in the form of *S*-nitrosopro-

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1570-0232/02/$ – see front matter
PII: S1570-0232(02)00121-6

teins, *S*-nitrosoalbumin (SNALB) being the most abundant of them [2]. SNALB plasma concentrations in healthy volunteers have been reported for the first time by Stamler et al. to be 7 μ*M* [2]. The discovery of the long-lived SNALB in human circulation and its putative physiological function as a reservoir for ·NO initiated much scientific research in this area in various disciplines including analytical chemistry.

Many different analytical methods have been developed for the determination of SNALB and other *S*-nitrosoproteins in vitro and in vivo. Table 1 shows that highly contradictory data were observed for basal plasma levels of *S*-nitrosoproteins, in particular of SNALB, with a range of three orders of magnitude, i.e., between 10 n*M* and 10 μ*M*. The main reason for these discrepancies could be the inability to measure directly intact SNALB, i.e., the absolute necessity to perform additional analytical procedures, such as isolation of SNALB from plasma, conversion of the *S*-nitroso group of SNALB into ·NO or nitrite (NO_2^-), and their further derivatization prior to detection. SNALB cannot be separated from its precursor albumin neither by chromatographic nor by mass spectrometric methods. In the presence of albumin and ubiquitous nitrite, sample acidification leads to artifactual formation of SNALB, and must therefore be avoided. Also, functional groups of albumin and other proteins distinct from *S*-nitroso groups may artifactually lead to SNALB-unrelated ·NO and/or NO_2^- formation, as a result of unspecificity of the treatment used to produce these species.

Table 1
Summary of basal levels of *S*-nitrosoalbumin (SNALB) and total *S*-nitrosoproteins (PSNOs) in plasma of healthy subjects reported in the literature[a]

Ref.	Concentration (n*M*) (mean±SD, or range)
[2] Stamler et al., 1992	7000±5000 (SNALB)
[3] Butler and Rhodes[b], 1997	250–1000 (SNALB)
[4] Tsikas et al., 1999	181±150 (SNALB)
[5] Marley et al., 2000	28±7 (PSNOs)
[6] Tyurin et al., 2001	4200±1000 (SNALB)
[7] Cannon et al., 2001	24–35 n*M* (PSNOs)
[8] Moriel et al., 2001	250±200 (PSNOs?)
[9] Rossi et al., 2001	30–40 (PSNOs)

[a] References are reported in chronological order starting with the first report by Stamler et al. in 1992.

[b] Personal communication of J.S. Stamler to the authors; the range is valid for samples that have been stored for 2 to 3 weeks.

The $HgCl_2$-catalysed conversion of the *S*-nitroso group to NO_2^-, first reported by Saville [10], is generally accepted to be specific for thionitrites (R–S–N=O). On the basis of this principle, we have developed a gas chromatography–mass spectrometry (GC–MS) method for the quantitative determination of SNALB in human plasma, which involves selective extraction of SNALB by affinity-column chromatography [4]. By means of this method, we detected endogenous SNALB in plasma of healthy humans at a mean concentration of 181 n*M*, which is severalfold smaller than the value originally reported by Stamler et al. [2] (Table 1).

Due to the potential significance of SNALB as a the most abundant physiological reservoir and carrier of ·NO and the analytical difficulty in measuring accurately circulating SNALB, which is manifested by greatly differing basal SNALB levels reported by various investigators (Table 1), we were interested in an alternative method to quantitate SNALB in human plasma. This method should not, however, dispense with the selectivity of the affinity-column chromatography of SNALB and the accuracy of the GC–MS analysis of NO_2^- [4]. For the conversion of SNALB into the final analyte NO_2^- we replaced the reaction involving $HgCl_2$ with two well-known specific reactions, i.e., (1) transfer of the *S*-nitroso group of SNALB to the sulfhydryl group of cysteine to form *S*-nitrosocysteine (CySNO) [11–13], and (2) Cu^{2+}-catalysed release of ·NO from CySNO [12,13]. Lastly, ·NO is oxidized to NO_2^- in aqueous phase by molecular oxygen and water [14]. The previously reported [4], fully validated, $HgCl_2$ utilizing GC–MS method was used as a reference procedure for the newly developed method. We found that both methods reveal similar results with respect to accuracy and precision as well as to the basal plasma levels of endogenous SNALB. Our methods show that normal SNALB plasma levels in healthy humans are of the order of 150–200 n*M*.

2. Experimental

2.1. Materials

2,3,4,5,6-Pentafluorobenzyl bromide was obtained

from Aldrich (Steinheim, Germany). L-Cysteine was purchased from Sigma (Munich, Germany). HiTrap-Blue Sepharose affinity columns (1 ml and 5 ml) were obtained from Pharmacia Biotech (Freiburg, Germany). Centrisart I ultrafiltration cartridges (pore size 4 μm, molecular mass cut-off 20 000) were bought from Sartorius (Göttingen, Germany). SNALB and S^{15}NALB standards were prepared from freshly obtained human plasma using unlabeled and ^{15}N-labeled butylnitrite, respectively, purified and isolated by affinity-column chromatography (5 ml column) and centrifugal ultrafiltration, and standardized as described previously [15]. Typically, SNALB and S^{15}NALB preparations contained the compounds at concentrations ranging between 100 and 160 μ*M*, and were stored at 4 °C until use.

