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Review

Measurement of circulating nitrite and *S*-nitrosothiols by reductive chemiluminescence☆

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Abstract

Considerable disparities in the reported levels of basal human nitrite and *S*-nitrosothiols (RSNO) in blood have brought methods of quantifying these nitric oxide (NO) metabolites to the forefront of NO biology. Ozone-based chemiluminescence is commonly used and is a robust method for measuring these species when combined with proper reductive chemistry. The goal of this article is to review existing methodologies for the measurement of nitrite and RSNO by reductive chemiluminescence. Specifically, we discuss in detail the measurement of nitrite and RSNO in biological matrices using tri-iodide and copper(I)/cysteine-based reduction methods coupled to chemiluminescence. The underlying reaction mechanisms, as well as the potential pitfalls of each method are discussed.

Keywords: Reviews; Nitric oxide; *S*-Nitrosohemoglobin; Tri-iodide; Copper(I)/cysteine

Contents

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1. Introduction

Nitric oxide (NO) is a reactive gaseous molecule that plays a critical role in both physiological and pathological vascular signaling. In blood vessels, the diatomic free radical is synthesized by the endothelial isoform of the enzyme nitric oxide synthase (eNOS) using L-arginine as a substrate and NADPH, tetrahydrobiopterin, and oxygen as cofactors [1–3]. Once synthesized in the endothelium, NO can diffuse into underlying smooth muscle cells where it activates soluble guanylyl cyclase, ultimately resulting in the relaxation of the smooth muscle cells and promoting vasodilation. The majority of the NO that does not diffuse abluminally reacts rapidly ($k = 2 \times 10^7\ M^{-1}\ s^{-1}$) with hemoglobin by the following equations (Eqs. (1) and (2)):

$$\text{NO} + \text{oxyhemoglobin}(Fe^{2+}) \rightarrow \text{nitrate}(NO_3^-) + \text{methemoglobin}(Fe^{3+}) \quad (1)$$

$$\text{NO} + \text{deoxyhemoglobin}(Fe^{2+}) \rightarrow \text{iron-nitrosylhemoglobin(Hb-NO)} \quad (2)$$

NO is oxidized to the inert metabolite nitrate by its reaction with oxyhemoglobin, while reaction of NO with deoxyhemoglobin results in the formation of iron-nitrosyl hemoglobin [4]. A fraction of NO produced by eNOS in the vasculature (approximately 20%) escapes inactivation by hemoglobin and is oxidized to nitrite (NO_2^-) in the plasma by a reaction catalyzed by ceruloplasmin [5], modifies proteins and lipids to form low concentrations of *N*-nitrosamines, *S*-nitrosothiols (RSNO), and nitrated lipids. The significance of these lower-yield reaction products is the subject of active investigation, as they may serve as stable storage forms of NO that may later be bioactivated to mediate endocrine NO signaling, distal from the original site of NO production.

A growing number of studies in human subjects and animal models suggest that NO may indeed mediate endocrine signaling. For example, inhaled NO, in addition to mediating pulmonary vasodilation, has been shown to limit myocardial infarction in mice [6], increase urinary blood flow in anesthetized pigs [7], decrease systemic vascular resistance in anesthetized sheep [8], and increase blood flow in the human forearm when eNOS is inhibited [9]. However, the circulating NO species responsible for this endocrine behavior remains unclear. Stamler and co-workers proposed that NO-dependent modification of the β-cysteine-93 of hemoglobin to form *S*-nitrosated hemoglobin is responsible for this activity [10]. We have recently proposed that the NO oxidation product, nitrite, mediates this effect and is a vascular store of NO that is enzymatically reduced to NO by deoxygenated hemoglobin along the physiological oxygen and pH gradient [11,12].

The development of accurate and reproducible methodologies to isolate and quantify NO species is paramount to the ongoing debate surrounding the identity of the endocrine storage form and endocrine NO signaling in vivo. In the last decade, published levels of NO species have been as variable as the different methods used to measure them [13,14], reinforcing the need for extensively validated and robust methodologies. For example, basal concentrations of *S*-nitrosohemoglobin (SNO-Hb) in blood have been reported to range from undetectable [15] to 7 μM [16], and in plasma, reported levels of nitrite have ranged from undetectable [17] to 26 μM [18]. The vast differences in reported levels may be largely attributed to variations in sample processing techniques, the different assays employed, and nitrite contamination during sample preparation. Also inherent to the problem are the debated effects of sample pretreatments used to isolate specific species and the specificity of assays for the range of NO species [19,20].

Quantification difficulties arise when measuring NO species in blood for a variety of reasons. The pervasive nature of nitrite in the laboratory, present in everything from glassware to tap water, poses countless contamination problems leading to overestimation of nitrite concentration. However, the transient nature of nitrite in blood due to its reaction with hemoglobin leads to rapid degradation and underestimated values if not experimentally addressed [14,21,22]. Further, many NO-modified proteins are present at low levels in blood, and their measurement requires the use of highly sensitive methods with corresponding low detection limits. For example, the low basal levels of SNO-Hb and SNO-albumin preclude the use of the traditional Griess–Saville assay for their measurement in plasma and blood as this assay has quantitation limits in the lower μM-range in biological matrices. Thus, the ideal assay would be a method that possesses high sensitivity and specificity coupled with sample treatment that limits the possibility for contamination and degradation in biological samples. Ozone-based chemiluminescence coupled with a variety of reductive methods satisfies these characteristics and will be the focus of the rest of this review.

