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A Chemiluminescense-based Assay for S-nitrosoalbumin and Other Plasma S-nitrosothiols

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The lack of a simple assay for the quantification of S-nitrosothiols in complex biological matrices has hampered our understanding of their contribution to normal physiology and pathophysiological states. In this paper we describe an assay based upon the release of nitric oxide by reaction with a mixture consisting of Cu(I), iodine and iodide with subsequent quantification by chemiluminescense. With this method we can detect levels of S-nitrosothiols down to 5 nM in plasma. Following alkylation of free thiols with N-ethylmaleimide, and removal of nitrite with acidified sulfanilamide, we were able to measure known amounts of S-nitrosoalbumin added to plasma or whole blood, with an inter-assay variation for plasma S-nitrosothiols of ~4%. Further studies showed that the mean concentration of circulating S-nitrosothiols in venous plasma of healthy human volunteers was 28 ± 7 nM.

Keywords: S-nitrosothiols, S-nitrosoalbumin, nitric oxide, chemiluminescence

Abbreviations: HPLC, high performance liquid chromatography; NO, nitric oxide; PBS, phosphate buffered saline; DTPA, diethylenetriaminepentaacetic acid; EDTA, ethylene diaminetetraacetic acid; NEM, N-ethylmaleimide; RSNO, S-nitrosothiol; S-NO-albumin, S-nitrosoalbumin

INTRODUCTION

The pharmacological properties of S-nitrosothiols (RSNOs) resemble those of nitric oxide (NO), including but not limited to vasodilatation,[1] smooth muscle relaxation[2,3] and inhibition of platelet aggregation.[4,5] However, their biological half-life is considerably longer than that of authentic NO. Low molecular weight RSNOs have been detected in plasma and airway lining fluid.[6,7] It has been hypothesised that their presence in blood may represent a buffer or storage system for NO or be used to transport NO to sites distal to those of its production.[6,8] The contribution of RSNOs to both normal physiology and pathophysiological events is, however, still unclear. This is primarily due to the lack of sensitive assays for their detection in complex biological matrices such as blood.

A method originally described for the quantification of thiols, the Saville assay,[9] is based on the mercuric ion (Hg^{2+})-catalyzed release of

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nitrosonium ions (NO^+) from the S-nitrosated thiol, which then reacts with the aromatic amine, sulfanilamide to form a diazonium salt, followed by coupling to another aromatic amine, *N*-1-naphthylethylenediamine HCl. The second step of the Saville reaction is thus identical to the classical Griess[10] assay for quantification of nitrite. Although this method can be used for the detection of RSNOs in simple aqueous systems it is not sufficiently sensitive to detect endogenous RSNO levels in plasma. Furthermore, it depends on the measurement of differences between comparably high levels of endogenous nitrite (which are subject to large inter- and intraindividual variation depending on the dietary intake of nitrate) and small incremental changes caused by the Hg^{2+}-catalyzed liberation of nitrite from RSNOs. Modifications of the original Saville method aimed at increasing sensitivity and separating the final reaction product from interfering nitrite have been described in combination with HPLC[11,12] and direct UV/VIS detection or, more sensitive fluorometric detection.[13,14] Concentrations as low as 10 nM of individual low molecular weight RSNOs can be measured by combining HPLC separation with either direct electrochemical detection or photolytic cleavage of RSNOs followed by chemiluminescent detection of released NO.[15] However, these methods are limited by the sample volume that can be applied (which limits sensitivity) and have only been described for the measurement of low molecular weight compounds such as S-nitrosocysteine and S-nitrosoglutathione. These, however, probably represent only a minor component of the total circulating RSNO pool, the majority of which exists as S-nitrosoalbumin (SNO-albumin).[6] Furthermore, photolysis is not absolutely specific in releasing NO from RSNOs; it can also generate NO from nitrite, nitrosyl iron complexes and nitrosamines.[16]

Recently, two chemiluminescense-based assays have been described for the direct measurement of RSNOs under both acidic and alkaline conditions, with a reported sensitivity of 10 nM.[17] However, these studies did not report RSNO concentrations in normal plasma. It is not clear whether this is because plasma concentrations were below their limit of detection or whether they are not suitable for measurements in complex biological matrices such as plasma. We here describe the development and validation of a chemiluminescense-based assay with a detection limit of 5 nM which was successfully employed for the measurement of total plasma RSNOs in normal healthy volunteers. The determined concentration of total plasma RSNOs was found to be considerably lower than previously reported values.