2.2. Procedures for the quantification of SNALB in human plasma

Plasma was obtained from healthy volunteers who were not taking any drugs within the preceding 2 weeks. Blood was withdrawn from antecubital veins using 5-ml monovettes containing citrate as anti-coagulating 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 as follows: to 400-μl aliquots of plasma samples S^{15}NALB was added to achieve relevant final concentrations. Extraction of proteins from plasma samples was performed on 1-ml Hi-TrapBlue Sepharose affinity columns preconditioned with 2 ml of buffer A (50 m*M* KH_2PO_4, pH 7.0). Cartridges were washed with 4 ml of buffer A, and proteins were eluted from the columns with 2-ml aliquots of buffer B (50 m*M* KH_2PO_4, 1.5 *M* KCl, pH 7.0). In some experiments, eluates were ultra-filtered by centrifugation up to a protein fraction of approximately 400 μl. Aliquots (100 μl) of the eluates or the protein fractions from ultrafiltered samples were treated with an aqueous $HgCl_2$ solution (10 μl, 10 m*M*) and incubated for 1 h at room temperature. Other 100-μl aliquots were spiked with aqueous buffered cysteine solution (10 μl, 1 m*M*) and subsequently with aqueous $CuSO_4$ solution (10 μl, 1 μ*M*) and incubated for 30 min at room temperature. All samples were analyzed for nitrite by GC–MS after derivatisation with pentafluorobenzyl (PFB) bromide and extraction with toluene (300-μl aliquots) as described [4].

2.3. Measurement of ·NO

·NO in aqueous buffered solutions was measured by an ISO-NO meter equipped with a 200-μm diameter shielded microsensor ISO-NOP200 and a Duo-18 data recording system from World Precision Instruments (Berlin, Germany). The ·NO electrode was inserted into 1-ml aliquots of aqueous buffered solutions which were placed in 1.5-ml glass vials and constantly mixed by a magnetic stirrer at 350 rpm at room temperature.

2.4. Experiments on the method's accuracy and precision

Plasma from healthy volunteers was pooled, aliquoted (1 ml) and spiked with S^{15}NALB (3.5 μ*M* for treatment with $HgCl_2$ or 5.1 μ*M* for cysteine/$CuSO_4$) and varying concentrations of SNALB (0, 0.55, 1.1, 2.2, 3.3, 4.4, 5.5 μ*M* for treatment with $HgCl_2$; and 0, 0.7, 1.4, 2.8, 4.2, 5.6, 7.0 μ*M* for treatment with cysteine/$CuSO_4$). Samples were processed on different days in triplicate for each concentration. Analyses were performed using 100-μl aliquots of the eluates as well as of the protein fractions generated by centrifugal ultrafiltration of the remaining 1.8-ml eluate portions.

2.5. S-Transnitrosylation of albumin by $CyS^{15}NO$ in the rat

S-Transnitrosylation of endogenous albumin by $CyS^{15}NO$ in vivo was investigated in male Sprague–Dawley rats (weighing approx. 400 g). Five minutes after intravenous (i.v.) injection into a tail vein of saline solutions of $CyS^{15}NO$ (476, 952, 1904 nmol, one rat each) or $CyS^{14}NO$ (476 nmol, three rats) serving as control, the rats were decapitated and the blood was collected in a citrate-containing monovette placed on ice. Plasma was immediately generated by centrifugation at 4 °C (1800 *g*, 5 min). Proteins from 400-μl aliquots of rat plasma were extracted by affinity-column chromatography and eluates were analysed by GC–MS for nitrite after treatment with $HgCl_2$ (1 m*M*) or cysteine (100 μ*M*)/$CuSO_4$ (500 n*M*) for 1 h, and derivatisation with PFB bromide. In

addition, plasma nitrite was determined in the respective samples by GC–MS after derivatisation with PFB bromide. The study had been approved by the local supervisory committee for studies in animals (Hannover).

2.6. GC–MS conditions

GC–MS analyses were 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 [4]. Selected ion detection 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. Optimisation of ·NO release and nitrite formation from S-nitrosoalbumin

Fig. 1 shows that addition of CySNO to phosphate buffer (pH 7.0) resulted in immediate ·NO formation. ·NO curves show maxima which are dependent upon the concentration of CySNO initially added. The kinetics of ·NO formed from CySNO was found to depend on Cu^{2+} and cysteine (Fig. 2). Cu^{2+} ions (at nanomolar concentrations) accelerated both release and decline of ·NO released from CySNO. Therefore, traces of Cu^{2+} ions present as contamination in the CySNO solution could have caused the apparent spontaneous release of ·NO from CySNO (Fig. 1; see also below). Unlike Cu^{2+} ions, cysteine (at micromolar concentrations) delayed both release and decline of CySNO-derived ·NO (Fig. 2). In these measurements, the ·NO signal reached baseline values after 5 to 15 min.

Measurement of nitrite in buffered solutions of CySNO showed that nitrite values increased with incubation time, in particular in dependence on the presence and concentration of $CuSO_4$ (Fig. 3A) and $HgCl_2$ (Fig. 3B). The highest nitrite concentrations, which correspond to a recovery of 90% with respect to CySNO, were obtained from CySNO with the use of $CuSO_4$ at 500 n*M* after 3 min of incubation and did not change until 30 min of incubation (Fig. 3A). Use of $HgCl_2$ resulted in lower nitrite concentrations, i.e., smaller recovery values. Constant maximum recovery of nitrite from CySNO of about 80% was obtained using 100 n*M* of $HgCl_2$ after incubation times ranging between 5 to 30 min (Fig. 3B). Without external addition of $CuSO_4$ or $HgCl_2$ maxi-

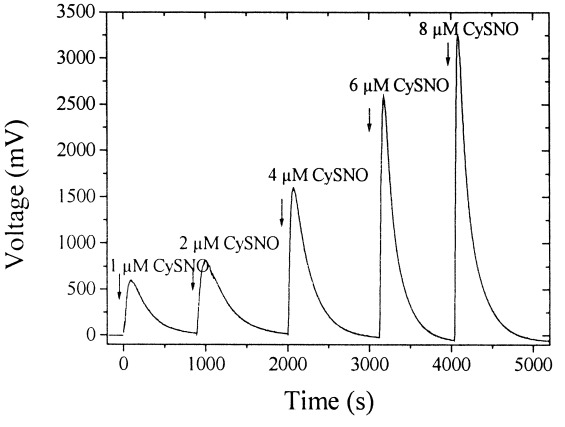


Fig. 1. Formation of ·NO from *S*-nitrosocysteine (CySNO) in 50 m*M* potassium phosphate buffer, pH 7.0, at room temperature. Arrows indicate the time points of consecutive addition of varying SNC concentrations to the same buffer solution.

