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Application of a stable-isotope dilution technique to study the pharmacokinetics of human ^{15}N-labelled *S*-nitrosoalbumin in the rat: Possible mechanistic and biological implications☆

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ABSTRACT

In the year 1992, *S*-nitrosoalbumin (SNALB) has been proposed to be the most abundant physiological carrier and pool of nitric oxide (NO) activity in human circulation, by which NO-dependent biological functions are regulated. The concentration, the metabolism and the mechanisms of the biological actions of SNALB are controversial and still incompletely understood. Moreover, the suitability of SNALB as a biomarker of diseases associated with altered NO bioactivity in human circulation has not been demonstrated convincingly so far. In the present study, we report on the development and application of a stable-isotope technique to study the pharmacokinetics of ^{15}N-labelled SNALB (S^{15}NALB) in anesthetized rats. S^{15}NALB was synthesized from albumin isolated by affinity chromatography from freshly prepared human plasma. This technique was also applied to study and quantify the formation of S^{15}NALB from endogenous rat plasma albumin and intravenously applied *S*-[^{15}N]nitrosoglutathione ($GS^{15}NO$) or *S*-[^{15}N]nitrosocysteine ($S^{15}NC$) in anesthetized rats. In these investigations the mean arterial pressure (MAP) was monitored continuously. The elimination half-life ($t_{1/2}$) of S^{15}NALB from rat plasma was determined to be 4.1 min ($t_{1/2}\alpha$) and 9.4 min ($t_{1/2}\beta$). S^{15}NALB (125 nmol) produced long-lasting decreases in MAP (by 49% for 18 min). Thirty minutes after intravenous (i.v.) injection of S^{15}NALB (125 nmol), repeated i.v. injection of L-cysteine or D-cysteine (10 μmol each) produced repeatedly potent (by 44–55%) but short-lasting (about 4 min) MAP falls. Intravenously administered $GS^{15}NO$ and $S^{15}NC$ (each 500 nmol) could not be isolated from rat blood. ^{15}N-Labelled nitrite and nitrate were identified as the major metabolites of all investigated *S*-nitrosothiols in rat plasma. The results of this study suggest that in the rat S^{15}NALB is a potent *S*-transnitrosylating agent and that the blood pressure-lowering effect of S^{15}NALB and other *S*-nitrosothiols are mediated largely by L-cysteine via *S*-transnitrosylation to form $S^{15}NC$ that subsequently releases ^{15}NO. Our results also suggest that *S*-transnitrosylation of the single reduced cysteine moiety of albumin by endogenous GSNO or SNC in blood is possible but does not represent an effective mechanism to produce SNALB in vivo. This stable-isotope dilution GC–MS technique is suitable to perform in vivo studies on SNALB using physiologically and pharmacologically relevant doses.

1. Introduction

S-Nitrosoalbumin (SNALB) is one of the most abundant physiological high-molecular-mass (HMM) *S*-nitrosothiols (RSNO) and has been proposed to be a biomarker of NO bioactivity in human circulation. First analyses of plasma from healthy humans by chemiluminesence provided SNALB levels of the order of 1–7 μM [1,2], whereas the concentration of low-molecular-mass (LMM) *S*-nitrosothiols, presumably *S*-nitrosoglutathione (GSNO) and *S*-nitrosocysteine (SNC), was reported to be considerably lower, i.e., of the order of 0.2 μM [1]. In plasma of healthy humans SNALB levels were found by a GC–MS method to be of the order of 160 nM [3,4]. Further data on SNALB levels in human plasma are very rare. Most investigators usually measure the sum of *S*-nitrosothiols in plasma, not distinguishing between SNALB and other HMM *S*-nitrosothiols. So far, plasma levels of HMM *S*-nitrosothiols in humans and animals have been reported to cover almost four orders of magnitude. The concentration of SNALB and other *S*-nitrosothiols including *S*-nitrosohaemoglobin in plasma and erythrocytes and the biological functions of *S*-nitrosothiols are currently controversially discussed [5–12]; for recent reviews see refs. [13,14].

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The mechanisms of formation and biological actions of circulating SNALB in humans are still poorly understood. Reaction of nitric oxide (NO), a putative endothelium-derived relaxing factor (EDRF) [15,16], with sulfhydryl groups of reduced thiols of peptides and proteins has been reported to lead in part to *S*-nitrosylated compounds by nitrosylation of the sulfhydryl (SH) groups, and in part to sulfenic acid formation by oxidation of the sulfhydryl groups [17]. It should be pointed out that NO itself does not directly react with SH groups, but formation of RSNO requires production of nitrosating species such as N_2O_3. A further mechanism for the formation of SNALB could be the reaction with LMM *S*-nitrosothiols of the sole sulfhydryl group of Cys-34 of albumin (ALB), the most abundant reduced thiol in human blood [18]. This has been shown for various physiological and non-physiological compounds [1,2,14,19–22]. LMM *S*-nitrosothiols such as GSNO and SNC have been reported to be present in the circulation and in various tissues of humans and animals [1,23–25]. GSNO has been detected in GSH-containing incubates of isolated NO synthases [26,27]. Production of an *S*-nitrosothiol would initiate formation of numerous *S*-nitroso compounds in thiols-containing matrices via *S*-transnitrosylation reactions [19,22]. It can, therefore, be assumed that the arrival in the circulation of a LMM *S*-nitrosothiol such as GSNO or SNC, for example from intra-cellular sources, would result in formation of circulating SNALB by *S*-transnitrosylating albumin. Since the equilibrium constants for the reversible reactions (1) and (2) are both in favour of SNALB, i.e., K_{eq} = 1.69 and 1.33, respectively [22], and the concentration of albumin greatly prevails over GSH and Cys in plasma (about 400, 4 and 8 μM, respectively), circulating SNALB concentrations should be, at least in theory, considerably higher than those of GSNO and SNC.

$$\text{GSNO} + \text{ALB} \Leftrightarrow \text{GSH} + \text{SNALB} \quad (1)$$

$$\text{SNC} + \text{ALB} \Leftrightarrow \text{Cys} + \text{SNALB} \quad (2)$$

In vivo, intravenous (i.v.) bolus injection of SNALB has been reported to cause a greater duration of blood pressure fall than SNC or glycerol trinitrate (GTN) and to produce marked prolongation of the template bleeding time associated with inhibition of platelet aggregation [28]. More recently, Orie et al. [29] have demonstrated that in rats infused SNALB (prepared from human albumin) acts primarily as a venodilator at high concentrations. Also, this group has suggested that SNALB represents as stable reservoir of NO that can release NO when the concentrations of LMM thiols are elevated [29]. Generally, *S*-transnitrosylation reactions have been appreciated to regulate protein function, biological activities and metabolic fate both of endogenous NO (reviewed in refs. [5,30]), exogenous NO donors [31] and of *S*-nitrosothiols [32,33]. Thus, *S*-transnitrosylation reactions could be promising targets for the development of new medical therapies.

Until now, most investigations on formation and reactions of SNALB have been performed in aqueous buffered solutions [19–22], presumably due to the lack of reliable analytical methods suitable for specific quantification of SNALB in plasma. We have reported on accurate and specific GC–MS methods for the quantification of endogenous SNALB in human plasma using ^{15}N-labelled SNALB (S^{15}NALB) as internal standard [3,4]. These methods are based on the affinity column chromatography extraction of SNALB and S^{15}NALB, their conversion to nitrite and ^{15}N-labelled nitrite ([^{15}N]nitrite), respectively, either by $HgCl_2$ [3] or by a combination of Cu^{2+} and L-cysteine [4], their derivatization to the pentafluorobenzyl (PFB) derivatives, and GC–MS quantification by selected-ion monitoring (SIM) of the ions at *m*/*z* 46 for nitrite (i.e., [^{14}N]nitrite) and *m*/*z* 47 for [^{15}N]nitrite [3,4,13]. Besides accurate quantitative determination of SNALB in plasma of healthy and diseased subjects [3,4], this method was found to be useful to investigate S^{15}NALB stability in human blood in vitro [34] and *S*-transnitrosylation reactions in vitro and in vivo in the rat [35]. One of these studies revealed that the half-life ($t_{1/2}$) of S^{15}NALB at the very high concentration of 25 μM in human blood is about 5.5 h [34]. The half-life of S^{15}NALB formed from i.v. infusion of $GS^{15}NO$ in the rat was estimated to be 10–20 min [35].

In the present study we report on the modification of the GC–MS method previously reported by our group [4] and the development of a stable-isotope dilution technique allowing performance of pharmacokinetic studies in vivo in anesthetized, operated but otherwise untreated rats using low doses of S^{15}NALB. This technique was also applied in vivo in the rat to investigate whether the *S*-transnitrosylation of endogenous albumin by putative endogenous LMM *S*-nitrosothiols may be an efficient mechanism leading to formation of SNALB. In this article, possible mechanistic and biological implications of the findings are discussed.

2. Experimental

2.1. Materials and chemicals

Sodium [^{15}N]nitrite (98% at ^{15}N) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Sodium [^{15}N]nitrate (99% at ^{15}N) was supplied from MSD Isotopes Merck Frosst Canada (Montreal, Canada). Glutathione (GSH), L-cysteine, D-cysteine and *N*-acetyl-L-cysteine (NAC) were purchased from Sigma (Munich, Germany). HiTrapBlue Sepharose affinity columns (1 ml for quantitative analyses and 5 ml cartridges for isolation of freshly prepared SNALB and S^{15}NALB) were obtained from Pharmacia Biotech (Freiburg, Germany). Centrisart I® ultrafiltration cartridges (pore size 4 μm, cut-off 20 kDa) were obtained from Sartorius (Göttingen, Germany). NO gas and other chemicals including sodium nitrite and buffer salts were purchased from Merck (Darmstadt, Germany). ODQ, a putatively selective inhibitor of soluble guanylyl cyclase, was obtained from ALEXIS Corporation (San Diego, CA, USA). Stock solutions of ODQ were prepared in DMSO. Collagen was obtained from Hormonchemie (Munich, Germany).