2. Ozone-based chemiluminescence is a sensitive method for NO measurement

Although many methods and analytical techniques are employed to measure NO and its metabolites, in recent years gas-phase ozone-based chemiluminescence, utilizing machines such as Sievers' nitric oxide analyzer (NOA) (Model 280i) and Eco Physics' CLD 60 Series NO_x analyzers, has emerged as the most commonly used assay for NO detection due to its specificity, sensitivity, versatility and dependable operating performance. The principle of chemiluminescent NO detection is based on the rapid reaction of NO in the gas phase with ozone (O_3), which yields NO_2^* in an excited state. As the excited electron returns to its ground state, a photon is emitted and is detected as chemiluminescence ($h\nu$). (Eqs. (3) and (4)).

$$\text{NO} + O_3 \rightarrow NO_2^* + O_2 \quad (3)$$

$$NO_2^* \rightarrow NO_2 + h\nu \quad (4)$$

This emitted light is detected and amplified by a photomultiplier tube (PMT), to generate an electrical signal. The specificity of this method for NO is due to the unique properties of the NO molecule, including its ability to exist as a gas and its rapid reaction rate with ozone [23].

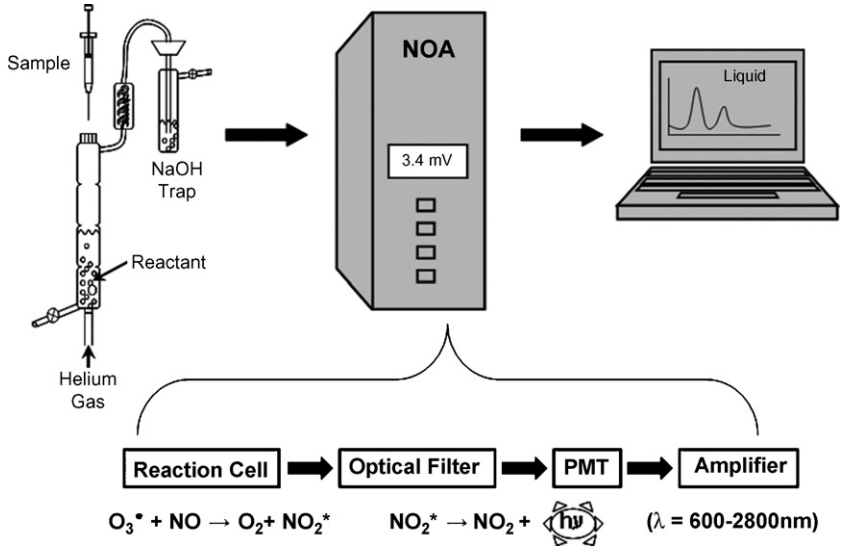


Fig. 1. Chemiluminescence mechanism and apparatus setup. Samples are injected into the glass purge vessel containing reductive solutions which is bubbled with helium gas. NO in the headspace is carried through a NaOH-trap into the NOA, which generates ozone to react with NO. The reaction of NO with ozone produces chemiluminescence which is detected by the photomultiplier tube and the signal is recorded by data recording software. NOA, nitric oxide analyzer; PMT, photomultiplier tube.

Since the chemiluminescent reaction is rapid and takes place in the gas phase, the NOA can be connected directly in-line with a glassware system in which NO gas is formed. The glass purge vessel contains a redox active chemical solution that can selectively reduce NO-modified species (i.e. *S*-nitrosothiols, iron-nitrosyls, *N*-nitrosamines) to release NO gas into solution. In order to measure the NO produced in the chemical solution, an inert gas, usually helium, is purged through the mixture to force NO out of solution and into the headspace, so that it can subsequently be carried by the helium into the reaction chamber of the NOA to be measured (Fig. 1). The versatility of chemiluminescence is due in part to the development of a number of chemical reductive assays used in conjunction with chemiluminescence to selectively measure different nitrogen species [24–28]. Using the appropriate reductive methods, chemiluminescence can be a highly specific and reproducible method of detecting not only pure NO, but also other species such as nitrite, nitrate, *S*-nitrosothiols and iron-nitrosylated proteins (Fe-NO), with detection limits recorded as low as 1 nM [23,28] in purified standards and imprecision less than 5% [27]. The following review outlines the procedural methods, underlying reaction mechanisms, and potential problems of some of the most commonly employed methodologies used in conjunction with chemiluminescence to measure nitrite and *S*-nitrosothiols in blood.

3. Measurement of nitrite by tri-iodide-based gas phase chemiluminescence

Acidic tri-iodide (I_3^-) reagent (to be prepared fresh daily: 2.0 g potassium iodide and 1.3 g iodine, 40 ml distilled H_2O and 140 ml glacial acetic acid) is perhaps the most commonly used reductant and is capable of reducing a number of NO metabolites to NO for chemiluminescent detection [26–29]. Biological samples containing even considerable amounts of protein, such as serum or plasma, can be injected directly into the purging I_3^- (7–9 ml) reagent and nitrite, *S*-nitrosothiols, *Fe*-nitrosyls and/or *N*-nitrosamines are reduced to NO gas, which is then detected by the NOA. Tri-iodide, present as I_3^- or HI_3, is also available in solution as free iodine and iodide (I^- and I_2) due to its redox activity. Free iodine then reacts with nitrite as a reducing agent, producing stoichiometric amounts of NO and iodide side product (Eqs. (5)–(7)).

$$I_3^- \rightarrow I_2 + I^- \quad (5)$$

$$KI \rightarrow K^+ + I^- \quad (6)$$

$$2NO_2^- + 2I^- + 4H^+ \rightarrow 2NO + I_2 + 2H_2O \quad (7)$$

I_3^- also readily reduces RSNO, releasing free iodine, thiyl radicals (which combine to form the disulfide, i.e. RSSR), and nitrosonium cation [27] (Eq. (8)). By reaction with free iodine, nitrosonium cation is readily converted to NO gas, resulting in quantitative yield of NO from nitrosothiols [30,31] (Eq. (9)).