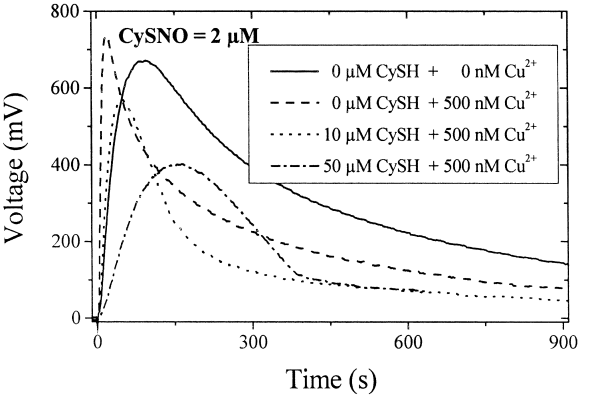


Fig. 2. Effects of cysteine (CySH) and Cu^{2+} (supplied as $CuSO_4$) on ·NO release from *S*-nitrosocysteine (CySNO) in 50 m*M* potassium phosphate buffer, pH 7.0, at room temperature. Reactions were started by adding under stirring to the indicated solutions a constant amount of an aqueous solution of CySNO to achieve a final concentration of 2 μ*M*.

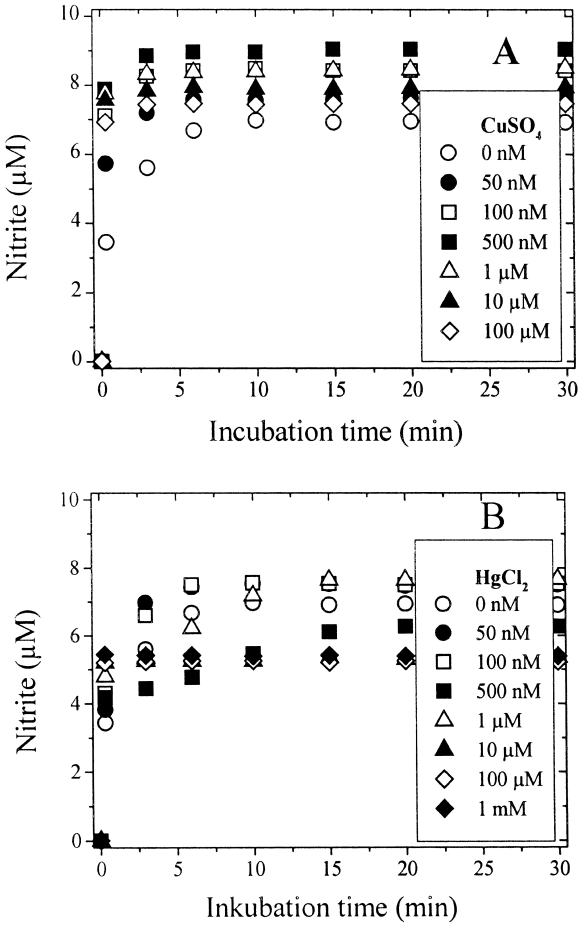


Fig. 3. Effects of $CuSO_4$ (A) and $HgCl_2$ (B) on nitrite formation from CySNO (10 μ*M*) in 50 m*M* potassium phosphate buffer, pH 7.0, at room temperature. Incubations were performed in 1.5-ml glass vials.

mum recovery of nitrite from SNC amounted to approximately 70% and was obtained after 10 min of incubation. At $HgCl_2$ concentrations of 10, 100 and 1000 μ*M*, nitrite was recovered from CySNO by 50%.

Authentic SNALB did not release detectable amounts of ·NO in phosphate buffer (Fig. 4), suggesting that the *S*-nitroso group of the SNALB solution is much more stable than that of CySNO and apparently insensitive to traces of transition metal ions (present both in solution and bound to SNALB). However, addition of cysteine to SNALB caused a weak increase in the ·NO signal, most

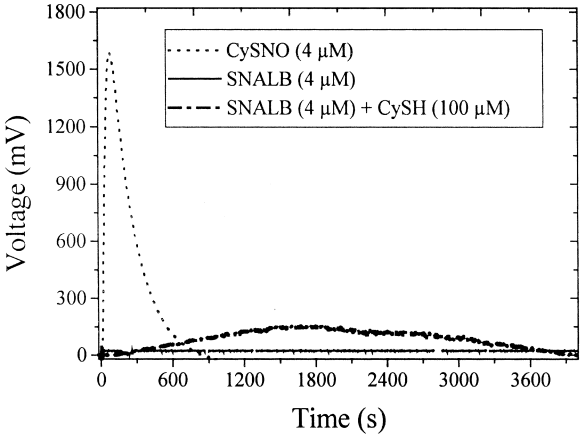


Fig. 4. Release of ·NO from CySNO (4 μ*M*) and authentic SNALB (4 μ*M*) in the absence and in the presence of cysteine (CySH, 100 μ*M*) in 50 m*M* potassium phosphate buffer, pH 7.0, at room temperature.

likely due to intermediate formation of CySNO from *S*-transnitrosylation of cysteine by SNALB. In the absence of cysteine, 500 n*M* of $CuSO_4$ converted only about 20% of SNALB to nitrite (Fig. 5). However, recovery of SNALB-derived nitrite was increased by cysteine in a concentration-dependent fashion, reaching maximum values of about 75% with 40 to 50 μ*M* cysteine (Fig. 5).