2.2. Synthesis of S-nitrosothiols

SNALB and S^{15}NALB standards were prepared by incubating albumin extracted from freshly obtained human plasma with unlabeled and ^{15}N-labelled butylnitrite, respectively, and were isolated, characterized and standardized by GC–MS as described previously [34]. Typical SNALB concentrations in stock solutions in physiological saline were about 150–170 μM. SNC, GSNO, *S*-nitroso-*N*-acetyl-L-cysteine (SNAC), S^{15}NC, $GS^{15}NO$ and ^{15}N-labelled SNAC (S^{15}NAC) were prepared by mixing equimolar solutions in physiological saline of L-cysteine, GSH or NAC and unlabeled or ^{15}N-labelled nitrite and by acidifying with HCl (at a final concentration of 50 mM) as described elsewhere [22] and stored in an ice-bath in the dark until immediate use. The structure of the LMM *S*-nitrosothiols in their stock solutions was elucidated by electrospray ionization-mass spectrometry [36]. Stock solutions of LMM *S*-nitrosothiols usually contained the compounds at 4.8 mM.

2.3. Measurement of SNALB-derived NO in vitro

NO in aqueous buffered solutions (potassium phosphate buffer, 50 mM, pH 7.4) was measured by an ISO-NO meter equipped with a 200 μm diameter shielded micro-sensor ISO-NOP200 and a Duo-18 data recording system (World Precision Instruments, Sarasota, Florida). The NO electrode was inserted into 1 ml aliquots of buffer placed in 1.5 ml brown glass vials which were constantly mixed by a magnetic stirrer at 350 rpm at room temperature (20–25 °C). NO derived from S^{15}NALB (0–20 μM or 20 μM) was recorded continuously prior to and after addition of L- or D-cysteine at varying final

concentrations (0–50 μM or 500 μM). Dilutions of S^{15}NALB and L- or D-cysteine for these experiments were freshly prepared. Calibration (in the range of 0–5 μM) was carried out by using freshly prepared saturated aqueous solutions of NO (assumed to contain approximately 2 mM of NO) which were prepared by introducing authentic NO gas into buffer previously deoxygenated by argon. For calculation of NO concentrations, the maximal levels of the continuously recorded current were used. NO was released from S^{15}NALB immediately after addition of L- or D–cysteine or after addition of S^{15}NALB to the buffered thiol solutions. Maximum currents occurred between 90 and 120 s after addition of S^{15}NALB or the thiols.

2.4. Analysis of SNALB and S^{15}NALB in rat plasma by GC–MS

Quantification of SNALB in unspiked and spiked rat plasma samples was performed as described elsewhere [3]. Briefly, plasma samples (400 μl) were spiked with the internal standard S^{15}NALB and diluted with buffer A (1.6 ml; 50 mM potassium phosphate buffer, pH 7.0). These solutions were applied to 1 ml HiTrap-Blue Sepharose affinity columns preconditioned with buffer A (2 ml). Cartridges were washed with buffer A (4 ml), and proteins were eluted from the columns with buffer B (2 ml; 50 mM potassium phosphate buffer, 1.5 M KCl, pH 7.0). Subsequently, eluates were ultrafiltered by centrifugation (1800 × *g*, 20 min, 4 °C) in order to obtain a protein fraction of about 800 μl. Two 100 μl aliquots of these fractions were treated with $HgCl_2$ (final concentration of 1 mM) and analysed by GC–MS after derivatization with PFB bromide as described [3]. Freshly prepared, unspiked rat plasma samples used in this study contained endogenous SNALB at 250 ± 120 nM (n = 5) by GC–MS and not detectable concentrations of SNC (≤1 μM) and GSNO (≤0.1 μM) as measured by HPLC [22]. Plasma nitrite and nitrate were determined by GC–MS as described elsewhere [37].

In studies on pharmacokinetics and on in vitro and in vivo S^{15}NALB formation from S^{15}NC or $GS^{15}NO$ and albumin in rat plasma, the same procedure was used as described above, with the exception that no S^{15}NALB or S^{14}NALB were added externally to plasma samples.

2.5. GC–MS conditions

A Hewlett-Packard MS engine 5890 connected directly to a gas chromatograph 5890 series II equipped with an autosampler (sample tray capacity for 100 vials) Hewlett-Packard model 7673 (Waldbronn, Germany) was used for GC–MS analyses. The gas chromatograph was equipped with a fused-silica capillary column Optima 17 (30 m × 0.25 mm i.d., 0.25 μm film thickness) from Macherey-Nagel (Düren, Germany). Aliquots (1 μl) of toluene extracts were injected in the splitless mode by means of the autosampler. The following oven temperature program was used with helium (50 kPa) as the carrier gas: 1 min at 70 °C, then increased to 280 °C at a rate of 30 °C/min. Interface, injector and ion source were kept at 280, 200 and 180 °C, respectively. Electron energy and electron current were set to 200 eV and 300 μA, respectively. Electron-capture negative-ion chemical ionization (ECNICI) with methane (200 Pa) as the reagent gas was used. Routinely, electron multiplier voltage was set to 1.8 kV. Quantification by GC–MS in the ECNICI mode was performed by SIM of the ions at m/z 45.95 for unlabelled nitrite and m/z 46.95 for ^{15}N-labelled nitrite using a dwell-time of 50 ms for each ion [37].

2.6. Animal studies

These studies required the participation of at least three investigators. Each one experiment was performed per day and GC–MS analyses were performed within the same day or overnight.

2.6.1. Ethics

The studies described below had been approved by the local supervisory committee for studies in animals (Hannover, Germany). The maximum blood loss in our animals was estimated to be about 30%. All animal experiments reported here were final experiments, i.e., with a lethal end for the rats.

2.6.2. Pharmacokinetics of S^{15}NALB in the rat

Three male Sprague Dawley rats (weighing 540, 490 and 430 g) were anesthetized with xylazine (2 mg/kg body weight) and ketamine (70 mg/kg body weight). Aliquots {250 μl (41.5 nmol), 750 μl (125 nmol) or 1355 μl (225 nmol)} of a freshly prepared 166 μM solution of S^{15}NALB in saline were injected i.v. into the right jugular vein of the rats, respectively. Before and at various times after injection (i.e., 1, 3, 5, 8, 12, 20, 30, and 45 min), about 1 ml aliquots of venous blood from the right femoral vein was collected into pre-cooled 2.3 ml EDTA monovetes, plasma was immediately prepared by centrifugation (800 × *g*, 5 min, 2 °C), samples were immediately processed, and endogenous SNALB and exogenous S^{15}NALB were analysed by GC–MS [3].

Mean arterial pressure (MAP) was measured by using a catheter made of polyethylene (outer diameter 0.9 mm; inner diameter 0.58 mm) which was treated with heparin sulphate before placement into the right femoral artery of the animals. The catheter was fixed using suture material to avoid displacement. Connecting the catheter to a pressure transducer (Cobe Laboratories Inc., Lakwood, CO, USA) allowed direct and continuous measurement of the blood pressure. Monitoring and documentation of the MAP was performed by means of the pressure monitor BP1 (Word Precision Instruments, Berlin, Germany) and the flat-bed recorder L6512 (Linseis, Princeton Jet., NJ, USA).

2.6.3. Formation of S^{15}NALB from i.v. administered $GS^{15}NO$ or S^{15}NC in the rat

Six male Sprague Dawley rats were anesthetized with xylazine (2 mg/kg body weight) and ketamine (70 mg/kg body weight). Three rats (weighing 338, 340 and 380 g) received i.v. each 500 nmol of S^{15}NC (105 μl aliquots of 4.76 mM). Three rats (weighing 310, 330 and 350 g) received i.v. each 500 nmol of $GS^{15}NO$ (105 μl aliquots of 4.76 mM). Ten minutes before and 3 min after injection, about 2 ml aliquots of venous blood from the right femoral vein were collected into pre-cooled (in ice bath) 2.3 ml EDTA monovetes, and plasma was immediately prepared by centrifugation (800 × *g*, 5 min, 2 °C). In addition, 250 μl aliquots of the plasma samples generated from blood taken before and after administration of S^{15}NC and $GS^{15}NO$ were incubated for 10 min at room temperature each with 2.4 nmol of S^{15}NC and $GS^{15}NO$, respectively, in order to measure in vitro formation of S^{15}NALB. These amounts correspond to final added concentrations each of 9.6 μM for S^{15}NC and $GS^{15}NO$. All samples were immediately analysed for endogenous SNALB and exogenous S^{15}NALB by GC–MS as described above. Prior to derivatization for GC–MS, a half of the affinity chromatography sample extracts (each 100 μl aliquots) were treated with 10 μl aliquots of a 10 mM solution of $HgCl_2$ and incubated for 60 min at room temperature in order to convert the *S*-nitroso groups to nitrite, i.e., to the final analyte [3].