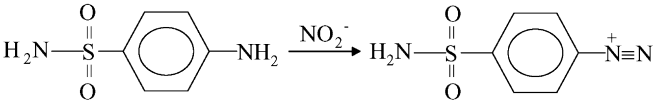
$$I_3^- + 2RS\text{-}NO \rightarrow 3I^- + RSSR + 2NO^+ \quad (8)$$

$$2NO^+ + 2I^- \rightarrow 2NO + I_2 \quad (9)$$

Detection of nitrite by this method is highly sensitive, with limits of detection less than 1 pmol and approaching limits of quantitation of 1 nM in biological matrices depending on sample injection volume [27–29]. Investigators have found NO detection from nitrite by tri-iodide chemiluminescence to be linear over a broad range of nitrite concentrations (10 nM to 1 mM) with a high level of intra-assay reproducibility (0.5%) [27]. Furthermore, measurement of nitrite by tri-iodide chemiluminescence has been validated against nitrite measurement by both flow injection analysis combined with the Griess reaction and HPLC assays ($R^2 = 0.9$) [21]. However, since tri-iodide reduces

several NO-containing species, pretreatment chemistry must be performed to ensure the signal of interest is indeed due to nitrite. One method of ensuring specificity for nitrite employs the use of acidified sulfanilamide (5% wt./vol. in 1 M HCl) to convert nitrite into a diazonium cation that is undetectable by chemiluminescence [26–28] (Eq. (10)).

$$NO_2^- + \text{sulfanilamide} \rightarrow \text{diazonium cation} \quad (10)$$



By the comparison of two injections of the same sample, one untreated and the other pretreated for 3 min (9:1, v/v) with a solution of acidified sulfanilamide (5% wt./vol. in 1 M HCl) to deplete the sample of free nitrite, it is possible to calculate the amount of nitrite in the sample. Subtraction of the signal remaining after acidified sulfanilamide treatment (which represents the signal from *S*-nitrosothiols, *Fe*-nitrosyls, and *N*-nitrosamines) from the signal of the untreated sample (which include all the aforementioned NO metabolites plus nitrite) yields the signal corresponding to the amount of nitrite in the sample [26,28] (Fig. 2).

Quantification of nitrite in a sample involves the conversion of the NOA signal into actual concentration using a standard curve. Known amounts of nitrite (0–1000 pmol) are prepared in phosphate buffered saline and 100 μl volumes are subsequently injected into the I_3^- solution for measurement. The peaks attained from these injections (Fig. 3A) are integrated and the area under each peak is plotted against the corresponding amount of nitrite in order to construct a standard curve (Fig. 3B). In this concentration range (0–10 μM), the chemiluminescent signal is linear with increasing concentration ($R^2 = 0.999$) and the slope of the line has units of area/pmol. For any given NOA machine, we have observed that this slope is relatively constant over a number of months and can be used to calculate nitrite concentrations in biological samples by the following equation

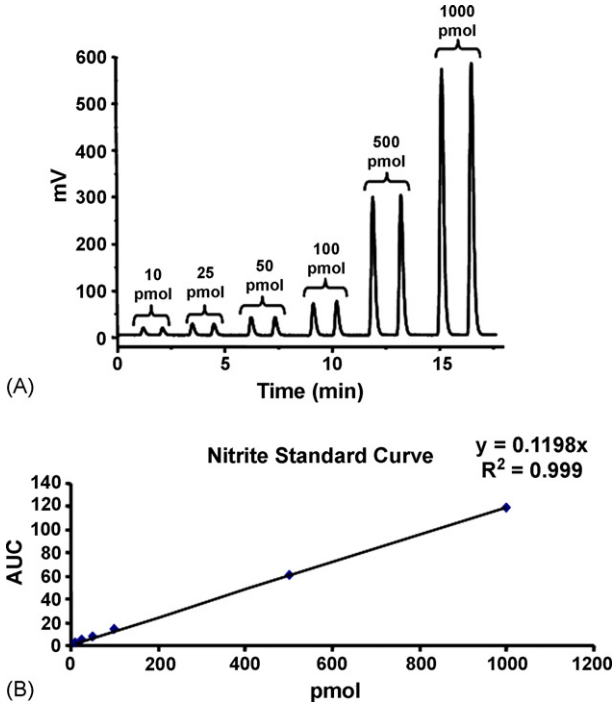


Fig. 3. Nitrite standard curve in tri-iodide method. Known amounts of nitrite (0–1000 pmol) were injected in duplicate into the I_3^- solution. Panel A is depicts the resulting peaks from these injections. Panel B is a standard curve constructed by plotting the area under each peak shown in (A) against the corresponding amount of nitrite.

(Eq. (11)):

$$[\text{Nitrite}] = \frac{(\text{Area})}{(\text{Slope}) \times (\text{Volume injected})} \quad (11)$$

The area under the curve of the sample to be analyzed is divided by the slope from the standard curve (area/pmol) and then divided by the volume of the sample injected (μl). This calculation provides the concentration of nitrite in the sample (μM) [32].

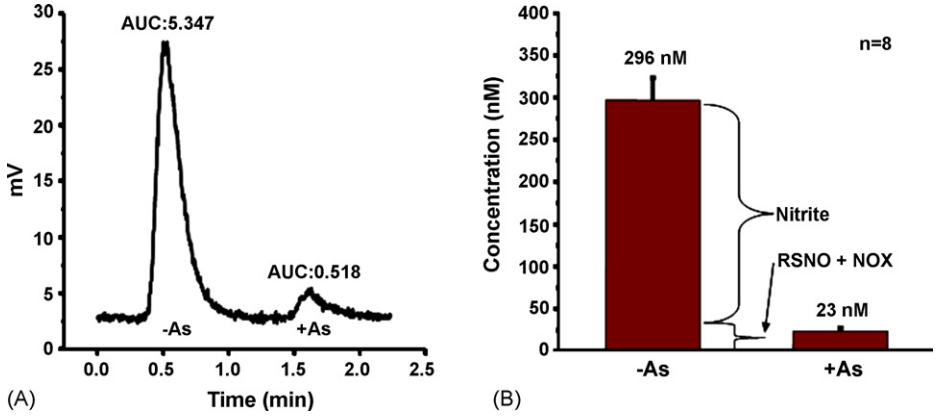


Fig. 2. Treatment with acidified sulfanilamide eliminates nitrite. Red blood cell samples were lysed 1:9 (v/v) in 0.5 mM EDTA, immediately treated either with or without acidified sulfanilamide (AS, 5 wt.%; 9:1, v/v) for 3 min, and then analyzed by tri-iodide chemiluminescence. In this experiment, 1.0 ml of untreated sample and 1.1 ml of treated sample were injected. Panel A is the raw data depicting peaks produced with and without AS treatment. Panel B is the quantification of the data shown in panel A with the concentration of nitrite equivalent to the difference of the concentration with and without AS treatment.