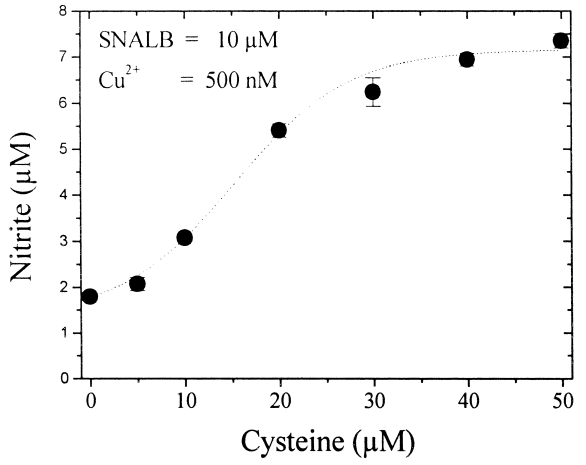


Fig. 5. Dependence of nitrite formation from authentic SNALB (10 μ*M*) in 50 m*M* potassium phosphate buffer, pH 7.0, at room temperature, on cysteine concentration in the presence of externally added Cu^{2+} (500 n*M*). Incubations (10 min) were performed in 1.5-ml glass vials, and reaction was started by addition of cysteine. Data are shown as mean±SD from duplicate incubates.

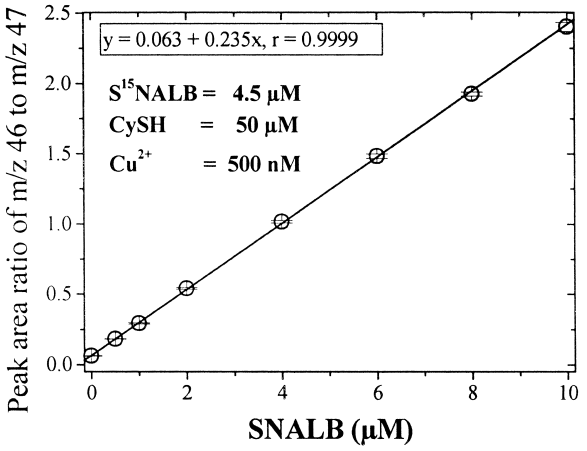


Fig. 6. GC–MS standard curve for authentic SNALB in 50 m*M* phosphate buffer, pH 7.0, containing 1.5 *M* KCl (i.e., elution buffer B). S^{15}NALB was used as internal standard, and cysteine/$CuSO_4$ to convert SNALB and S^{15}NALB into nitrite and ^{15}N-labeled nitrite, respectively. Data are shown as mean±SD from triplicates.

3.2. Quantitative determination of S-nitrosoalbumin in human plasma

Fig. 6 shows a standard curve which was constructed from data obtained from GC–MS analyses of incubates of authentic SNALB and S^{15}NALB in the elution buffer using optimum concentrations of cysteine and $CuSO_4$ (i.e., 50 μ*M* and 500 n*M*, respectively). A highly linear relationship (r= 0.9999) was observed between the peak area ratio of m/z 46 (for SNALB-derived nitrite) to m/z 47 (for S^{15}NALB-derived ^{15}N-nitrite) (y) and the SNALB concentration (x), suggesting that the method should be useful to quantitate by GC–MS endogenous SNALB after its affinity-column extraction from plasma and treatment of the eluate with cysteine/$CuSO_4$.

Indeed, standard curves prepared by spiking SNALB to human citrated plasma samples up to 7 μ*M*, extracting SNALB and its internal standard S^{15}NALB by affinity-column chromatography, converting these compounds into nitrite and ^{15}N-nitrite by using cysteine/$CuSO_4$ as well as $HgCl_2$, preparing the PFB derivatives and analysing them by GC–MS, were linear in the whole concentration range. The regression equations observed from linear regression analysis between the peak area ratio of m/z 46 to m/z 47 measured in the eluate (y) and the concentration of SNALB added to plasma (x) were: $y=1.036+0.180x$, $r=0.9987$ for cysteine/$CuSO_4$ and $y=1.509+0.236x$, $r=0.9985$ for $HgCl_2$. The peak area ratio of m/z 46 to m/z 47 was also determined in the respective protein fractions of ultrafiltered eluates. The regression equations were: $y=0.22+0.187x$, $r=0.9963$, for cysteine/$CuSO_4$, and $y=0.32+0.285x$, $r=0.9996$, for $HgCl_2$. Ultrafiltration of the eluates did not considerably change the slopes of the straight lines (0.180 vs. 0.187, and 0.236 vs. 0.285), but lowered the y-axis intercepts each by a factor of approximately 5. This factor corresponds to the enrichment of the protein fraction from 2 ml (eluate) to 0.4 ml (protein fraction of ultrafiltered eluate). Similar data were obtained previously for SNALB using $HgCl_2$ [4]. The decrease in the y-axis intercepts upon ultrafiltration indicates that the pooled plasma used in this experiment was practically free of endogenous SNALB.

The data on accuracy and precision of both procedures are summarized in Table 2. Both procedures possess comparable accuracy and precision in the concentration range investigated with mean recovery values of 103 and 95%, respectively, and mean RSD values below 10%.