2.7. Measurement of platelet aggregation

Blood platelets were isolated from EDTA-anticoagulated venous blood from nine healthy volunteers who had not received aspirin or other non-steroidal antiphlogistic drugs for at least 10 days. Platelet aggregation measurements were performed using freshly

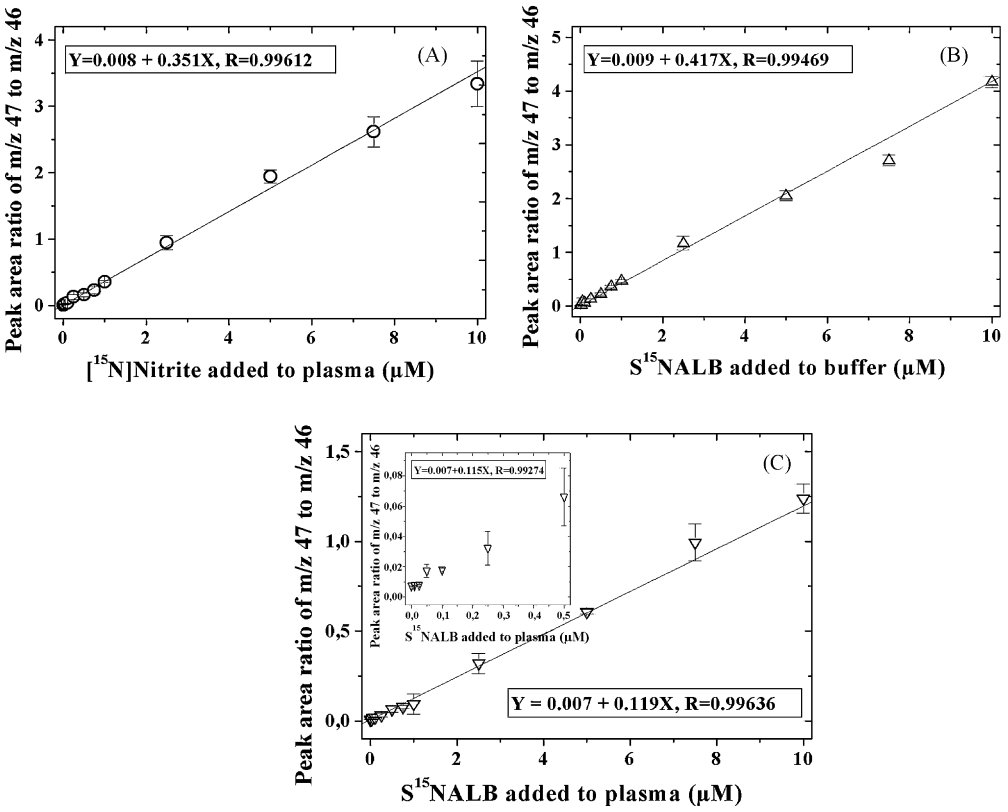

Fig. 1. Standard curves of [^{15}N]nitrite in human plasma (A), S^{15}NALB in elution buffer B (B), and of S^{15}NALB in human plasma (C). Neither nitrite (i.e., [^{14}N]nitrite) nor SNALB (i.e., S^{14}NALB) were externally added to buffer or plasma. Standard curves in (A) and (B) were prepared without affinity column chromatography extraction. Standard curve in (C) was prepared after affinity column chromatography extraction as described [3]. All S^{15}NALB analyses were performed in the elution buffer after incubation with $HgCl_2$ (1 mM). Data are shown as mean ± SD from duplicate analyses by two investigators (i.e., $n = 4$ in total). Inset in (C) is an amplification of the lower concentration range. Please note that the extraction yield of SNALB (about 45% [34]) and the sample dilution factor of 2 (400 μl plasma versus 800 μl elution buffer), i.e., a total dilution factor of about 4, have not been considered in this figure.

prepared washed platelet suspensions as described elsewhere [38]. Briefly, washed platelet suspensions (250 μl aliquots containing approximately 10^8 cells) were incubated in duplicate at 37 °C with 0.9 wt.% NaCl (i.e., the control) with SNALB (2 μM) alone, with SNALB (2 μM) plus L-cysteine (10 μM), or SNALB (2 μM) plus L-cysteine (10 μM) plus ODQ (10 μM). Platelet aggregation was induced by collagen (1.0 μg/ml) and was monitored for 5 min with an Apact dual-channel aggregometer (LAbor, Hamburg, Germany). The final portion of DMSO in the platelet suspension was less than 1 vol.%; at these portions DMSO did not influence platelet aggregation.

2.8. Statistical analysis

Values are expressed as mean ± SD except in platelet aggregation measurements which are presented as mean ± SEM. The significance of differences was determined with the unpaired *t*-test. A *P* value of <0.05 was considered significant.

3. Results

3.1. Analysis of S^{15}NALB by GC–MS linearity, precision and limit of quantitation

Fig. 1 shows standard curves of [^{15}N]nitrite and S^{15}NALB prepared in buffer B and in human plasma in the concentration range of 0–10 μM each considered to be relevant in the present study. In these experiments no [^{14}N]nitrite or S^{14}NALB were added externally to buffer of plasma. Thus, the measured peak area ratio of *m*/*z* 47 to *m*/*z* 46 (R_{PA}) reflects the concentration ratio of added [^{15}N]nitrite or S^{15}NALB to the basal or blank concentration of nitrite (i.e., [^{14}N]nitrite) present in the plasma or in the buffer B used. In buffer and plasma linear relationships were observed between R_{PA} (y) and the respective analyte concentration (x). From the reciprocal of the slope of the regression equation of Fig. 1A, a mean basal plasma nitrite concentration of 2.85 μM is estimated. From the reciprocal of the slope of the regression equation of Fig. 1B, a mean blank nitrite concentration of 2.4 μM in buffer B is estimated.

A linear relationship was observed between R_{PA} and S^{15}NALB added to human plasma after affinity chromatography column extraction (Fig. 1C). However, the slope of the regression equation is about 3.5 times smaller than that observed in buffer (Fig. 1B), although in both cases the final matrix analysed was the elution buffer B. This apparent discrepancy is due to the fact that the ratio R_{PA} was plotted versus the concentration of S^{15}NALB added to plasma but not versus the S^{15}NALB concentration in the elution buffer B after extraction. Thus, this experiment does not consider the extraction recovery of SNALB, which is of the order of 45% [34], and the sample dilution factor of 2 (400 μl plasma versus 800 μl elution buffer), i.e., a total dilution factor of about 4.

The results shown in Fig. 1 suggest that the expectedly constant nitrite concentration present in the elution buffer B can be used to roughly estimate S^{15}NALB concentrations in vitro and in vivo experiments. These findings also suggest that instead of calculating absolute concentrations, the peak area ratio R_{PA} may serve as a rough estimate of S^{15}NALB present in rat plasma in vivo or in vitro in the present study. For the sake of simplicity, we decided to measure and use the peak area ratio R_{PA} as a rough estimate of S^{15}NALB concentration in vitro experiments and in vivo

pharmacokinetic studies. Calculation of absolute concentrations would require knowledge of exactly measured volumes of extracts from affinity chromatography extraction and/or of protein fractions of these solutions after protein concentration by ultrafiltration [3,34].

Analyses of S^{15}NALB and [^{15}N]nitrite in the experiments described above (Fig. 1) were performed each in duplicate by two investigators. These analyses were performed with an intra-day precision (RSD) ranging between 0.5% and 15%. In unspiked elution buffer the peak area ratio R_{PA} was of the order of 0.006 which is close to the *y*-axis intercepts of the standard curves for S^{15}NALB and [^{15}N]nitrite (Fig. 1). The value of R_{PA} for blank [^{15}N]nitrite, i.e., R_{PAo}, is mainly determined by the extent of [^{15}N]nitrite-contamination in solutions and materials used including buffers and GC–MS instrument. Theoretically, the R_{PAo} value for nitrite is calculated to be 0.0036 because of the natural abundance of the ^{15}N isotope of 0.36%. However, this value has never been measured by us using our GC–MS instrument when nitrite was analysed as its PFB derivative. For [^{15}N]nitrite in plasma the R_{PAo} value was measured to be (mean ± SD, $n = 4$) 0.00764 ± 0.0005, whereas the R_{PA} value for [^{15}N]nitrite added at 10 nM, i.e. R_{PA10}, was determined to be 0.01152 ± 0.00137. Paired *t*-test analysis for R_{PAo} and R_{PA10} revealed that these values were statistically significantly different ($P = 0.004$). For S^{15}NALB in plasma the R_{PAo} value was measured to be (mean ± SD, $n = 4$) 0.00733 ± 0.00058 (RSD, 7.9%), whereas the R_{PA} value for S^{15}NALB added at 50 nM to plasma, i.e. R_{PA50}, was determined to be 0.02067 ± 0.00404 (RSD, 19.5%). Paired *t*-test analysis for R_{PAo} and R_{PA50} revealed that these values were statistically significantly different ($P = 0.031$). Thus, we defined 50 nM as the LOQ of the method for S^{15}NALB added to plasma, and the presence of S^{15}NALB in rat plasma in vivo and in vitro experiments was considered for R_{PA} values above the R_{PA50} value of 0.020 (see insertion in Fig. 1C).

3.2. Pharmacokinetics of S^{15}NALB in the rat

Before i.v. injection of S^{15}NALB in the rats, the peak area ratio R_{PA} in the protein fraction of ultrafiltered eluate amounted to 0.0058 ± 0.004. Injection of S^{15}NALB resulted in dose-dependent clear increases in R_{PA} measured in the first blood sample taken about 1 min after injection, i.e., to 0.3 for 41.5 nmol of S^{15}NALB, to 1.8 for 125 nmol of S^{15}NALB, and to 4.7 for 225 nmol of S^{15}NALB, indicating high enrichment of exogenous S^{15}NALB in rat plasma over endogenous SNALB plus blank nitrite (Fig. 2A). These R_{PA} values are within the ranges measured for [^{15}N]nitrite and S^{15}NALB concentrations added to buffer or plasma (Fig. 1). In the blood samples taken thereafter, R_{PA} fell and reached values of 0.012, 0.035 and 0.037 at the end of the experiment, respectively. Representative chromatograms from GC–MS analyses of S^{15}NALB in rat plasma before and at selected time points after i.v. injection of 225 nmol of S^{15}NALB are shown in Fig. 3. The peaks obtained at the trace of *m*/*z* 46 (i.e., [$^{14}NO_2$]$^-$) were relatively constant and independent of the use of $HgCl_2$ (to convert *S*-nitroso groups to nitrite), indicating that the major compound contributing to these peaks is blank nitrite. By contrast, the peaks obtained at the trace of *m*/*z* 47 (i.e., [$^{15}NO_2$]$^-$) were considerably greater in the samples treated with $HgCl_2$ as compared to the respective $HgCl_2$-untreated samples, strongly indicating that the major compound contributing to these peaks is S^{15}NALB.