3.1. Measurement of plasma nitrite

While nitrite is relatively stable in physiological buffers and in plasma, nitrite has a half-life of about 11 min in blood due to its reaction with oxyhemoglobin [13,14,21]. Thus, accurate measurement of nitrite levels in blood is difficult and time-sensitive. Further complicating matters is the ubiquitous nature of nitrite and the constant threat of contamination. In order to accurately measure nitrite concentration in plasma, either the plasma must be immediately separated from the red blood cells by centrifugation or the reaction between nitrite and hemoglobin must be chemically oxidized (i.e. by ferricyanide) or blocked (i.e. by carbon monoxide). For the former determination of plasma nitrite we suggest that blood samples are drawn from a patient (or animal) and immediately centrifuged at the bedside ($750 \times g$ for 2 min at 4 °C) and frozen on dry ice. These samples can later be thawed and injected directly into tri-iodine for measurement. Although it should be recognized that the short half-life of nitrite in blood makes a certain amount of degradation of plasma nitrite during centrifugation unavoidable, rapid sample processing (less than 3 min) and centrifugation at low temperature ensures that only a limited amount (less than 10%) of nitrite is lost during this time. We have measured nitrite in vacutainers at the NIH and it is important to be aware that almost all clinical vacutainers contain contaminating nitrite. We recommend collecting blood in heparinized plastic syringes and then transferring the blood to plastic Falcon or Eppendorf tubes (containing 10 units of heparin sodium per ml of whole blood to prevent clotting) for centrifugation. This protocol limits nitrite contamination. To further safeguard against contamination, it is important to test glassware, Eppendorf tubes, and other laboratory equipment periodically by rinsing with nitrite-free, distilled, filtered water (we use the Milli-Q, Millipore system) and injecting that water into the NOA system using I_3^-. While tap water contains trace levels of nitrite (137 ± 45 nM), we have found that nitrite is undetectable in Millipore and molecular biology-grade water [32].

3.2. Measurement of whole blood nitrite

To measure whole blood nitrite, whole blood is lysed at the bedside in a chemical cocktail that oxidizes all the hemoglobin to methemoglobin, such that it will not rapidly react with nitrite. We have developed a preservation solution that oxidizes hemoglobin to stabilize nitrite in whole blood until the time of measurement. This solution consists of potassium ferricyanide ($K_3Fe(CN)_6$, 800 mM) to oxidize ferrous heme to ferric heme, Nonidet-40 substitute (NP-40) (10%, v/v) to solubilize red cell membranes, and *N*-ethylmaleimide (NEM, 100 mM) to block free thiols, all dissolved in Millipore water [21]. Since NP-40 is a detergent that suds, $K_3Fe(CN)_6$ and NEM should be dissolved in water before adding NP-40 to the solution. After drawing blood, it should be immediately mixed into this solution at a ratio of four parts blood to one part preservation solution as this ratio is sufficient to stabilize nitrite in the blood for future measurement [21]. Samples should be stored at −80 °C along with a undiluted sample of the preservation solution that will be used later to measure and correct for contaminating nitrite in the solution [21,32].

At the time of measurement, the preserved blood is thawed at room temperature, diluted with ice cold methanol (1:1, v/v), vortexed, and centrifuged at $750 \times g$ for 2 min. The centrifugation step should be repeated until the supernatant appears clear. This methanol precipitation step deproteinates the sample in order to limit foaming during injections into tri-iodide solution. Once clear of protein, the supernatant can be injected into the tri-iodide solution to determine apparent nitrite concentration ($[\text{Nitrite}]_{\text{measured}}$). In order to calculate actual nitrite concentration in the blood ($[\text{Nitrite}]_{\text{actual}}$), dilutions and contaminating nitrite levels must be taken into consideration according to the following equation (Eq. (12)):

$$[\text{Nitrite}]_{\text{actual}} = \{[\text{Nitrite}]_{\text{measured}} - ([\text{Nitrite}]_{\text{MeOH}} \times 0.5 + [\text{Nitrite}]_{\text{Pres.Soln.}} \times 0.1)\} \times 2.5 \quad (12)$$

By this equation, the concentration of nitrite contaminating the methanol ($[\text{Nitrite}]_{\text{MeOH}}$) and the preservation solution ($[\text{Nitrite}]_{\text{Pres. Soln.}}$) are corrected for by multiplying $[\text{Nitrite}]_{\text{MeOH}}$ by a dilution factor of 0.5 (to account for the 1:1 (v/v)-dilution in whole blood), and $[\text{Nitrite}_{\text{Pres. Soln.}}]$ by a dilution factor of 0.1 (because of the 1:4 (v/v)-dilution in whole blood and subsequent 1:1 (v/v)-dilution in methanol). The sum of these contaminating values is then subtracted from $[\text{Nitrite}]_{\text{measured}}$, and this corrected concentration is multiplied by a dilution factor of 2.5 (to account for the 4:1 (v/v)-dilution of blood into preservation solution and the 1:1 (v/v)-dilution into methanol) to find $[\text{Nitrite}]_{\text{actual}}$. It is important to note that the preservation and methanol stock solutions should be tested for nitrite contamination before use. While it is expected that these solutions will have low levels of nitrite present, nitrite concentrations over 75 nM each should be avoided and different stock solutions may be required [21,32]. We check the nitrite levels in our methanol and preservation solutions before each use and prepare fresh preservation solution weekly; solutions with greater than 75 nM contaminating nitrite are discarded. Common values for the terms in Eq. (12) have been previously reported (i.e. $[\text{Nitrite}]_{\text{MeOH}}$, 38 ± 11 nM; $[\text{Nitrite}]_{\text{Pres. Soln.}}$, 23 ± 20 nM; $[\text{Nitrite}]_{\text{actual}}$ 181 ± 29; $[\text{Nitrite}]_{\text{measured}}$ 202 ± 37) [32].