Endogenous SNALB was determined in freshly obtained citrated plasma samples of 10 healthy humans using S^{15}NALB at 1000 n*M*. After affinity-column extraction of 400-μl plasma aliquots and centrifugal ultrafiltration of the eluates to protein fraction volumes of about 400 μl, 100-μl aliquots of these fractions were treated with $HgCl_2$ (1 m*M*) or cysteine (100 μ*M*)/$CuSO_4$ (500 n*M*), incubated for 60 and 30 min, respectively, and processed further as described. Endogenous SNALB was detected in all plasma samples by both procedures. Plasma concentrations were (mean±SD) 156±64 n*M* with $HgCl_2$ and 205±96 n*M* with cysteine/$CuSO_4$.

3.3. S-Transnitrosylation of albumin by S^{15}NC in vivo in the rat

Intravenous bolus injection of CyS^{15}NO in rats did not result in detectable amounts of circulating S^{15}NALB both using $HgCl_2$ and cysteine/$CuSO_4$, but yielded ^{15}N-labeled nitrite in a dose-dependent manner (Fig. 7). Comparable values for the peak

Table 2
Accuracy and precision of the method for SNALB using $HgCl_2$ or cysteine/$CuSO_4$

SNALB added (n*M*) $HgCl_2$[a]/CySH/Cu^{2+}[b]	SNALB measured (n*M*, mean±SD, *n*=3) $HgCl_2$/CySH/Cu^{2+}	Recovery (%) $HgCl_2$/CySH/Cu^{2+}	Precision (RSD, %) $HgCl_2$/CySH/Cu^{2+}
0/0	4±0.1/20.4±1.9	n.a./n.a.[c]	2.50/9.31
550/700	592±58/653±73	106.9/90.4	9.80/11.1
1100/1400	1288±213/1346±128	116.7/94.7	16.5/9.51
2200/2800	2202±295/2718±325	99.9/99.2	13.4/11.9
3300/4200	3388±113/4131±401	102.5/99.5	3.34/9.71
4400/5600	4246±108/5146±419	96.5/91.5	2.54/8.14
5500/7000	5523±154/6768±394	100.3/96.4	2.79/5.82

[a] S^{15}NALB, 3500 n*M*.
[b] S^{15}NALB, 5100 n*M*.
[c] n.a., Not applicable.

area ratios of *m*/*z* 47 to *m*/*z* 46 were obtained for circulating S^{15}NALB with both methods of treatment.

4. Discussion

4.1. Physiological levels of S-nitrosoalbumin in human plasma

Irrespective of the mechanisms leading to formation of *S*-nitroso compounds in vivo, appearance of a single *S*-nitroso compound in the circulation would initiate formation of *S*-nitroso compounds from all thiols present in blood as well as in the vascular tissue via rapidly and reversibly running *S*-transnitrosylation reactions [13,16]. In equilibrium, the concentrations of the thiols and the corresponding *S*-nitroso compounds will be determined by the respective equilibrium constants [11,13].

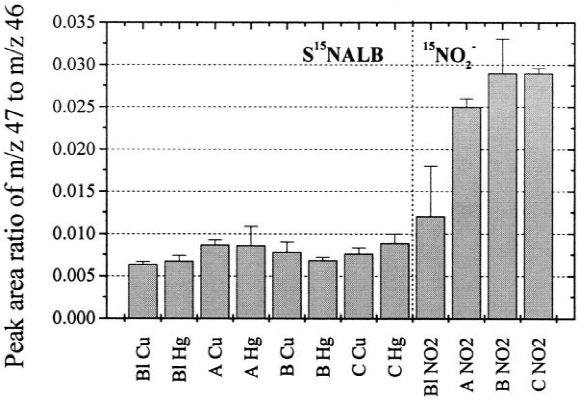


Fig. 7. In vivo metabolism of $CyS^{15}NO$ in rats following separate i.v. injection into a tail vein of $CyS^{15}NO$ [476 nmol (A), 952 nmol (B), 1904 nmol (C), one rat each] or $CyS^{14}NO$ [476 nmol (Bl), three rats] serving as a control. Left, peak area ratio of *m*/*z* 47 for S^{15}NALB to *m*/*z* 46 for SNALB in plasma. Right, peak area ratio of *m*/*z* 47 for [^{15}N]nitrite and *m*/*z* 46 for nitrite in plasma. Bl, Blank; Hg, $HgCl_2$ (1 m*M*); Cu, cysteine (100 μ*M*)/$CuSO_4$ (500 n*M*). NO_2, nitrite.

The most important endogenous thiol in humans, i.e., L-cysteine, exists as free amino acid as well as a moiety in peptides, proteins and enzymes. The most abundant endogenous thiol in human circulation is albumin which possesses a single sulfhydryl group in Cys-34 [17]. The plasma concentration of this albumin (i.e., ALB–CySH) is of the order of 300 μ*M* [15]. Low-molecular-mass cysteinyl-thiols also exist in the circulation. These thiols are cysteine (CySH), cysteinylglycine and glutathione (GSH) and occur physiologically in plasma at concentrations in the range of 4 to 10 μ*M* [18]. Considering that equilibria of *S*-transnitrosylation reactions containing albumin, cysteine, glutathione and the corresponding *S*-nitrosothiols are in favor of *S*-nitrosoalbumin (SNALB, i.e., ALB–CyS–N=O) [11,13], it can be expected that SNALB will be the most abundant circulating *S*-nitrosothiol in human plasma.

In 1992, Stamler et al. reported that SNALB physiologically occurs in human circulation at concentrations of the order of 7000 n*M*, whereas low-molecular-mass *S*-nitrosothiol, presumably *S*-nitrosoglutathione and *S*-nitrosocysteine, and ·NO concentrations are of the order of 200 n*M* and 1 n*M*,

respectively [2]. On the basis of these findings it was postulated that circulating SNALB serves as a reservoir and carrier for L-arginine-derived ·NO, by which ·NO-related functions such as vasodilation and inhibition of platelet aggregation are regulated in vivo [2].