The integral method was applied to determine the pharmacokinetics of S^{15}NALB in the rat. On the assumption that the decay of the concentration of S^{15}NALB in rat plasma follows the law of the one-compartment model with a first order kinetics, the concentration of S^{15}NALB at time point *t*, i.e., C_t, can be described by the equation $C_t = C_o \times e^{-k \times t}$, whereas C_o is the concentration of S^{15}NALB at the time point 1 min after injection and *k* is the elimination rate

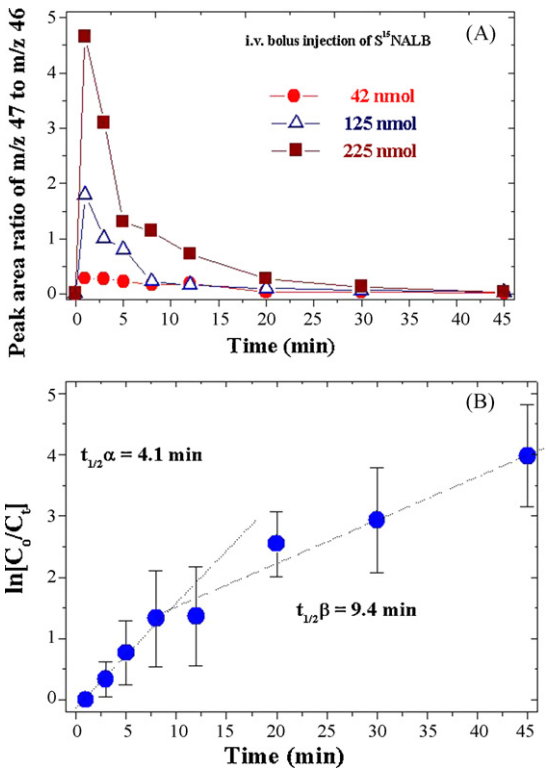

Fig. 2. Pharmacokinetics of S^{15}NALB in three rats administered intravenously at three different doses. (A) Time course of the peak area ratio of *m*/*z* 47 to *m*/*z* 46. (B) Mathematical analysis (integral method) of the data shown in (A), i.e., plotting of $\ln[C_o/C_t]$ versus time. C_o represents the maximum values obtained for the peak area ratio of *m*/*z* 47 to *m*/*z* 46 1 min (t_{max}) after S^{15}NALB administration. C_t represents the values for the peak area ratio of *m*/*z* 47 to *m*/*z* 46 measured at various time points following t_{max}. Data in (B) are shown as mean ± SD ($n = 3$).

constant. Linearization of this equation by taking the logarithm yields the equation $\ln[C_o/C_t] = k \times t$. Plotting of $\ln[C_o/C_t]$, actually of the natural logarithm of the corresponding mean R_{PA} for the three doses, versus the time *t* did not result in a straight line across the whole observation time (Fig. 2B). This finding suggests that the one-compartment model is not applicable to the whole observation time (Fig. 2B). Nevertheless, the integral method revealed two linear regimes suggesting that the pharmacokinetics of S^{15}NALB in the rat is a biphasic process, with the one-compartment model being formally valid in each of these two regimes (Fig. 2B). From this plot the elimination half-life ($t_{1/2}$) of S^{15}NALB from plasma is estimated to be 4.1 min in the early phase ($t_{1/2}\alpha$, time interval: 0–8 min) and 9.4 min in the later phase ($t_{1/2}\beta$, time interval: 8–45 min).

3.3. S-transnitrosylation of endogenous albumin by i.v. injection of $GS^{15}NO$ or $S^{15}NC$ in the rat

Bolus i.v. injection of $GS^{15}NO$ or $S^{15}NC$ in rats resulted in small increases in R_{PA} measured in the protein fractions of the affinity chromatography extraction as compared with the R_{PA} measured before i.v. injection (Fig. 4), indicating formation of S^{15}NALB from the reaction between endogenous albumin and exogenous $GS^{15}NO$ or $S^{15}NC$. In most cases, the R_{PA} measured in samples treated with $HgCl_2$ was higher than in the corresponding $HgCl_2$-untreated samples, strongly suggesting presence of the intact *S*-[^{15}N]nitroso group in albumin. Considerably higher increases in R_{PA} in the protein fractions were obtained from (in vitro) addition to plasma of equivalent amounts (with respect to the estimated whole blood volume of the rats) of $GS^{15}NO$ and $S^{15}NC$ before as well as after injection of the compounds (Fig. 4). In consideration of the rapidity and

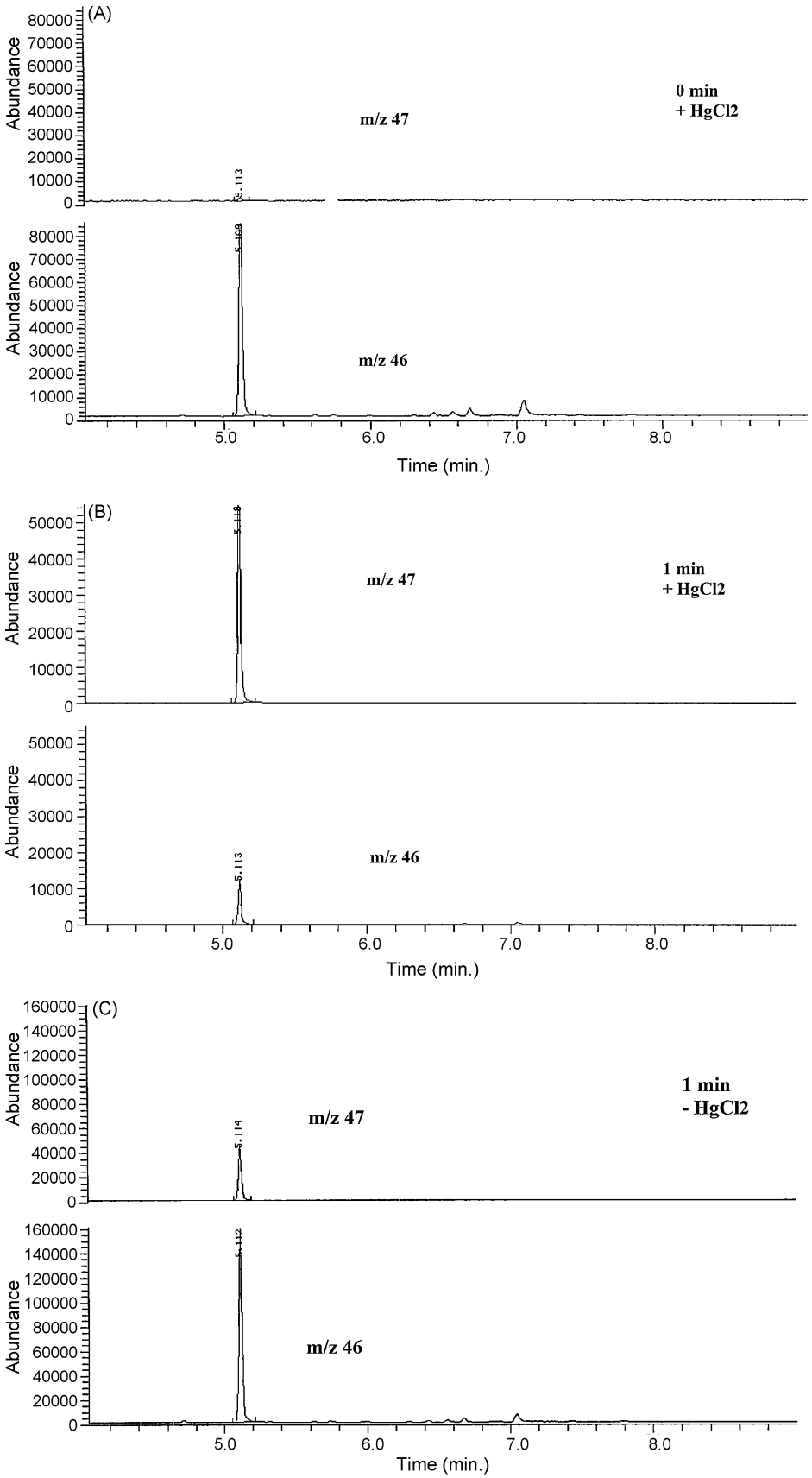

Fig. 3. Partial chromatograms from the GC–MS analysis of S^{15}NALB in rat plasma before (A, 0 min) and at various times (B and C: 1 min; D and E: 12 min; F and G: 30 min) after intravenous administration of 225 nmol of S^{15}NALB into a 430 g weighing rat. The labelling "+$HgCl_2$" and "−$HgCl_2$" indicates that samples were treated or not treated with $HgCl_2$. The pentafluorobenzyl derivatives of [^{14}N]nitrite (*m*/*z* 46, lower tracings) and [^{15}N]nitrite (*m*/*z* 47, upper tracings) emerged from the GC column almost at the same time, i.e., at 5.11 min.