MATERIALS AND METHODS

Chemicals

PapaNONOate (*N*-propyl-1,3-propanediamine diazeniumdiolate) was purchased from Cayman Chemicals Company (Ann Arbor, MI, USA). All other chemicals were from Sigma-Aldrich Company Ltd. (Poole, UK).

Preparation of S-nitrosoalbumin

Human serum albumin (20 mg/ml) was treated with 2 mM dithiothreitol in phosphate buffered saline (PBS) together with 100 μM diethylenetriaminepentaacetic acid (DTPA) and gently stirred at room temperature for 2 h in order to ensure that the Cys-34 thiol group was fully reduced. This was followed by extensive dialysis in Visking tubing (Medicell, London, UK, molecular weight cutoff 14,000) at 4°C against 3 × 3 l PBS, pH 7.4 containing 100 μM DTPA. S-nitrosocysteine (10 mM) was freshly prepared by reacting equimolar sodium nitrite with L-cysteine hydrochloride at pH 2 and incubated with reduced albumin (150 μM or 10 mg/ml) at room temperature for 30 min in the dark to allow transnitrosation, and formation of SNO-albumin, respectively (yield > 95%). Any unreacted thiol was alkylated

with N-ethylmaleimide (NEM; 1 mM) at room temperature, followed by dialysis at 4°C against 3 × 3 l PBS supplemented with 100 μM DTPA. The stock solution of SNO-albumin (≈ 145 μM) was stored at −20°C and the concentration of SNO-albumin was determined immediately prior to use by measurement of mercury-displaceable nitrite using the Saville reaction.

Plasma Samples

Venous blood was collected into tubes containing EDTA (final concentration 2 mM), and centrifuged for 10 min at 1300*g* and 4°C. Samples used for measurement of endogenous RSNO levels in normal healthy subjects were collected into pre-chilled tubes containing EDTA (final concentration 2 mM) and NEM (final concentration 5 mM) and centrifuged at 1300*g* for 10 min at 4°C. To obtain the low molecular weight fraction plasma was centrifuged through a 30,000 molecular weight cut off filter (Millipore, Bedford, MA, USA) for 30 min at 13,000*g*.

Chemiluminescense-based Assay for S-nitrosothiols

S-nitrosothiols were determined by a copper(I)/iodide/iodine-mediated cleavage of RSNOs to form NO, which was quantified by its gas phase chemiluminescent reaction with ozone. The reaction chamber was custom designed to accommodate larger sample volumes but otherwise was identical in design to a commercially available glass reaction chamber (Sievers Radical Purger™). It contained 8 ml glacial acetic acid and 2 ml potassium iodide (50 mg/ml) and was kept at 70°C via a water jacket. One minute prior to injection of the sample 200 μl of copper(II) sulphate (200 mM) was added. This solution was constantly purged with nitrogen, and used only once per sample. The outlet of the gas stream was directly passed into a Sievers Instruments Model 280 Nitric Oxide Analyzer (NOA™; Sievers, Boulder, Colorado, USA). Data collection and analysis was performed using the NOAnalysis™ software (Sievers, Boulder, Colorado, USA).

Unless stated otherwise, NEM (5 mM) was added to all plasma samples in order to alkylate free thiol groups and both stabilize RSNOs and prevent any further S-nitrosation. One of the problems of assays for S-nitrosothiols has been the comparably high background concentration of nitrite present in biological samples, which is also converted to NO by the above described reaction mixture. Nitrite was therefore removed by reaction with 0.5% sulfanilamide in 0.1 M hydrochloric acid to form a stable diazonium salt which was not converted to NO under the applied conditions. This method effectively removes nitrite up to a concentration of 10 μM, with no effect on the standard curve for SNO-albumin, whereas the presence of NEM prior to addition of sulfanilamide prevents artifactual RSNO generation from nitrite. This was confirmed by experiments showing that sulfanilamide completely removed nitrite added to plasma at concentrations of 1, 2.5 and 5 μM ($n = 3$). By comparison with addition of acidified ammonium sulfamate (0.5% in 0.045 M hydrochloric acid) in the presence of exogenous nitrite we observed a significant NO signal (equivalent to 970 nM, $n = 3$) following addition of 5 μM nitrite, rendering the use of this reagent unsuitable for quantification of low concentrations of RSNOs. To prevent foam formation in the reaction chamber, antifoam™ (Sievers, Boulder, Colorado, USA) that has been validated for use with the NO analyzer was diluted 1 : 30 in deionized water, and added to the plasma at a ratio of 1 : 9, immediately prior to sample injection. Plasma volumes of 100–2000 μl were injected rapidly into the reaction chamber by means of a Hamilton syringe and the release of NO was quantified by its chemiluminescent reaction with ozone. Addition of SNO-albumin either on its own or added to plasma caused an immediate release of NO. Using this method the intra-assay coefficient of variation was 3.7% and the interassay coefficient of variation was 4.3% ($n = 8$).