Recently, Tyurin et al. have reported that they detected SNALB in plasma of preeclamptic pregnant women at higher concentrations (6300 n*M*) than in healthy pregnant women (5100 n*M*) or in nonpregnant healthy women (4200 n*M*) [6]. These levels are very close to those originally reported by Stamler et al. [2]. Higher levels of SNALB in preeclampsia are in contradiction to the previously and convincingly shown impaired ·NO production and hypertension in this disease [20]. To overcome this contradiction, Tyurin et al. have assumed that circulating SNALB levels are elevated in preeclampsia not due to enhanced ·NO production, but rather due to attenuated ·NO release from SNALB as a result of decreased ascorbic acid levels in preeclampting pregnant women. This conclusion is based on the observations that ascorbic acid is able to release ·NO from SNALB in vitro [19], and that circulating ascorbate levels may be reduced in preeclampsia [21]. However, this conclusion has not been supported by presenting own data on ascorbic acid levels measured in plasma samples of the patients and controls examined. Furthermore, ascorbic acid is an exogenous compound the concentration of which in plasma depends mainly on nutrition. Also considering that other circulating endogenous compounds, notably thiols and transition metal ions, may also catalyse ·NO release from SNALB [20], it is highly unlikely that an exogenous compound such as ascorbate will be the key compound that regulates and modulates ·NO release from SNALB in vivo in humans.

The basal concentrations of SNALB determined by Stamler et al. [2] and Tyurin et al. [6] in plasma of healthy volunteers (i.e., 4000–7000 n*M*) are highly contradictory to those measured by other groups including our own, which are severalfold smaller, ranging between 30 and 250 n*M* (Table 1). In our opinion, the SNALB levels originally reported by Stamler et al. [2] and recently by Tyurin et al. [6] are incorrect [22]. Interestingly, Stamler's newer data for normal SNALB plasma concentrations, ranging between 200 and 1000 n*M* [3], are considerably lower than the originally reported levels [2].

4.2. GC–MS analysis of S-nitrosoalbumin using $HgCl_2$ or cysteine/Cu^{2+}

In previous work [4], we have described a GC–MS method which allows accurate and precise quantitation of SNALB in human plasma. This method involves use of S^{15}NALB as internal standard, affinity-column extraction of endogenous SNALB and S^{15}NALB from nonmodified plasma, $HgCl_2$-catalysed conversion of the *S*-nitroso groups to nitrite and [^{15}N]nitrite, respectively, preparation of the PFB derivatives and their GC–MS analysis (Fig. 8, route 1). By means of this method we found that SNALB is an endogenous constituent in plasma of healthy and ill humans, and that its concentration is of the order of 200 n*M*. The highly divergent SNALB plasma levels in healthy humans obtained from the use of different analytical methods (Table 1) and the potential importance of SNALB as a physiological reservoir for ·NO from endogenous as well as exogenous sources, e.g., ·NO donating drugs [23], prompted us to develop an alternative method without, however, loosing the inherent accuracy and selectivity of the affinity-column chromatography and GC–MS and technologies. The main modification carried out concerns the replacement of $HgCl_2$ by the cysteine/Cu^{2+} reagent for the conversion of the *S*-nitroso group into nitrite (Fig. 8, route 2). The results of the present study show that the use of cysteine in combination with Cu^{2+} ions is comparably specific and efficient to the use of $HgCl_2$ with respect to the conversion of the *S*-nitroso group into nitrite, although the procedures are most likely based on different mechanisms, i.e., intermediate formation of the nitrosonium $^{+}$NO (using $HgCl_2$) and ·NO itself (using cysteine/Cu^{2+}), respectively (Fig. 8).

Approximately 90% of the plasma proteins eluted by affinity-column chromatography is albumin, i.e., ALB–CySH; the eluate of this extraction also contains endogenous SNALB (i.e., ALB–CyS–N=O) and the externally added internal standard S^{15}NALB (ALB–CyS–^{15}N=O) [4]. The recovery rate for albumin and S^{15}NALB of the affinity chromatography step is about 50% [4]. Thus, protein fraction samples