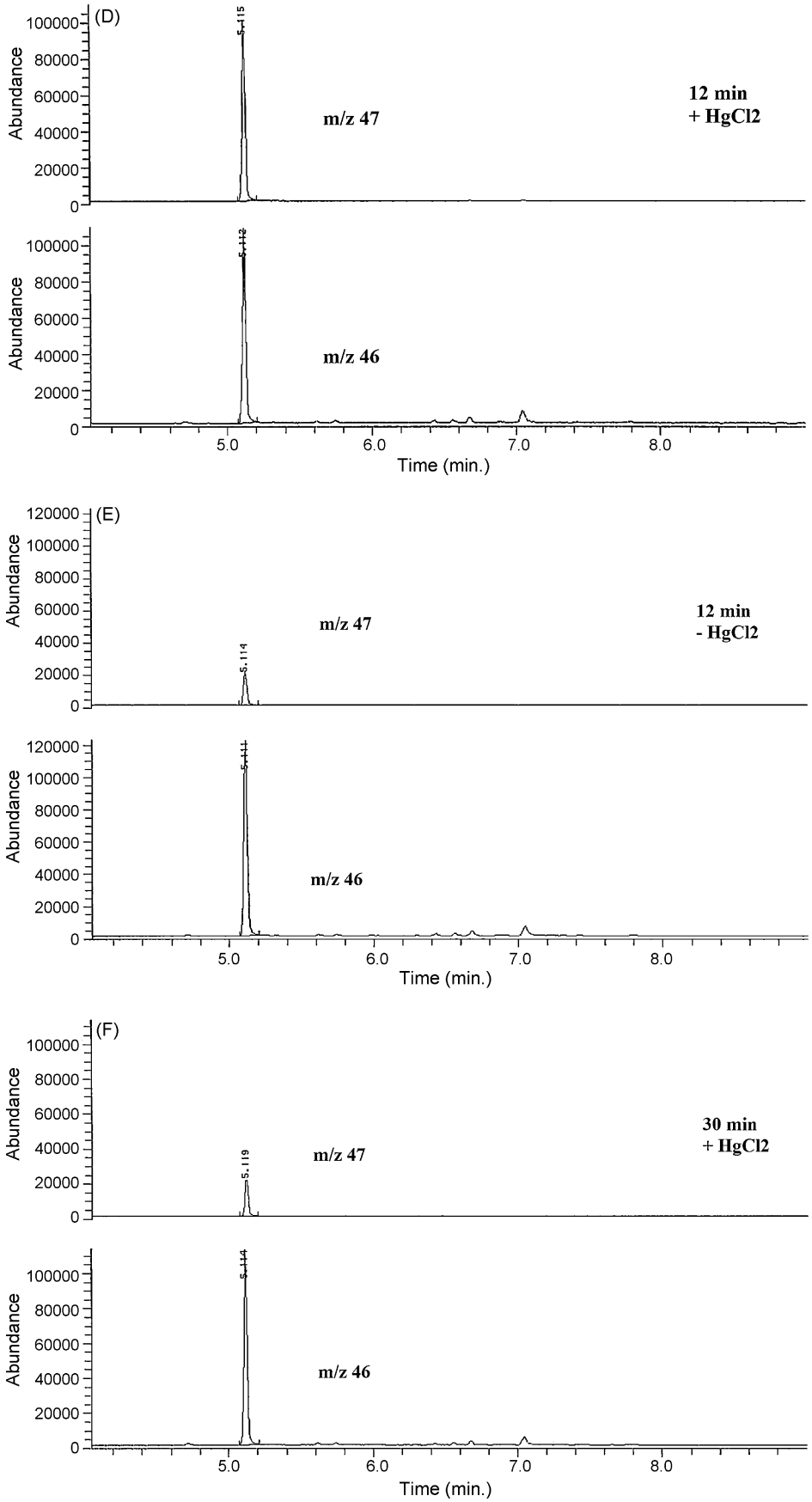

Fig. 3. (*Continued*)

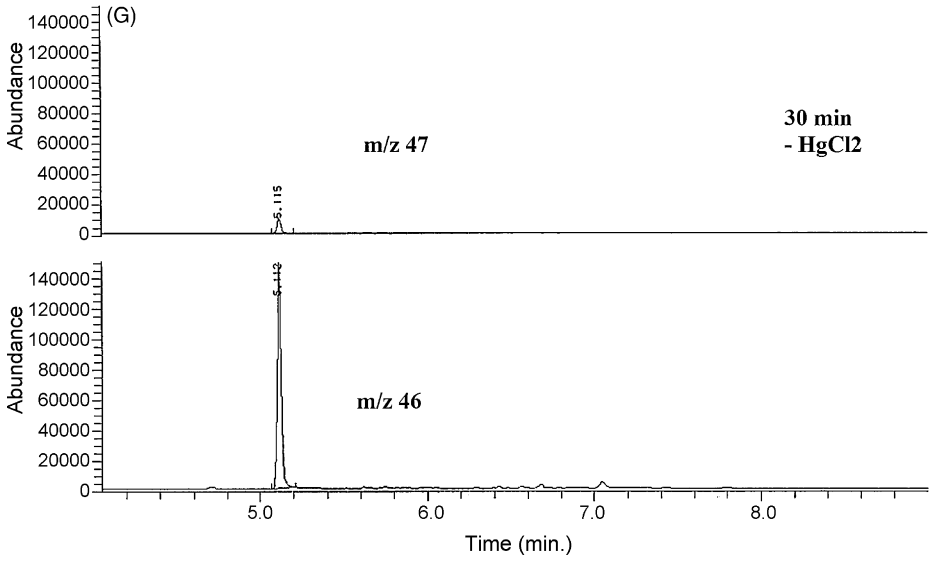

Fig. 3. (*Continued*).

unselectivity of the *S*-transnitrosylation reaction with regard to thiols, the finding that the yield of S^{15}NALB from *S*-transnitrosylation of endogenous albumin by $GS^{15}NO$ and $S^{15}NC$ in vivo is much lower than in vitro in plasma suggests that $GS^{15}NO$ and $S^{15}NC$ are rapidly metabolized in blood, presumably by releasing ^{15}NO and *S*-transnitrosylating extra- and intra-cellular SH groups. This assumption is supported: (i) by the inability to isolate and detect authentic $GS^{15}NO$ and $S^{15}NC$ from rat blood after i.v. injection and (ii) by measuring ^{15}N-labelled nitrite and nitrate in plasma and different tissues including liver and kidney (R_{PA} up to 0.2 for [^{15}N]nitrite; data not shown). Furthermore, it is interesting that considerably lower R_{PA} values were measured from i.v. injected $GS^{15}NO$ and $S^{15}NC$ (Fig. 4; each 500 nmol) as compared with the i.v. injection of the about 12-fold lower dose of 42 nmol of S^{15}NALB, i.e., the lowest dose used in the present study (Fig. 2A). In vivo as well as in vitro, $GS^{15}NO$ and $S^{15}NC$ produced very similar R_{PA} values suggesting comparable *S*-transnitrosylation potency of these species. These findings collaborate with previous observations in human plasma and blood in vitro [35]. Also, the finding that similar R_{PA} values were observed for S^{15}NALB in vitro before and after i.v. injection both of $GS^{15}NO$ and $S^{15}NC$ (Fig. 4; "in vitro") suggests that these *S*-nitrosothiols did not alter considerably the SH group of cysteine in rat albumin.

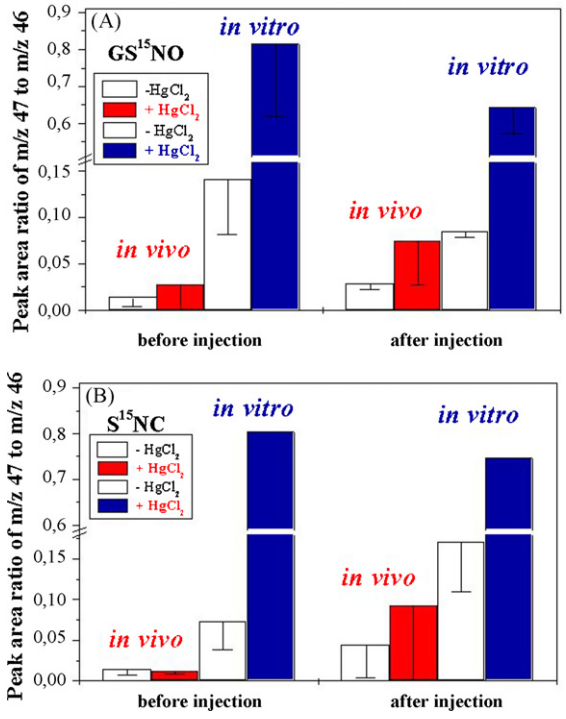

Fig. 4. Peak area ratio of *m*/*z* 47 to *m*/*z* 46 measured in the S^{15}NALB fraction extracted from rat plasma before and 3 min after intravenous injection of $GS^{15}NO$ (A) or $S^{15}NC$ (B) each in three rats. Open columns indicate no sample treatment with $HgCl_2$ and closed columns indicate sample treatment with $HgCl_2$. Labelling "in vivo" indicates in vivo formation of S^{15}NALB after i.v. administration of 500 nmol of $GS^{15}NO$ or $S^{15}NC$. Labelling "in vitro" indicates in vitro formation of S^{15}NALB after incubation of the plasma samples obtained from the "in vivo" experiments for 10 min with $GS^{15}NO$ or $S^{15}NC$ at final concentrations of 9.6 μM each.

3.4. Blood pressure-lowering effects of S^{15}NALB, $S^{15}NC$, $GS^{15}NO$ and $S^{15}NAC$ in the rat

Bolus i.v. injection of S^{15}NALB, $S^{15}NC$, $GS^{15}NO$ or $S^{15}NAC$ in the rats resulted in immediate and dose-dependent fall in the mean arterial pressure (MAP). In all rats investigated, baseline MAP amounted to approximately 70–80 mmHg, and MAP did not fall below approximately 30 mmHg upon i.v. administration of *S*-nitrosothiols. A representative tracing of the MAP from i.v. injection of S^{15}NALB (125 nmol) is shown in Fig. 5. As can be seen in this figure, MAP fell by 49% after injection of S^{15}NALB, with the baseline MAP being recovered 18 min after injection. The MAP fall caused by S^{15}NALB and all other investigated LMM *S*-nitrosothiols typically showed two peaks, of which the first peak required less than 30 s to reach its minimum, while the subsequent second minimum was greater in extent and required up to about 240 s to reach its minimum. Thirty-three minutes after the i.v. injection of S^{15}NALB, repeated i.v. bolus injection of L-cysteine (each 10 μmol, i.e., 1 ml aliquots of a freshly prepared 10 mM solution of L-cysteine in saline) caused falls in MAP (ΔMAP) of comparable extent (by 55%, 45% and 44%) as S^{15}NALB. However, baseline MAP was recovered much faster, i.e., about 4 min after injection of L-cysteine (Fig. 5). Injection of D-cysteine (each 10 μmol, i.e., 1 ml aliquots of a freshly prepared 10 mM solution of D-cysteine in saline) instead of L-cysteine also caused ΔMAP of the same extent (by 50%, 45% and 40%) and comparable MAP recovery time within 4 min (data not shown) when followed S^{15}NALB i.v. injection (not shown). Repeated i.v.