This method provides a measure of the total nitrite in both red blood cells and in plasma. One can measure the plasma nitrite concentration, the whole blood nitrite concentration, and the patient's hematocrit, and then calculate the red blood cell nitrite concentration using the following equation (Eq. (13)):

$$[\text{Whole blood nitrite}] = [\text{Red cell nitrite}](\text{Hematocrit}) + [\text{Plasma nitrite}](1 - \text{Hematocrit}) \quad (13)$$

Utilizing this method, we have measured the basal concentration of nitrite in whole blood to be 176 ± 17 nM [21], the plasma level to be 114 ± 11 nM [32], and the calculated red blood cell nitrite to be 288 ± 47 nM [32], with significant artery-to-vein gradients in all species (i.e. arterial whole blood nitrite levels

Table 1
Summary of published nitrite concentrations in red blood cells, plasma, serum and whole blood of healthy humans by different groups and different methodologies

Matrix	Nitrite (nM)	LOD	Methodology	Reference
RBC	263 ± 150	1 nM	I_3^- CL	[34]
	288 ± 47	1 nM	I_3^- CL	[32]
Plasma	114 ± 11	1 nM	I_3^- CL	[32]
	172 ± 35	5 nM	Copper/Iodide/Iodine CL	[48]
	200 ± 20	10 nM	FIA with Griess	[49,50]
	205 ± 21	1 nM	I_3^- CL	[34]
	200 ± 100	100 nM	HPLC with Griess	[50,51]
	221 ± 72	50 nM	Griess	[22,50]
	305 ± 23	10 nM	I_3^- CL/HPLC/FIA with Griess	[52]
	550	50 nM	HPLC	[53]
	1300–13000	1500 nM	Griess	[50,54]
	1800 ± 400	22 fmol	GC-MS	[55]
	23500–31000	50 nM	HPLC	[18]
Serum	523 ± 72	50 fmol	GC-MS	[56]
	3700 ± 600	1000 nM	Griess; Cu-Cd alloy	[50,57]
	4900 ± 1200	500 nM	Griess; VCl_3	[50,58]
	5000–20000	700 nM	Griess; enzymatic	[50,59]
Whole blood	100–400	2 nM	FIA with Griess	[50,60]
	143 ± 7 (venous)	1 nM	I_3^- CL	[21]
	176 ± 10 (arterial)	1 nM	I_3^- CL	[21]
	181 ± 29	1 nM	I_3^- CL	[32]
	240 ± 98	1 nM	I_3^- CL	[34]

RBC, red blood cells; CL, chemiluminescence; FIA, flow injection analysis.

were 176 ± 10 and venous levels were 143 ± 7 in the human forearm) [21,33{Cannon, 2001 #28}]. It should however, be noted that other groups have found different levels of plasma and whole blood nitrite using other methods (Table 1).

We were surprised that healthy human red blood cells contain significant concentrations of nitrite, approaching 300 nM. This same observation has been reported by Feelisch and co-workers [34]. Thus, nitrite is concentrated and relatively stable in red blood cells at these low concentrations [21]. Furthermore, whole blood nitrite levels have been demonstrated to increase upon NOS stimulation or inhibition and can be considered a marker of eNOS activity [21,35].

4. Measurement of *S*-nitrosothiols by reductive chemiluminescence

The measurement of *S*-nitrosothiols (RSNO) in blood, particularly *S*-nitrosohemoglobin (SNO-Hb), is the subject of considerable controversy [22,31,34,36–38]. The controversy centers on the wide variety of methodologies employed for the measurement of RSNO, and the drastically different conclusions these methods yield (Table 2).

One of the great challenges in *S*-nitrosothiol determination is distinguishing between nitrite and RSNO. The most common method uses I_3^- as a reducing agent. As mentioned above, untreated samples injected into I_3^- solution will produce a signal equivalent to the concentration of all reducible NO species present (i.e. nitrite + *S*-NO + *Fe*-nitrosyl + *N*-nitrosamine). To differentiate between nitrite and RSNO, nitrite must first be eliminated from the sample. In our method nitrite is eliminated by treatment with (9:1, v/v) 5 wt./vol.% acidified sulfanilamide. Samples should be pretreated with acidified sulfanilamide for 3 min, vortexed, and then injected into tri-iodide solution to produce a signal corresponding to the sum of the RSNO and NO_x species (i.e. *Fe*-nitrosyl, *N*-nitrosamine) remaining in the sample. A comparison of the peaks produced from samples injected with and without acidified sulfanilamide pretreatment provides an accurate and reproducible method of measuring nitrite [30,34].

Elimination of RSNO from biological samples requires pretreatment with mercuric chloride ($HgCl_2$, 5 mM final) for 2 min and subsequent treatment with acidified sulfanilamide (as described above) before injection into tri-iodide solution. Mercury ions (Hg^{2+}) convert RSNO to NO^+ (Eq. (14)).

$$Hg^{2+} + 2RSNO \rightarrow Hg(RS)_2 + 2NO^+ \quad (14)$$

However, NO^+ is quickly converted to NO_2^- and hence acidified sulfanilamide treatment is needed to eliminate nitrite. Using this type of sample treatment, calculating RSNO levels in a sample is possible by subtracting the concentration of the sample treated with acidified sulfanilamide and $HgCl_2$ from the sample treated with acidified sulfanilamide alone [26–28].