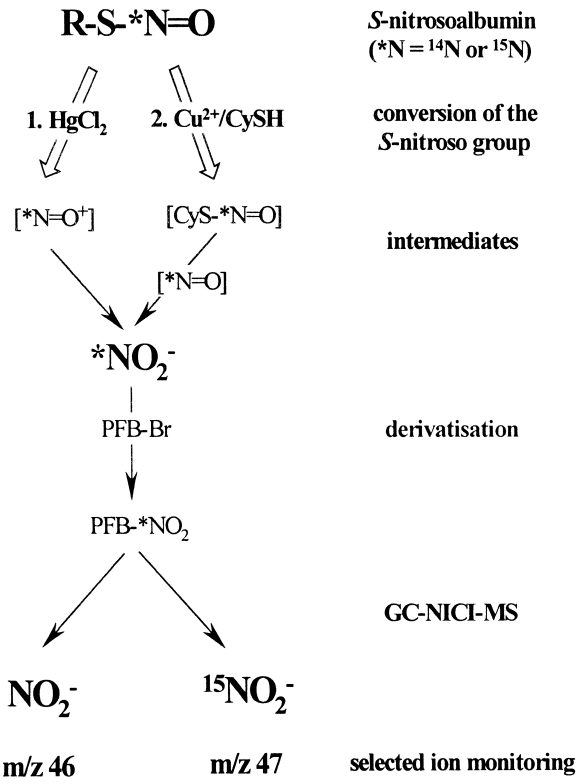


Fig. 8. Schematic drawing of the principle of the GC–MS methods for the analysis of *S*-nitrosoalbumin (RSNO, R = albumin–Cys-34) using ^{15}N-labeled *S*-nitrosoalbumin ($RS^{15}NO$, R = albumin–Cys-34) as internal standard. Endogenous plasma RSNO and externally added $RS^{15}NO$ are extracted from plasma by affinity-column chromatography, and the *S*-nitroso groups are converted by $HgCl_2$ (1) [4] or cysteine/Cu^{2+} (2) (this study) to NO_2^- and $^{15}NO_2^-$, respectively. NO_2^- and $^{15}NO_2^-$ are further derivatized with pentafluorobenzyl bromide (PFB-Br) to the corresponding PFB derivatives. PFB-NO_2 and PFB-$^{15}NO_2$ are separated by capillary GC, ionized by negative-ion chemical ionization (NICI) 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 selected ion monitoring. Asterisks indicate the ^{14}N and ^{15}N isotopes of the nitrogen atoms of the *S*-nitroso group and nitrite.

(400 μl) from ultrafiltered eluates (2 ml) contain the above mentioned compounds at concentrations half of those of whole plasma, e.g., ALB–CySH at 150 μ*M*. Addition of cysteine (CySH) to these samples leads to rapid formation of *S*-nitrosocysteine (CyS–N=O) and ^{15}N-labeled *S*-nitrosocysteine (CyS–^{15}N=O) according to Eq. (1) (Fig. 8):

$$4\text{ALB–CyS–N=O} + 4\text{CySH} \Leftrightarrow 4\text{ALB–CySH} + 4\text{CyS–N=O} \quad (1)$$

The high sensitivity of CyS–N=O towards Cu^{2+}, the catalytical amounts of Cu^{2+} needed, and the concomitant formation of ·NO (Fig. 4) suggest that the following reactions could take place according to Eq. (2a) (i.e., reduction of Cu^{2+} to Cu^+), Eq. (2b) (i.e., reduction of the N atom of the *S*-nitroso group to nitric oxide) and Eq. (2c) (i.e., combination of thiyl radicals) with the netto reaction expressed by Eq. (2):

$$4\text{CySH} + 4\text{Cu}^{2+} \Leftrightarrow 4\text{CyS}\cdot + 4\text{Cu}^{+} + 4\text{H}^{+} \quad (2a)$$

$$4\text{CyS–N=O} + 4\text{Cu}^{+} + 4\text{H}^{+} \Rightarrow 4\text{CySH} + 4\cdot\text{NO} + 4\text{Cu}^{2+} \quad (2b)$$

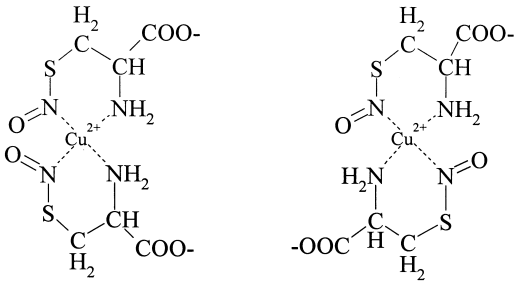
$$4\text{CyS}\cdot \Rightarrow 2(\text{CyS})_2 \quad (2c)$$

$$4\text{CyS–N=O} \overset{\text{CySH, Cu}^{2+}}{\Rightarrow} 2(\text{CyS})_2 + 4\cdot\text{NO} \quad (2)$$

An alternative mechanism for ·NO formation from CySNO could involve Cu^{2+}-catalysed transfer of one electron from the S atom to the N atom of the *S*-nitroso group of CySNO in two six-membered complexes formed between two molecules of CySNO and one Cu^{2+} ion as shown in Fig. 9, without formation of isolable Cu^+ ions. In this mechanism, no cysteine is required to start the reaction. *S*-Nitrosohomocysteine (hCySNO), *S*-nitroso-*N*-acetylcysteine (SNAC), *S*-nitrosoglutathione (GSNO) and SNALB are considerably more stable and much poorer ·NO donors than CySNO [11], suggesting that formation of the Cu^{2+}/*S*-nitrosothiol complexes is dependent on (1) the substitution status of the α-amino group of the *S*-nitroso compound and (2) the distance of the α-amino group from the *S*-nitroso group of the *S*-nitroso compound. SNAC, GSNO and SNALB contain CySNO the α-amino group of which is substituted; on the other hand, hCySNO is longer than CySNO by a CH_2 group. In both cases, formation of the Cu^{2+}/*S*-nitrosothiol complexes is hindered.

Autoxidation of ·NO in aqueous solution yields nitrite according to Eq. (3) [14]:

$$4\cdot\text{NO} + \text{O}_2 + 2\text{H}_2\text{O} \Rightarrow 4\text{NO}_2^- + 4\text{H}^+ \quad (3)$$



cis[Cu(CySNO)$_2$] *trans*[Cu(CySNO)$_2$]

Fig. 9. Proposed mechanism for the Cu^{2+}-catalysed release of ·NO from *S*-nitrosocysteine (CySNO). Two molecules of CySNO form a six-membered complex with one Cu^{2+} ion through its interactions with the N atom of the *S*-nitroso group and the α-amino group of CySNO. This complex may exist in two different forms, the *cis*[Cu(CySNO)$_2$] isomer (left) and *trans*[Cu(CySNO)$_2$] isomer (right). By this mechanism, Cu^{2+} ions catalyse the transfer of one electron from S to N of the *S*-nitroso group, producing ·NO and two cysteinyl radicals (CyS·) which combine to form the cysteine disulfide [i.e., cystine, (CyS)$_2$].