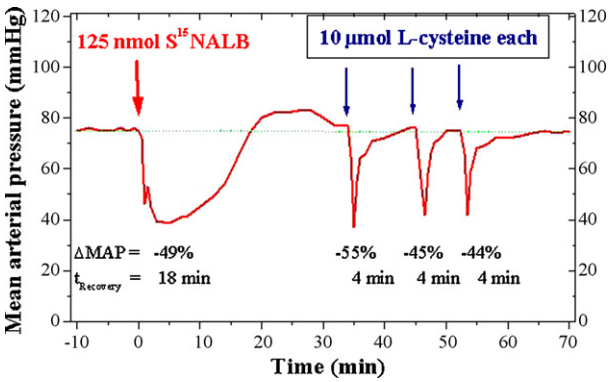

Fig. 5. Time course of the mean arterial pressure (in mmHg) in a rat (490 g) before and after i.v. bolus injection of S^{15}NALB (125 nmol) followed by repeated i.v. injection each of 10 µmol of L-cysteine as indicated by arrows. ΔMAP means change in mean arterial pressure.

bolus injection of increasing amounts (100, 400, 500, 750 nmol) of L-SNC into a rat (weight, 420 g) revealed almost linear relationships between dose and time to reach the maximum of MAP fall as well as for time to reach the baseline MAP with approximate rates of 0.03 s/nmol L-SNC and 0.6 s/nmol L-SNC, respectively. Qualitatively similar results were also observed with i.v. injection of GSNO and SNAC. Bolus i.v. injection of up to 10 µmol of freshly prepared solutions in saline (1 ml, 10 mM) of L-cysteine, D-cysteine, GSH or NAC alone did not produce any falls in MAP (data not shown).

3.5. Cysteine-mediated release of ^{15}NO from S^{15}NALB

In aqueous buffered solution the reaction of L-cysteine or D-cysteine and S^{15}NALB produced ^{15}NO that was detected and quantitated by an NO electrode. L- and D-cysteine produced very similar amounts of ^{15}NO (shown only for L-cysteine in Fig. 6). ^{15}NO formation from the reaction of L-cysteine and S^{15}NALB was found to depend upon the concentration of S^{15}NALB at a fixed L-cysteine concentration of 500 µM (Fig. 6A) and upon L-cysteine concentration at a fixed S^{15}NALB concentration of 20 µM (Fig. 6B). At L-cysteine concentrations far exceeding those of S^{15}NALB (Fig. 6A)–a condition similar to that prevailing in the experiment reported in Fig. 5–measured ^{15}NO concentration in the buffer (y) was found to depend linearly upon the S^{15}NALB concentration (x). Linear regression analysis between y and x revealed the regression equation $y = 0.02 + 0.15x$ ($R = 0.99973$), indicating average L-cysteine-mediated conversion of 15% of S^{15}NALB to ^{15}NO. At L-cysteine concentrations comparable to those of S^{15}NALB, less than 10% of S^{15}NALB released detectable amounts of ^{15}NO (Fig. 6B), suggesting that even low amounts of L-cysteine may produce considerable amounts of NO from SNALB.

3.6. Inhibition of platelet aggregation by SNALB

SNALB, added at 2 µM to washed human platelets, inhibited collagen-induced platelet aggregation; however, the effect of SNALB was not statistically significant from that of physiological saline (SNALB: 74 ± 2% versus the control NaCl: 80 ± 3%, $P = 0.116$) (Fig. 7). Incubation of washed platelets with SNALB (2 µM) and L-cysteine (10 µM) clearly inhibited platelet aggregation as compared both to 0.9 wt.% NaCl ($P = 3.7 \times 10^{-7}$) and to SNALB alone ($P = 4.6 \times 10^{-9}$). Co-incubation of washed platelets with SNALB (2 µM), L-cysteine (10 µM) and ODQ (10 µM) (i.e., "+Cys + ODQ"; Fig. 7) reversed almost completely the inhibitory effect of the combination of SNALB and L-cysteine (i.e., "+ Cys"; platelet aggregation: 71 ± 3% versus 27 ± 5%, $P = 7.3 \times 10^{-7}$), but did not completely

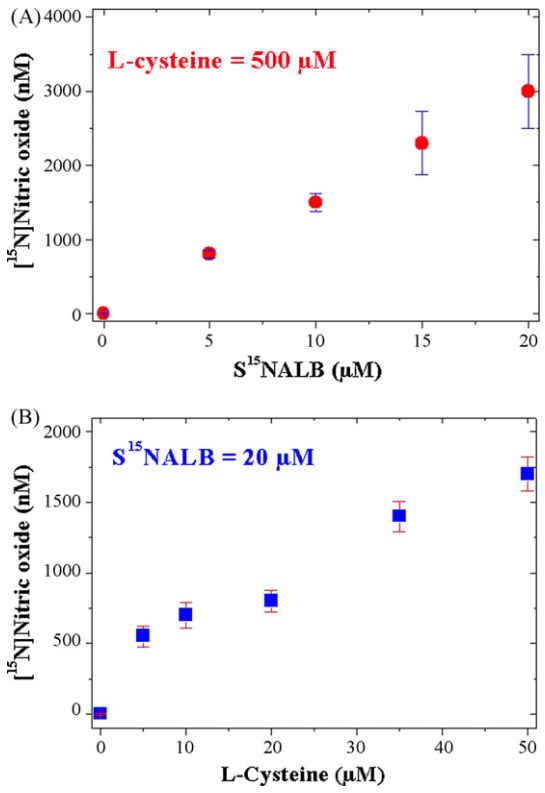

Fig. 6. L-Cysteine-mediated release of [^{15}N]nitric oxide from S^{15}NALB in 50 mM potassium phosphate buffer, pH 7.4, at room 25 °C. (A) S^{15}NALB concentration varied as indicated and L-cysteine was used at the fixed concentration of 500 µM. (B) L-cysteine concentration varied as indicated and S^{15}NALB was used at the fixed concentration of 20 µM. For calculation of NO concentrations, the maximal levels of the continuously recorded current were used. Maximum currents occurred between 90 and 120 s after addition of the thiols or S^{15}NALB.

reverse the weak inhibitory effect of SNALB as compared to control (NaCl, $P = 0.029$). That ODQ inhibited the action of the combination of SNALB and L-cysteine on platelet aggregation suggests that the mechanism of the inhibition of platelet aggregation involves soluble guanylyl cyclase. Quantitatively similar results were also obtained using D-cysteine instead of L-cysteine, suggesting that most likely free NO mediated these anti-platelet effects.

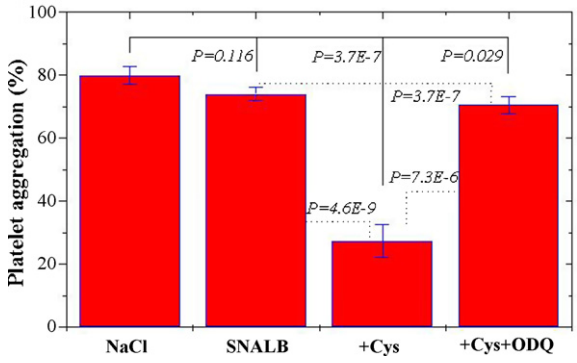

Fig. 7. Collagen-induced (1 µg/ml) aggregation of washed human blood platelets (approximately 10^8) incubated with physiological saline (NaCl) serving as the control ("NaCl"), SNALB (2 µM) alone ("SNALB"), SNALB (2 µM) plus L-cysteine (10 µM) ("+Cys"), or SNALB (2 µM) plus L-cysteine (10 µM) plus ODQ (10 µM) ("+Cys + ODQ"). Data are shown as mean ± SEM. P values were obtained from statistical t-test analysis of the various combinations. EDTA venous blood was obtained from nine healthy volunteers. The means of 9 for "NaCl" and "+Cys + ODQ", 16 for "SNALB" and 19 for "+Cys" independent measurements were used in this figure and in statistics.

4. Discussion

4.1. Quantitative analysis of S-nitrosothiols by mass spectrometry

Mass spectrometry-based approaches such as GC–MS and LC–MS and their variants GC–MS/MS and LC–MS/MS emerged indispensable analytical tools to discover, establish and quantitate LMM and HMM biomarkers in biological fluids and tissues. These approaches have also been widely applied to investigations of the chemistry, biology and pharmacology of the L-arginine/NO pathway, in particular in combination with the use of stable-isotope labelled analogs (reviewed in refs. [13,39,40,41]). Previous studies from our group showed that GC–MS in combination with the use of ^{15}N-labelled *S*-nitrosothiols offers a powerful analytical approach to investigate various aspects of biologically and pharmacologically relevant *S*-nitrosothiols (reviewed in ref. [13]).