4.1. Measurement of SNO-hemoglobin

The measurement of SNO-Hb in blood is relatively more complex than the measurement of purified RSNO or RSNO in plasma. One technique for the quantification of SNO-Hb levels in vivo employs the use of Sephadex G-25 sizing columns, to remove low-molecular-mass (<5 kDa) molecules including nitrite, followed by treatment with $HgCl_2$ and 5 wt./vol.% acidified sulfanilamide [30] (Fig. 4). In this procedure, whole blood

Table 2
Summary of published *S*-nitrosothiol (RSNO) levels in red blood cells, plasma, serum and whole blood of healthy humans by various methodologies

Matrix	RSNO (nM)	LOD	Methodology	Reference
RBC	47–69	1 nM	I_3^- CL	[30,34]
	55	1 nM	I_3^- CL	[21]
	445–600	1–5 pmol	I_3^- CL (with $K_3Fe(CN)_6$)	[37]
	3060	1 nM	UV Photolysis CL	[44]
Plasma	15 ± 6	5 nM	Copper/Iodide/Iodine CL	[61]
	28 ± 7	5 nM	Copper/Iodide/Iodine CL	[26]
	30–40	3.4 pmol/g cells	HPLC-Fluorescence	[38]
	40 ± 7	5 nM	Copper/Iodide/Iodine CL	[48]
	62 ± 24	20 nM	HPLC with Griess	[62]
	156 ± 64	100 nM[a]	GC-MS	[63]
	220 ± 190	100 nM	HPLC with Griess	[64]
	250 ± 200	5 nM	I_3^- CL	[65]
	321 ± 139	20 pmol	VCl_3 CL	[24]
	5930 ± 750	100–500 nM	Saville/Griess	[66]
	7190 ± 5730	1 nM	UV Photolysis CL	[16]
Serum	35 ± 13	1 nM	I_3^- CL	[9]
	45 ± 14 (arterial)	1 nM	I_3^- CL	[33]
	63 ± 13	1 nM	I_3^- CL	[33]
	450 ± 400	20 nM	Saville/diaminonaphthalene	[67]
	930 ± 360	10 pmol	Cu(I)/Cys CL	[25]
Whole blood	N.D.	1 nM	I_3^- CL	[15]
	45–325 (venous)	2 pM	CO/Copper CL	[47]
	250–560 (arterial)	2 pM	CO/Copper CL	[47]

RBC, red blood cells; CL, chemiluminescence; N.D., not detectable.
[a] LOQ value.

is drawn fresh into a syringe from healthy volunteers and transferred to heparinized conical tubes. The blood is then centrifuged at 750 × *g* for 10 min at 4 °C and the plasma is discarded. The red blood cell pellet (1 ml) beneath the buffy coat is extracted and lysed 1:9 (v/v) in a SNO stabilization solution containing 1% Nonidet P-40 detergent, $K_3Fe(CN)_6$ (12 mM), NEM (10 mM), and DTPA (100 μM; to chelate copper and other trace metals that contaminate labware and degrade RSNO) in PBS at pH 7.2. After 5 min, the solution is passed through a pre-rinsed Sephadex G-25 column to remove nitrite, low-molecular-mass molecules and excess reagent. The major *S*-nitrosated protein at this stage of sample processing is hemoglobin. Hemoglobin concentration is then measured in Drabkin's Reagent (outlined below), and the samples are treated with and without $HgCl_2$ (5 mM final) and 5 wt./vol.% acidified sulfanilamide (9:1, v/v) as described above. NO levels in all of the samples are measured using the I_3^- chemiluminescent assay and the RSNO levels are quantified by taking the difference between NOA signals produced by $HgCl_2$-treated and $HgCl_2$-untreated samples. The flow diagram in Fig. 4 illustrates the preparation of erythrocytic samples and the quantification of SNO-Hb by this type of reductive chemiluminescence method. This technique has been validated by at least nine independent NO biochemistry laboratories [26–29,33,39–43]. The method is highly sensitive (as low as 5 nM SNO in whole blood) [30], and specific for the measurement of RSNO in biological matrices. Using this method, we have previously demonstrated quantitative recovery of SNO-Hb (200–3200 nM) spiked in fresh red blood cell lysates (before dilution in preservation solution) ($R^2 = 0.996$) [30,31].

Nevertheless, some investigators have suggested in editorial forum that the acidified sulfanilamide method of measuring nitrite has the unwanted side effect of concomitantly degrading RSNO [20,37,44]. Rogers et al. reported that acidified sulfanilamide pretreatment poses a greater risk of contamination as compared with other chemiluminescent methods, and increases susceptibility to the loss of RSNO [37]. However, considering the stability of *S*-nitrosothiols in acid and the historical use of acidified sulfanilamide in the classic Griess–Saville reaction, the claims by Rogers et al. [37] not only lack supporting data, but are also inconsistent. As shown in Table 3, the recovery of all tested *S*-nitrosothiols in tri-iodide solution is minimally affected

Table 3
S-Nitrosothiol stability in acidified sulfanilamide

S-NO species	Recovery (%)	R^2
Cys-SNO	99.5	0.9994
GSNO	96.8	0.9992
SNO-HSA	90.8	0.9976
SNO-Hb	87.6	0.9981

Purified RSNO standards (0.5–10 μM) were subjected to tri-iodide-based chemiluminescence before and after pretreatment with 5 wt./vol.% acidified sulfanilamide (9:1, v/v) for 3 min. The concentration of each RSNO after acidified sulfanilamide treatment is reported here as a percentage of the concentration measured without acidified sulfanilamide pretreatment. R^2 values are for the linearity of the recovery of each RSNO over the entire concentration range. Note that the apparent lower recovery of RSNO after treatment with acidified sulfanilamide is actually secondary to the quenching of protein-bound nitrite, which can lead to overestimates of RSNO levels for proteins not treated with acidified sulfanilamide [31].