Standard curves for SNO-albumin were obtained over the range of 5 nM to 5 μM by addition of known amounts of SNO-albumin to either plasma or PBS, both containing 5 mM NEM. In order to determine stability in plasma, SNO-albumin was added at a final concentration of 1 μM to 1 ml aliquots of untreated and NEM-containing plasma. Aliquots (100 μl) were removed at 120 min and 24 h for determination of the remaining concentration of SNO-albumin. To examine the stability in whole blood of RSNOs during the centrifugation period SNO-albumin (final concentration 0 to 400 nM) was added to the test tubes containing whole blood, that had been anticoagulated with EDTA and treated with NEM. Partitioning of the exogenous SNO-albumin into the plasma phase was corrected for the hematocrit. Plasma RSNOs were then assayed as above.

Generation of S-nitrosothiols in Plasma

RSNOs were generated by incubating native plasma with a short half life NO donor, papaNONOate, at a final concentration of 100 μM for a period of 15 min, at 37°C. Parallel incubations were carried out with plasma pre-treated with 5 mM NEM in order to confirm specificity of RSNO formation. Before assaying RSNO concentration in the treated plasma, any remaining papaNONOate in the samples was removed by extensive dialysis against 3×31 PBS containing 100 μM DTPA. Nitrite was removed by addition of sulfanilamide/HCl as described above. The specificity of the assay was further confirmed by the measurement of NO release in samples that had been incubated initially with papaNONOate, as described above, but subsequently treated with 0.1% mercuric chloride for 5 min prior to the addition of sulfanilamide/HCl and injection into the reaction vessel.

Subjects

Ten healthy volunteers aged 24–55 years (mean age 34 years) and comprising 6 males and 4 females were studied. Non-fasting blood samples were taken on the day of the study from the antecubital vein, and processed immediately.

RESULTS

S-nitrosothiol Assay Validation and Stability Studies

Using the method described, NO was readily released from SNO-albumin, giving a signal of 1584 integral units with 100 μl of a 1 μM solution (100 pmol RSNO), which corresponds to a 98.9% efficacy of NO cleavage compared to a response of 1602 integral units obtained with 100 pmoles sodium nitrite ($n = 3$). A linear standard curve was readily obtained for SNO-albumin in PBS over a concentration range of 5 nM to 5 μM (data not shown). Having established a means of measuring SNO-albumin the stability of this compound was studied in human plasma at 37°C in the presence and absence of NEM. SNO-albumin was found to be relatively unstable in plasma with 38% loss of bound NO within 2 h, and 81% loss by 24 h. However, addition of NEM to plasma before addition of SNO-albumin stabilized the compound such that only 11% and 22% of the signal was lost by 2 and 24 h, respectively, at 37°C. As described below, SNO-albumin was sufficiently stable under the conditions of sample preparation, i.e. during centrifugation and separation.

The most important test for any assay to be used with biological fluids such as plasma, is whether one can reliably measure known amounts of authentic standards added to such fluids at physiologically relevant concentrations and recover the added amount in a reproducible manner. Since preliminary studies had established that plasma RSNOs are present in the low nanomolar range, this study focused on being able to measure such low levels. Following the addition of SNO-albumin to plasma, there was an almost stoichiometric recovery of NO per given amount of added RSNO, approaching 92% of the signal obtained with SNO-albumin in PBS