To the best of our knowledge, thus far LC–MS and LC–MS/MS techniques have not been reportedly applied to the quantitative determination of SNALB, a putative biomarker of NO bioactivity in the human circulation. Many grave reasons may be responsible for this failure. Presumably, the most important reason concerns the relatively low concentration of SNALB (e.g., 0.2 μM [3]) in the presence of a very high excess of albumin (e.g., 400–700 μM) together with the relative small difference in the molecular-mass of SNALB and albumin which is estimated to only about 0.04%. This circumstance makes indirect analysis of SNALB in plasma mandatory. However, acid-catalyzed hydrolysis is de facto prohibited mainly because of potential and abundant artificial formation of SNC and/or complete degradation of SNC under the strong conditions for pH and temperature. Also, enzyme-catalyzed proteolysis would most likely produce species such as SNC which are by far much less stable than SNALB under the required conditions. Due to the considerably higher stability of the tripeptide GSNO and the potential of LC–MS/MS to analyze GSNO with high sensitivity, specific enzymatic hydrolysis of SNALB to produce an oligopeptide that contains the *S*-nitroso group at Cys-34, such as Tyr-Leu-Gln-Gln-**Cys(-SNO)**-Pro-Phe-Glu or Gln-**Cys(-SNO)**-Pro, could perhaps enable quantitative determination of SNALB by LC–MS/MS. Until this day, the GC–MS approach seems to be the single one MS-based technique that allows indirect quantitative analysis of SNALB in the presence of high molar excesses of albumin (e.g., 3500-fold in human plasma) [3,13,34].

In the present study we applied the GC–MS methodology to investigate the pharmacokinetics of S^{15}NALB and the formation of S^{15}NALB from i.v. injection in the rat of the LMM *S*-nitrosothiols L-S^{15}NC and GS^{15}NO, the ^{15}N-labelled analogs of the putative endogenous L-SNC and GSNO. The principle of this GC–MS technique is based on the Saville method [42], i.e., on the conversion of the *S*-[^{15}N]nitroso group to [^{15}N]nitrite by $HgCl_2$. [^{15}N]Nitrite formed by the Saville reaction is subsequently derivatized to its α-[^{15}N]nitro-pentafluorotoluene, i.e., the pentafluorobenzyl (PFB) derivative, which is finally analysed by GC–MS. Interestingly, under ECNICI conditions α-[^{15}N]nitro-pentafluorotoluene ionizes readily to form the [^{15}N]nitrite ion (with *m*/*z* 47) which is finally selected and monitored in the SIM mode.

The GC–MS analysis of [^{15}N]nitrite as PFB derivative allows to detect as less as 170 amol of [^{15}N]nitrite (i.e., the LOD value) [43]. Considering an almost quantitative recovery of [^{15}N]nitrite from $HgCl_2$-treated *S*-[^{15}N]nitrosothiols and an overall yield (i.e., derivatization/extraction) of 20–100% for α-nitro-pentafluorotoluene from aqueous buffered solutions [43], it can reasonably be assumed that the LOD of this GC–MS method for various *S*-[^{15}N]nitrosothiols will be of the same order as for [^{15}N]nitrite.

By far more relevant than LOD, however, is the LOQ value in quantitative analyses in biological fluids, i.e., the lowest added concentration of the analyte that can be accurately and precisely measured and discriminated from the blank or basal analyte concentration. The LOQ of this GC–MS method is mainly determined by the concentration of [^{14}N]nitrite present in the matrix that contains a particular *S*-[^{15}N]nitrosothiol, i.e., S^{15}NALB in the present study. [^{15}N]Nitrite produced in rat plasma from $HgCl_2$-independent decomposition of S^{15}NALB and eventually from LMM *S*-[^{15}N]nitrosothiols is a less important determinant of the methods LOQ. This is because both [^{15}N]nitrite and LMM *S*-[^{15}N]nitrosothiols are almost quantitatively eliminated by the selective affinity chromatography column extraction of S^{15}NALB from rat plasma. Like other *S*-[^{15}N]nitrosothiols, S^{15}NALB also decomposes in part to [^{15}N]nitrite during derivatization with PFB bromide, notably in the absence of $HgCl_2$ (Fig. 3). However, this apparent [^{15}N]nitrite should be assigned to in situ-decomposed S^{15}NALB rather than to [^{15}N]nitrite from other sources. Upon addition of $HgCl_2$ to S^{15}NALB-containing extracts, the GC–MS peak of [^{15}N]nitrite, i.e., of the ion with *m*/*z* 47, increased, unequivocally evidencing the presence of S^{15}NALB in plasma of rats receiving this substance (Fig. 3).

In the buffer B used to elute S^{15}NALB from the affinity chromatography column, nitrite was found to be present at about 2400 nM in the present study. Due to the composition of the N atom of the stable-isotopes ^{14}N and ^{15}N at natural abundances of 99.635% and 0.365%, respectively, it can be estimated that blank nitrite consists of 2391 nM of [^{14}N]nitrite and only 9 nM of [^{15}N]nitrite. In comparison to the relatively high level of 2391 nM for blank [^{14}N]nitrite, the value of 9 nM for blank [^{15}N]nitrite is about 266 times lower than the blank nitrite and allows, therefore, for a much more sensitive quantification of [^{15}N]nitrite and S^{15}NALB-derived [^{15}N]nitrite than that of [^{14}N]nitrite. Indeed, external addition of 10 nM of [^{15}N]nitrite to plasma was statistically significantly different from the basal [^{15}N]nitrite level, and this concentration was measured with analytically acceptable accuracy (100 ± 20%) and precision (RSD ≤ 20%). Therefore, 10 nM of [^{15}N]nitrite can be defined as the LOQ of this GC–MS method for [^{15}N]nitrite in plasma samples that contain normal levels of nitrite (e.g., 1–3 μM for this method [39]).

With respect to the quantitative determination of S^{15}NALB in plasma the LOQ of the method is higher, i.e., 50 nM, because of the extraction yield of about 50% and of a final sample dilution factor of two. Incomplete recovery lowers the final S^{15}NALB concentration in the elution buffer by a factor of about 4 as compared with non-extracted plasma. Thus, the major advantage of using ^{15}N-labelled *S*-nitrosothiols over use of unlabelled *S*-nitrosothiols in combination with the analysis of ^{15}N-labelled nitrite as PFB derivative is a considerably lower LOQ value. As the LOQ of the method for S^{15}NALB is mainly dependent upon the concentration of blank nitrite in the elution buffer, the LOQ of the method could be further lowered by decreasing the blank nitrite concentration, for instance by using distilled water, buffer salts and laboratory materials of lowest nitrite content. Worth mentioning advantages of the lower LOQ values in this stable-isotope technique are a more specific and accurate quantitative determination of S^{15}NALB, notably distinguishing of S^{15}NALB from blank nitrite, and use in vivo studies of ^{15}N-labelled *S*-nitrosothiols, which are potent vasodilators, at much lower doses such as in pharmacokinetic studies as demonstrated in the present study. It may be expected that the use of such a GC–MS approach would help delineate more reliably the pathophysiology of SNALB and other *S*-nitrosolthiols in humans without altering considerably the haemodynamics.

4.2. Possible mechanistic and biological implications of S-nitrosothiols within the vasculature

On the basis of a $t_{1/2}\alpha$ value of 4.1 min for the first 8 min and of a $t_{1/2}\beta$ value of 9.4 min for the subsequent 37 min after i.v. bolus injection of S^{15}NALB into the rat (Fig. 2), it can be calculated

that 18 and 30 min after i.v. bolus injection of S^{15}NALB approximately 12% and 6% of the initially administered amount of S^{15}NALB, respectively, will still circulate in the rat. This approximation collaborates with the MAP course in the rat that received intravenously 125 nmol of S^{15}NALB (Fig. 5). That i.v. injection of L-cysteine 33, 42 and even 52 min after i.v. injection of S^{15}NALB caused repeatedly considerable MAP falls suggests that L-cysteine reacted with *S*-[^{15}N]nitroso groups different from those of S^{15}NALB through *S*-transnitrosylation to form L-S^{15}NC which is highly unstable in blood and releases ^{15}NO that finally caused MAP to fall (Fig. 5). Because i.v. injection of L- or D-cysteine that followed i.v. bolus injection of S^{15}NALB in the rat produced comparable falls in MAP (not shown) and because D-cysteine is most likely not so rapidly and effectively transported into intra-cellular compartments, it can reasonably be assumed that extra-cellular rather than intra-cellular *S*-[^{15}N]nitroso groups were involved in this *S*-transnitrosylation reaction. The assumption of intermediate formation of L-S^{15}NC from S^{15}NALB or other *S*-[^{15}N]nitrosothiols and L-cysteine and the subsequent decomposition of L-S^{15}NC to ^{15}NO in rat circulation is supported by the finding that in aqueous buffered solution the reaction of L-cysteine and S^{15}NALB produced significant amounts of ^{15}NO (Fig. 6).

The course and shape of MAP in the rat depicted in Fig. 5 may gain further interesting insights into the potential mechanisms by which the interplay between SNALB or other *S*-nitrosothiols and thiols may regulate vascular tone. In this context, of particular interest is L-cysteine which is the most abundant LMM thiol in human plasma [44,45] and forms one of the most labile, apparently spontaneously NO-releasing LMM *S*-nitrosothiols, i.e., L-SNC, a putative endothelium-derived relaxing factor [46]. It should be, however, mentioned that there may be considerable differences between humans and rodents regarding concentrations of individual LMM thiols [47] as well as regarding reactivity of cysteine moieties in mammalian and rodent proteins [11]. The present findings suggest that SNALB itself is not a directly acting vasodilator, but its blood pressure-lowering effect is most likely mediated by plasma L-cysteine. The underlying mechanism could involve reaction of L-cysteine with SNALB by which the *S*-nitroso group of SNALB is transferred to the SH group of L-cysteine to form L-SNC and albumin (reaction (2)). In vitro, this *S*-transnitrosylation reaction proceeds very rapidly, and more importantly, it is reversible, with the equilibrium (K_{eq} = 1.33) being in favour of SNALB [22]. Because the concentration of cysteinyl-SH groups of albumin greatly prevails over L-cysteine in human plasma–a molar ratio of 36:1 can be estimated from literature data [35]–circulating SNALB concentrations should be considerably higher than those of L-SNC in human circulation. These considerations also apply to GSNO, however, our discussion will focus on L-SNC, because L-SNC, unlike GSNO, is a very potent NO donor and can be actively transported through membranes from various cells [48,49,12].