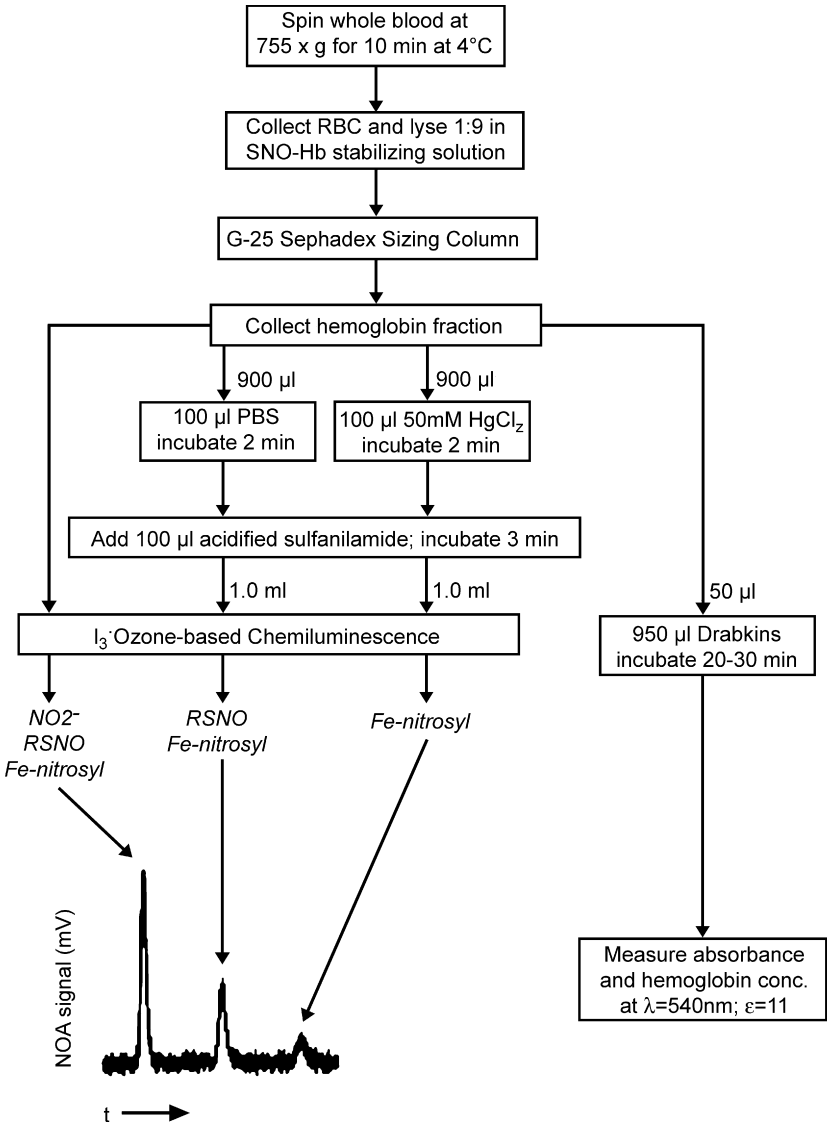


Fig. 4. Measurement of *S*-nitrosohemoglobin. The flow diagram illustrates the preparation of erythrocytic samples and the quantification of *S*-nitrosohemoglobin. Below the diagram is representative raw data from fresh human red cell preparations. Diagrammed in concert is the sample preparation and measurement of heme concentration using the Drabkin's Reagent.

by pretreatment with acidified sulfanilamide. In this experiment, several concentrations (0.5–10 μM) of the low-molecular-mass RSNO *S*-nitrosocysteine (Cys-SNO) and *S*-nitrosoglutathione (GSNO) and two purified *S*-nitrosated proteins, i.e. *S*-nitrosoalbumin (HSA-SNO) and SNO-Hb, were made (see ref. [30] for *S*-nitrosation method) and then subjected to tri-iodide-based chemiluminescence before and after pretreatment with 5 wt./vol.% acidified sulfanilamide (9:1, v/v). We found quantitative recovery of each RSNO tested over this concentration range and this was unchanged by pretreatment with acidified sulfanilamide (Table 3). It is important to note that a small concentration of nitrite associates with high molecular weight protein RSNOs, thus the signal from these RSNOs is slightly decreased after acidified sulfanilamide treatment. Indeed, the yield of NO from HMW protein RSNOs after treatment with acidified sulfanilamide is identical to the yield of RSNO with direct injection into solutions of Cu/cysteine, which will only release NO from RSNOs and not reduce nitrite. These data and the extensive validation of these assays using standards added to relevant biological matrices confirms that the use of acidified sulfanilamide does not degrade *S*-nitrosothiols and is required for accurate measurement of RSNO concentration [30,31].

4.2. Measurement of heme concentration

Drabkin's solution:

1. Pipette 50 μl hemoglobin solution into 950 μl Drabkin's solution {200 mg $K_3Fe(CN)_6$ (FW 329.26), 50 mg KCN (FW 65.12, Aldrich Chemicals), 140 mg KH_2PO_4 (FW 136.09), 0.5 ml NP-40, 1000 ml distilled or deionized water, adjusted to pH 7.4 in 1 ml cuvette}.