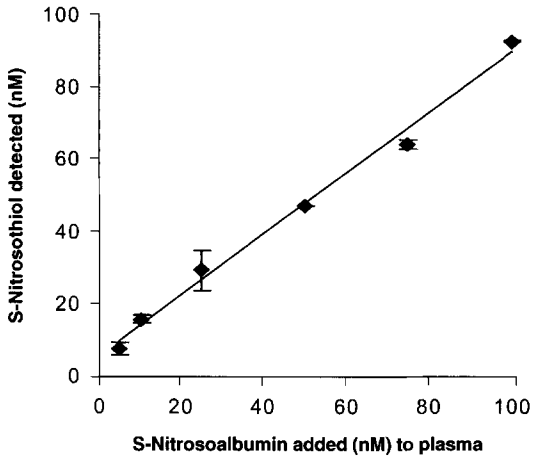

FIGURE 1 The detection of S-nitrosoalbumin (SNO-albumin) added to plasma. SNO-albumin was added to plasma to give a final concentration over a range of 2.5–100 nM ($n = 3$). The signal from the added SNO-albumin in plasma (y-axis) was compared to the known standard as measured in PBS (x-axis). Standard curves were linear in PBS from 5 nM to 5 μM, data not shown. The intercept across the y-axis (~8 nM) is lower than the values observed in normal controls, possibly because the plasma used for these spiking experiments was already several hours old and endogenous RSNOs largely decomposed.

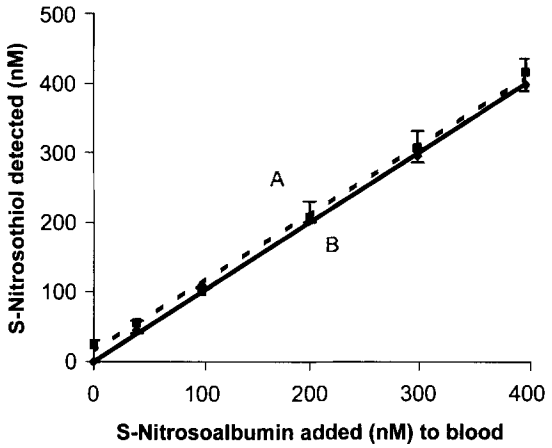

FIGURE 2 The detection of S-nitrosoalbumin (SNO-albumin) added to whole blood. SNO-albumin was added to blood containing 2 mM EDTA and 5 mM N-ethylmaleimide to give the final concentrations indicated ($n = 3$). Following centrifugation, the concentration of S-nitrosothiols was determined in plasma (y-axis) and compared to known standards in PBS (x-axis). For comparison, the standard curve from SNO-albumin in blood (A) is shown together with that obtained in PBS (B).

(i.e. in the absence of plasma constituents). The results, shown in Figure 1, represent the means of three experiments for the biologically relevant concentration range of 5–100 nM. To determine whether significant degradation of SNO-albumin occurred during the centrifugation period, the recovery of known amounts of SNO-albumin added to whole blood was determined in the presence of 2 mM EDTA and 5 mM NEM. As shown in Figure 2, over a wide range of concentrations (0 to 400 nM) the recovery of added SNO-albumin was stoichiometric, indicating stability during the period of sample preparation. The parallel shift of the standard calibration curve in whole blood compared to that obtained in PBS is likely to be caused by endogenously present RSNO.

S-nitrosation of Plasma Albumin by Exogenous NO

Addition of papaNONOate (final concentration 100 μM) to fresh human plasma and incubation for 15 min at 37°C, followed by a dialysis step, led to the generation of high molecular weight compounds (> 14,000) which released NO equivalent to an SNO-albumin concentration of 4.2 ± 0.6 μM. Parallel samples of plasma, in which thiol groups had been alkylated with NEM prior to addition of the NO donor, almost completely prevented formation of RSNOs, whereas addition of 0.1% mercuric chloride to the nitrosated plasma decreased the signal for RSNOs by 96% (see Figure 3), confirming the specificity of this assay for S-nitrosothiols.

Plasma Levels of Endogenous S-nitrosothiols

Human plasma was obtained from 10 healthy volunteers as described under Materials and Methods. RSNOs were detectable in the plasma of all subjects with a mean concentration of total RSNO of 28 ± 7 nM ($n = 10$). There was no detectable signal from the low molecular weight filtrate