Considering a whole rat plasma volume of about 10 ml, injection of 125 nmol of S^{15}NALB in the rat would result in a plasma S^{15}NALB concentration of about 12.5 μM immediately after injection. The peak area ratio R_{PA} of about 1.8 measured in rat plasma 3 min after i.v. injection of 125 nmol of S^{15}NALB (Fig. 2A), the standard curve of S^{15}NALB in Fig. 1C, strongly suggest that a large portion of injected S^{15}NALB is present unchanged in rat blood immediately after injection. Indeed, a rough estimate comes to a plasma concentration of 15 μM for S^{15}NALB 1 min after injection of 125 nmol of S^{15}NALB (125 nmol), suggesting that the administered dose of S^{15}NALB is still entirely present in rat plasma at this time. Most likely, however, a part of the administered dose of S^{15}NALB has already reacted directly with SH groups from various LMM thiols circulating in rat blood, including L-cysteine and GSH, as well as with protein SH groups abundantly present on the surface of various blood cells including thrombocytes and erythrocytes [12]. It is possible that these *S*-transnitrosylation reactions were in part facilitated by intermediately formed L-S^{15}NC. Because *S*-transnitrosylation reactions are very quick, the rapid decrease in the peak area ratio R_{PA} measured 5 min after i.v. injection of S^{15}NALB is most likely due to *S*-transnitrosylation of circulating SH groups, resembling the rapid disappearance of drugs due to distribution within the body, and may explain the calculated elimination half-life of S^{15}NALB of 4.1 min ($t_{1/2}\alpha$). A few seconds after i.v. injection of S^{15}NALB in the rat the MAP fell very sharply (Fig. 5). This initial, abrupt and short-lasting fall in MAP is most likely caused by ^{15}NO released from intermediately formed L-S^{15}NC. Likely, initially formed L-S^{15}NC disappears very rapidly from the blood leading to a temporary increase in MAP due to a reduced ^{15}NO release rate from L-S^{15}NC. Because initially formed L-S^{15}NC may potently *S*-transnitrosylate SH groups, subsequently occurring *S*-transnitrosylation reactions inside cells, notably red blood cells [50], may also led to a temporary decrease in L-S^{15}NC concentration in rat plasma. Assuming an equilibrium constant (K_{eq}) of 1.33 for the reaction between SNALB and L-cysteine [22], an initial S^{15}NALB concentration of 15 μM and initial albumin-SH and L-cysteine-SH groups concentrations in rat plasma of 300 and 10 μM, respectively, it can be calculated that the initially formed L-S^{15}NC would reach a maximum concentration of about 0.4 μM immediately after i.v. injection of 125 nmol of S^{15}NALB in rat plasma. Because of the high vasodilatory potency of L-SNC [46], it is quite possible that the initial and short-lasting fall in MAP of about 27 mmHg upon administration of 125 nmol of S^{15}NALB in our study had been caused by 0.4 μM of L-SNC. In previous study, using the same GC–MS methodology, we found that L-S^{15}NC added to human blood anticoagulated with EDTA could not be isolated after 10 min of incubation at added L-SNC concentrations below 10 μM [35]. This finding and the calculated L-S^{15}NC maximum rat plasma concentration of 0.4 μM may explain the failure to detect any L-S^{15}NC after i.v. injection of L-S^{15}NC or S^{15}NALB in rats in the present study.

In aqueous buffered solutions of LMM *S*-nitrosothiols such as SNC and GSNO and in the presence of their corresponding thiols, e.g., L-cysteine and GSH, we found stable equilibria for several *S*-transnitrosylation reactions persisting for up to 135 min, despite constant decreases in the concentration of the participating *S*-nitrosothiols [22]. Importantly, in such mixtures L-SNC turned out to be apparently considerably more stable than in the absence of other thiols and *S*-nitrosothiols, seemingly prolonging the half-life of L-SNC [22]. Nevertheless, it can reasonably be assumed that L-S^{15}NC is the most reactive and most potent blood pressure-lowering species participating in *S*-transnitrosylation reactions in rat circulation following i.v. injection of S^{15}NALB and L-cysteine. Our results suggest that MAP fall parallels L-S^{15}NC rat plasma concentration which, in turn, depends upon actual S^{15}NALB concentration in rat plasma. Given the considerably much lower stability of L-S^{15}NC in rat plasma as compared with that of S^{15}NALB and the striving for equilibrium concentrations of the *S*-transnitrosylation reaction involving S^{15}NALB, L-S^{15}NC, albumin and L-cysteine, the decrease in L-S^{15}NC plasma concentration will inevitably lead to concomitantly falls both in MAP and S^{15}NALB concentration. Most likely, this phenomenon predominates within the period of 8–45 min, i.e., in the late phase of the pharmacokinetic experiment, and is responsible for the considerably longer elimination half-life of S^{15}NALB of 9.4 min ($t_{1/2}\beta$) as compared to the $t_{1/2}\alpha$ of S^{15}NALB of 4.1 min in the early phase.

The course of R_{PA} in Fig. 2A suggests that the half-life of S^{15}NALB in the rat is even greater than 9.4 min within the observation period of 20–45 min. One possible explanation for this apparent prolongation of the half-life of S^{15}NALB in the rat could be re-*S*-transnitrosylation of albumin by *S*-[^{15}N]nitroso groups from extra- and intra-cellular sources including erythrocytic GS^{15}NO and *S*-[^{15}N]nitrosoheamoglobin (S^{15}NOHb). The formation of S^{15}NOHb

could have been mediated by L-S^{15}NC transported into the erythrocytes by a specific transporter system that exists within the red blood cell membrane [49]. This assumption is supported by the finding that incubation of fresh native human plasma with washed red blood cells having been pre-incubated with L-S^{15}NC led to formation of plasma S^{15}NALB and inhibition of platelet aggregation in a manner depending upon L-S^{15}NC concentration initially exposed to erythrocytes [49]. Further support of this assumption is provided by Fig. 5. Repeated i.v. injection of each 10 μmol of L-cysteine in the rat after i.v. bolus injection of S^{15}NALB led to immediate and short-lasting falls in MAP. We assume that these MAP falls were caused by low concentrations of L-S^{15}NC (about 0.4 μM) which was formed from the reaction of L-cysteine with remaining S^{15}NALB as well as with extra- and intra-cellular *S*-[^{15}N]nitroso groups inside and outside the vasculature driven by the very high doses of L-cysteine applied. On the basis of a rat blood volume of 30 ml, it is calculated that repeated administration of L-cysteine led to blood concentrations of about 0.7, 1.3 and 2 mM, respectively.

The recent study by Orie et al. [29] has demonstrated that infused SNALB causes venodilation in the rat at considerably high plasma concentrations (about 4 μM). The study by Orie et al. [29] and the present study suggest that endogenous circulating SNALB may have little impact on vascular function in vivo in the rat and presumably in humans. However, the latter remains to be demonstrated.

4.3. Possible implications for basal concentration of S-nitrosothiols in the human vasculature

We are aware of the differences which are likely to exist between humans and rats regarding *S*-nitrosothiols and reactivity of L-cysteine moieties in proteins including albumin [11], of the problematic extension of results from animal studies to humans, and of the high divergence in reported concentrations of SNALB and other *S*-nitrosoproteins in plasma of humans and other species [14]. However, in consideration of the overwhelming evidence in the literature we think that our results from the present investigation and from previous studies, which were all generated by using the GC–MS methodology [3,4], strongly suggest that the concentration of SNALB, and most likely of other *S*-nitrosoproteins, in plasma of healthy humans is presumably not higher than 160–200 nM. Reported concentrations for SNALB in plasma of humans in the basal state far above this value are, in our opinion, erroneously. SNALB concentrations in human plasma of the order of 200 nM and the need for equilibrium between thiols and *S*-nitrosothiols would also have the consequence that the concentration of LMM *S*-nitrosothiols including GSNO and SNC in plasma of healthy humans would be far below 10 nM.

5. Conclusions

In the year 1992, it has been reported for the first time that SNALB occurs in plasma of healthy humans at concentrations of the order of 7000 nM in the basal state as measured by chemiluminescence. Since then, SNALB and other HMM *S*-nitrosothiols have been reported to occur at concentrations ranging from about 10 to 10,000 nM and even higher. SNALB has been suggested to be a biomarker of NO bioactivity and to be involved in many diseases. However, high divergences, contradictory results and most importantly obvious analytical shortcomings in numerous basic and clinical studies challenge the concentrations and implications ascribed by many groups to SNALB and other *S*-nitrosoproteins in health and disease. By means of GC–MS we measured SNALB concentrations in the range 160–200 nM in plasma both of healthy subjects and of those suffering from hepatic and renal diseases.

At present, the GC–MS methodology is the single MS-based approach that allows quantitative but certainly indirect determination of unlabelled and ^{15}N-labelled SNALB (S^{15}NALB) in biological fluids including human and rat plasma. This methodology is selective for SNALB as it uses affinity chromatography for its isolation from plasma. The stable-isotope technique reported here has a low LOQ value which enables performance of pharmacokinetic studies at quasi physiologically relevant plasma concentrations. Our present study shows that S^{15}NALB is a relatively long-lived *S*-nitrosothiol in the rat and a potent *S*-transnitrosylating and a blood pressure-lowering species. Sixteen years after the discovery of SNALB in human circulation, we have until this day no dependable knowledge of the potential functions of SNALB in humans. Even if the present study was performed in rats, it suggests that endogenous SNALB in human plasma may be an interesting biomarker candidate for NO bioactivity in the human circulation. However, accurate and interference-free analysis of SNALB and S^{15}NALB still represents an analytical challenge. The GC–MS approach is useful for the quantitative analysis of SNALB and S^{15}NALB, but it suffers from being indirect. Actually, LC–MS-based techniques should be by nature the method of the choice for the quantitative determination in biological fluids both of LMM and of HMM *S*-nitrosothiols including SNALB and SNOHb. However, this remains to be demonstrated for these potential biomarkers.

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