Our results suggest that Cu^{2+} ions may catalyse this reaction, whereas cysteine seems rather to inhibit autoxidation of ·NO to nitrite, perhaps by inhibiting the catalytical action of Cu^{2+} ions (Fig. 2).

The overall reaction between SNALB and CySH in the presence of catalytical amounts of Cu^{2+} can be expressed by Eq. (4):

$$4\text{ALB–CyS–N=O} + 4\text{CySH} + \text{O}_2 + 2\text{H}_2\text{O} \overset{\text{Cu}^{2+}}{\Rightarrow} 4\text{ALB–CySH} + 2(\text{CyS})_2 + 4\text{NO}_2^- + 4\text{H}^+ \quad (4)$$

The equilibrium constant of reaction 1 amounts to 0.75 [11]. Considering an equilibrium concentration of ALB–CySH of about 150 μ*M* in the protein fraction of ultrafiltered eluates, it results that over-stoichiometric amounts of cysteine over ALB–CyS–N=O are needed to achieve maximum recovery of NO_2^- from ALB–CyS–N=O by means of the cysteine/Cu^{2+} reagent. However, the concentration of cysteine required can be lower than that of ALB–CySH (Fig. 5), because Cu^{2+} ions constantly shift reaction 1 in favor of CyS–N=O by converting CyS–N=O into ·NO through reaction 2 or through the alternative mechanism shown in Fig. 9.

We have previously investigated the effects of various factors on the measurement of SNALB in human plasma [4]. These parameters included nitrite, the physiological *S*-nitroso compounds *S*-nitrosocysteine, *S*-nitrosoglutathione and *S*-nitrosohemoglobin, the chemical nature of the anticoagulanting agent present in commercially available monovettes for blood sampling as well as the time point of addition of the internal standard S^{15}NALB to the blood or plasma sample. The most crucial parameter was recognised to be nitrite not from endogenous sources but rather from contaminations in the reagents used in the methods utilizing $HgCl_2$ [4] as well as cysteine/Cu^{2+} ions (in this study). In the absence of any nitrite our method would allow accurate quantification of SNALB in plasma at concentrations below 1 n*M* [4].

4.3. S-Transnitrosylation of albumin in vivo

The method utilizing $HgCl_2$ has been successfully applied in vivo to study formation of S^{15}NALB from i.v. infused ^{15}N-labeled *S*-nitrosoglutathione in the rat [16]. That study has shown that i.v. infusion of ^{15}N-labeled *S*-nitrosoglutathione into rats at infusion rates comparable with endogenous basal production rates of ·NO (i.e., 80 nmol/min for 150 min) led to formation of S^{15}NALB at plasma concentrations of 50 to 100 n*M* [16], which are very close to those found in healthy humans in the present and a previous study [4]. In the present study we could not detect considerable increases in circulating S^{15}NALB concentrations in the rat after i.v. bolus injection of CyS^{15}NO, but only dose-dependent increases in [^{15}N]nitrite plasma concentrations, indicating in vivo conversion of CyS^{15}NO into [^{15}N]nitrite. The differences between these studies concerning S^{15}NALB formation most likely result from the different application modes of the ^{15}N-labeled *S*-nitroso compounds, i.e., bolus injection into a tail vein (present study) versus constant infusion into the right jugular vein of the rat [16], rather than from differences in the *S*-transnitrosylation potency of *S*-nitrosocysteine and *S*-nitrosoglutathione [16]. i.v. injection into a tail vein may not ensure complete availability of

$CyS^{15}NO$ in the circulation as compared with i.v. infusion into a jugular vein of the rat.

5. Conclusions

S-Nitrosoproteins, i.e., proteins of which cysteine moieties are nitrosylated, occur physiologically in human circulation. Their physiological functions previously suggested, especially the regulation of ·NO-related actions in the circulation such as vasodilation and inhibition of platelet aggregation, are poorly investigated mainly because of the lack of reliable quantitative methods. SNALB carries a single free nitrosylable cysteine moiety on position 34. From several points of view, quantitative determination of SNALB in human plasma is highly preferred over determination of other structurally non-identified circulating proteins because they do not play a quantitatively important role as storage pool of ·NO. Mass spectrometry-based quantitative analytical methods, e.g., GC–MS, possess the unique advantage over non-mass spectrometric methods to use stable-isotope labeled analogs as internal standards. In our quantitative GC–MS methods we use $S^{15}NALB$ as the internal standard for endogenous SNALB. $S^{15}NALB$ can be added to the blood during its collection or to blood plasma immediately after its generation by rapid centrifugation under mild conditions. Endogenous SNALB and externally added $S^{15}NALB$ behave almost identically throughout the whole procedure, so that variations in extraction recovery, conversion of the *S*-nitroso groups into nitrite, either with $HgCl_2$ or cysteine/Cu^{2+}, and its derivatisation do not affect the methods reliability. These methods are useful to determine SNALB plasma levels in humans as well as to investigate formation and metabolism of ^{15}N-labeled *S*-nitroso compounds such as $S^{15}NALB$ in vivo. The methods reliability and sensitivity is almost exclusively limited by nitrite present as contamination in the reagents used. Elimination of contaminating nitrite by procedures avoiding artifactual formation of SNALB and other *S*-nitroso compounds seems to be the most promising way to greatly improve the methods efficiency. From own experience, the method involving use of $HgCl_2$ is more robust and yields more reliable results in quantitative measurements even in whole plasma than that utilizing cysteine/Cu^{2+} which could, furthermore, fail in the presence of chelators complexing Cu^{2+} ions, e.g., the anticoagulant EDTA.

Acknowledgements

This work was supported by a grant (TS 60/2-1) from the Deutsche Forschungsgemeinschaft. The authors thank F.-M. Gutzki for his excellent assistance in GC–MS analyses.

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