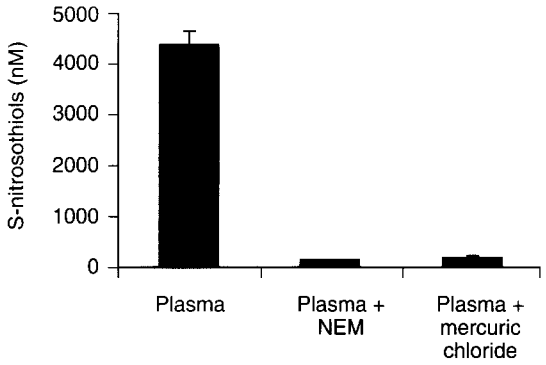

FIGURE 3 The generation of S-nitrosothiols by papaNONOate. PapaNONOate was added to plasma at a final concentration of 100 μM, and incubated for 15 min at 37°C. Plasma for these experiments was either (a) untreated, (b) pre-incubated with NEM for 30 min, or (c) treated with 0.1% mercuric chloride ($n = 3$) for 5 min following addition of papaNONOate, and stabilization by NEM, respectively.

in any of the samples analyzed, suggesting that the major forms of circulating RSNOs have a molecular weight of greater than 30,000.

DISCUSSION

In this paper we describe a novel assay for the measurement of plasma RSNOs, which has the advantage of being simple, highly sensitive and reproducible, and suitable for the analysis of a high number of samples within a short period of time. In particular, we have established that background nitrite can be removed quantitatively by reaction with sulfanilamide/hydrochloric acid after thiol groups have been blocked by NEM without affecting the subsequent breakdown of RSNOs to NO. Cleavage of the S–NO bond is achieved by reaction with a mixture consisting of copper(I)/iodine/iodide, and the NO which is generated in high yield is then detected by its chemiluminescent reaction with ozone. A reliable method for the removal of interfering nitrite, as described in the present study, represents a major methodological advancement in the quantitative analysis of low concentrations of RSNOs, since this is a common problem with most if not all other assays. This, together with the demonstration that we can stoichiometrically recover added SNO-albumin from plasma and blood enabled us to determine reliably the concentration of endogenous RSNOs in humans. Our results show that the basal plasma concentration of SNO-albumin is considerably lower than that suggested by previous studies.

The previous demonstration that alkylation of thiol groups by NEM stabilizes RSNOs in plasma by preventing transnitrosation reactions[17] and metal ions, even at low concentrations, can accelerate RSNO degradation[18,19] prompted us to collect blood onto solutions containing both NEM and EDTA. With this regime exogenous SNO-albumin added to whole blood was recovered almost quantitatively and could be readily detected down to low nanomolar concentrations.

Previous work has suggested that, under aerobic conditions, RSNOs can be generated from NO via the rate limiting step of nitric oxide's reaction with oxygen to form N_2O_3.[20] This hypothesis is, however, principally based upon experiments performed in physiological buffers. The current study confirms that incubation of plasma with papaNONOate, a NO donor with a relatively short half-life, lead to the production of compounds which gave rise to the release of NO under the reaction conditions described for RSNOs. The finding that treatment of plasma with NEM prior to incubation with papaNONOate almost completely abolished the signal suggests that the formed compounds are indeed RSNOs. Further confirmatory evidence of their nature comes from the observation that the signal can be largely attenuated by addition to the nitrosated sample of 0.1% mercuric chloride prior to addition of sulfanilamide/HCl, a treatment which is known to destroy RSNOs.

No attempts have been made to determine the exact mechanism by which RSNOs are cleaved to NO by the reagent mixture employed in our study. We reasoned, however, that addition of copper(II) sulphate to an acidic solution of potassium iodide may result in the oxidation of iodide

(I^-) to iodine (I_2), and – assuming stoichiometric oxidation of iodide by copper(II) – we hypothesize that this redox reaction resulted in a mixture of iodine/iodide and Cu^+ ions at approximate concentrations of 40/60 mM and 40 mM, respectively. This notion is supported by the observation that the reactant turns brown immediately on addition of the copper(II) solution, which is characteristic of aqueous solutions of I_3^- formed from complexation of I_2 by I^-. In view of the fact that both Cu^+ ions[19] and free iodine[21] have been shown to effectively generate NO from RSNOs we propose the following sequence of chemical reactions to account for the observed stoichiometric cleavage of RSNOs to NO:

$$I_2 + I^- \longrightarrow I_3^- \quad (1)$$

$$I_3^- + 2RSNO \longrightarrow 3I^- + RS^\bullet + 2NO^+ \quad (2)$$

$$2NO^+ + 2I^- \longrightarrow 2NO + I_2 \quad (3)$$

$$RSNO \longrightarrow RS^\bullet + NO \quad (4)$$

$$2RS^\bullet \xrightarrow{Cu^+} RSSR \quad (5)$$

We have previously extensively evaluated a method based upon removal of background nitrite by ammonium sulfamate under acidic conditions and back titration to neutral pH, followed by measurement of the increases in nitrite following addition of mercuric or cuprous ions. Although this method produced perfect standard curves in buffer, the slopes of the standard curves obtained from spiking experiments of plasma with known amounts of authentic SNO-albumin showed considerable inter-individual variations and poor reproducibility. It appeared that mercury did not stoichiometrically release NO from RSNOs when added to plasma, and this was related to the total concentration of thiols present in the plasma. This represents a general drawback of the classical Saville reaction. A more serious problem using this approach is observed in samples containing both nitrite and thiols, since thiols may compete with sulfanilamide for reaction with nitrite leading to artefactual S-nitrosation when thiol concentrations are relatively high (e.g. plasma). Although this problem can be avoided by prior alkylation of free thiol groups (as described in the present study) this potential pitfall appears not to be widely recognized.

Samouilov and Zweier[21] have tried to address the problem of contaminating nitrite in biological fluids. They also evaluated the efficacy of ammonium sulfamate for the elimination of nitrite, and observed greater efficiency of nitrite elimination than was observed in the current study. For the determination of S-nitrosothiols they compared two methods, namely the measurement of nitrite and nitrite plus S-nitrosothiols under acidic conditions, with subtraction of the level of nitrite from the total concentration. This method has the advantage of being carried out under acidic conditions in which the S-nitrosothiols are relatively stable. However, methods that rely on measuring differences of nitrite in the presence of a relatively high background level are more likely to suffer from problems of accuracy and reproducibility than those offering a direct approach, unless the concentration of S-nitrosothiols is high. Their second approach was an alkaline method based on reaction with hydroquinone/quinone. This method worked well for the release of NO from S-nitroso-glutathione or S-nitroso-penicillamine, but no data was given for S-nitrosoalbumin. This study did not report the concentrations of S-nitrosothiols in plasma.

The present study has not addressed the issue of whether the major circulating form of RSNOs are present as SNO-albumin or (an)other lower molecular weight species. However, the RSNO species formed by NO (generated from papaNONOate) was not removed by dialysis, indicating that it has a high molecular weight consistent with it being SNO-albumin. We have also determined that the RSNOs detected by this

method in human plasma are present primarily in the high molecular weight fraction, since there was no RSNO-related signal in the low molecular weight filtrate.

There have been relatively few reports on the concentration of RSNOs in human plasma. The initial report by Stamler and colleagues was based upon photolytic cleavage of the S–NO bond,[12] and levels of $\approx 7\,\mu M$ were observed with the majority being due to SNO-albumin. Photolysis has been shown to be non-specific for S-nitrosothiols, also causing NO release from compounds such as nitrite, nitrosamines, and dinitrosyliron complexes.[16] Thus, the micromolar concentrations reported by Stamler *et al.* are likely to be an overestimation of the true plasma RSNO concentration. Measurement of plasma RSNOs utilizing mercuric ion (Hg^{2+})-induced cleavage of the S-NO bond, reaction of nitrite with the Griess reagent and quantification of the formed azo dye by HPLC has reported values of 220 nM.[11] This method, however, also relies on difference measurements of nitrite cleaved from RSNOs by mercuric ion versus background nitrite which is determined in the absence of addition of mercury. For the reasons explained above, and contrary to the author's statement, this method may thus be subject to artefactual RSNO generation during sample processing.

Interestingly, from investigations on the concentration–response relationship of the synergism between NO and hydrogen peroxide in inhibiting platelet aggregation Naseem *et al.* estimated the concentration of endogenous S-nitrosothiols in human plasma to be in the range of 15–25 nM.[22] This concentration range falls well within the values we determined in blood from healthy volunteers utilizing the methodology described in this paper.

In conclusion, by combining a reagent mixture comprised of copper(I)/iodine/iodide for the specific and effective cleavage of RSNOs and a reaction chamber, which allows large volumes of plasma to be assayed, with a sensitive chemiluminescence technique to detect NO, the stoichiometric removal of endogenous nitrite by sulfanilamide, and the stabilization of RSNOs by NEM and EDTA, this method allows the reliable quantification of low nanomolar concentrations of endogenous RSNOs in complex biological matrices. This technique should be a useful tool for detailed quantitative and kinetic investigations on the formation, distribution and decomposition of S-nitrosated species in body fluids, isolated cells and tissues and help to gain a better insight into and broaden our understanding of the role of RSNOs in health and disease.

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