2. In separate cuvette, place 50 μl PBS in 950 μl Drabkin's solution.
3. Mix both cuvettes thoroughly and incubate in the dark at room temperature for 20–30 min.
4. Blank the spectrophotometer with the Drabkin's solution/PBS cuvette.
5. Measure the absorbance of cyanomethemoglobin at a fixed wavelength of 540 nm.
6. Calculate the hemoglobin concentration of the original hemoglobin solution using the following equation (Eq. (15)):

$$[\text{Heme}](\text{mM}) = \frac{(\text{Absorbance measured at 540 nm})(\text{dilution} = 20)}{(\text{Extinction Coefficient at 540 nm} = 11\ \text{mM}^{-1}\,\text{cm}^{-1})} \quad (15)$$

7. The percent SNO per heme can be calculated using Eq. (16):

$$\%\text{SNO per heme} = \frac{[\text{NO}]\ \text{using}\ I_3^-\ \text{method (mM)}}{[\text{Heme}](\text{mM})} \times 100\%. \quad (16)$$

4.3. Autocapture of NO by hemoglobin in tri-iodide

One criticism of methods utilized to measure RSNO in the presence of hemoglobin is the potential for deoxygenated hemoglobin to autocapture NO inside the reaction purge vessel, which potentially underestimates the concentration of RSNO present. In a recent publication, Rogers et al. developed a tri-iodide-based chemiluminescent method designed to eliminate autocapture [37]. In this method potassium ferricyanide ($K_3Fe(CN)_6$, 25 mM final) is added to the tri-iodide solution to limit NO-heme interaction through two mechanisms: (1) conversion of ferrous hemoglobin to its ferric form, which is a considerably less potent NO scavenger, and (2) release of NO bound to heme. Using this method, Rogers et al. reported levels of total Hb-bound NO to be 445–600 nM in whole blood [37], significantly higher than the concentrations reported by other groups (15–69 nM) [21,34]. However, this technique measures all NO species in blood, including nitrite, *S*-nitrosothiols, *Fe*-nitrosyls and *N*-nitrosamines. The levels Rogers et al. measured are actually consistent with the sum of these species measured by other groups [21,31,42], suggesting that much of what they measure as RSNO is in fact nitrite. Rogers et al. propose that this higher-level measure is due to elimination of autocapture of NO by heme in the purge vessel. To test whether autocapture was indeed a concern in the tri-iodide method, red blood cells were drawn fresh and lysed in 0.5 mM EDTA (1:9, v/v) then directly injected into tri-iodide solution with and without $K_3Fe(CN)_6$ (25 mM) [31]. Although the shape of the resulting peaks differed, with sharper and narrower peaks being produced in the presence of $K_3Fe(CN)_6$ (Fig. 5A), there was no significant difference in the area of the peaks. The concentration of total red blood cell NO species was 312 ± 17 nM with $K_3Fe(CN)_6$ treatment and 269 ± 15 nM with direct injection, suggesting that the effect of NO autocapture is minimal in this system (Fig. 5B). We recommend a similar assay which involves pretreatment of the sample with $K_3Fe(CN)_6$ [30], effectively also eliminating putative problems with autocapture and preventing the 10% loss of NO signal [31]. One can also modify the Rogers' assay to include the treatment of the sample with 5 wt./vol.% acidified sulfanilamide (9:1, v/v) to remove nitrite prior to injection of red blood cells into tri-iodide, thus providing an accurate measure of RSNO rather than the sum of all NO species.

Although Rogers et al. proposed that their higher red cell NO (Hb-NO + RSNO) concentration was due to the elimination of NO autocapture by heme, the actual factor responsible for these higher levels is red blood cell nitrite. Indeed when Rogers et al. experiment was repeated in our laboratory to include passage through a Sephadex G-25 column to remove nitrite, the level of red blood cell bound NO (*S*-nitrosothiol and *Fe*-nitrosyl) was determined to be 50 ± 5 nM [31] which is consistent with previous measurements using the $HgCl_2$ and acidified sulfanilamide pretreatment method in tri-iodide solution [30,34]. As illustrated in Fig. 5C and D, employment of our modification of the Rogers et al. assay before and after nitrite removal by Sephadex G-25 size exclusion chromatography, reveals 50% lower signals after column treatment [31]. This result suggests a substantial contribution of red blood cell nitrite to the signal observed by Rogers et al. [37]. Treatment with $HgCl_2$ followed by gel chromatography further degrades the signal by about 50%. Analysis of the area under the curve of the signals, with levels corrected for passage through the G-25 columns using measured heme concentrations, reveals a mean total NO value of 332 nM in the red blood cell, although only 121 nM of that signal is mercury-unstable (consistent with SNO-Hb). Fig. 5E depicts the levels of arterial SNO-Hb as measured by four different methods: (1) treating red cells lysed (1:9, v/v) in 0.5 mM EDTA with acidified sulfanilamide to remove nitrite, (2) the Rogers et al. method [37], (3) the levels published by Gladwin et al. [30], and (4) the levels published by McMahon et al. [44] (Fig. 5E). SNO-Hb levels were nearly identical using the first three reduction methods (i.e. 39–50 nM) [30], but inconsistent with the vastly higher level recorded using UV photolysis (i.e. 3060 nM) [44]. Fig. 5F displays the concentration of SNO-Hb in fresh arterial and venous blood samples using our modification of the Rogers et al. assay. Consistent with our earlier findings [28,30], no such artery-to-vein gradient was observed, with recorded mean values of 50.5 nM in the artery and 50.6 nM in the vein [31].

In contrast to the tri-iodide-based reduction, other groups utilize photolysis to measure SNO-Hb [20,44]. In this method, photolytic cleavage breaks the S-NO bond and the NO produced is detected by chemiluminescence with an NO-analyzer. Using this method, Stamler and co-workers have reported concentrations of human SNO-Hb significantly higher (i.e. 3 μM) [44] in red blood cells than those reported by groups using the I_3^- method (i.e. 50 nM) [30,34]. *S*-Nitrosothiol measurement by photolysis is thought to overestimate levels for a number of reasons. The primary concern is the assay's lack of specificity for *S*-nitrosothiols. Samouilov and Zweier proposed that the harsh photolytic exposure and secondary heating decom-

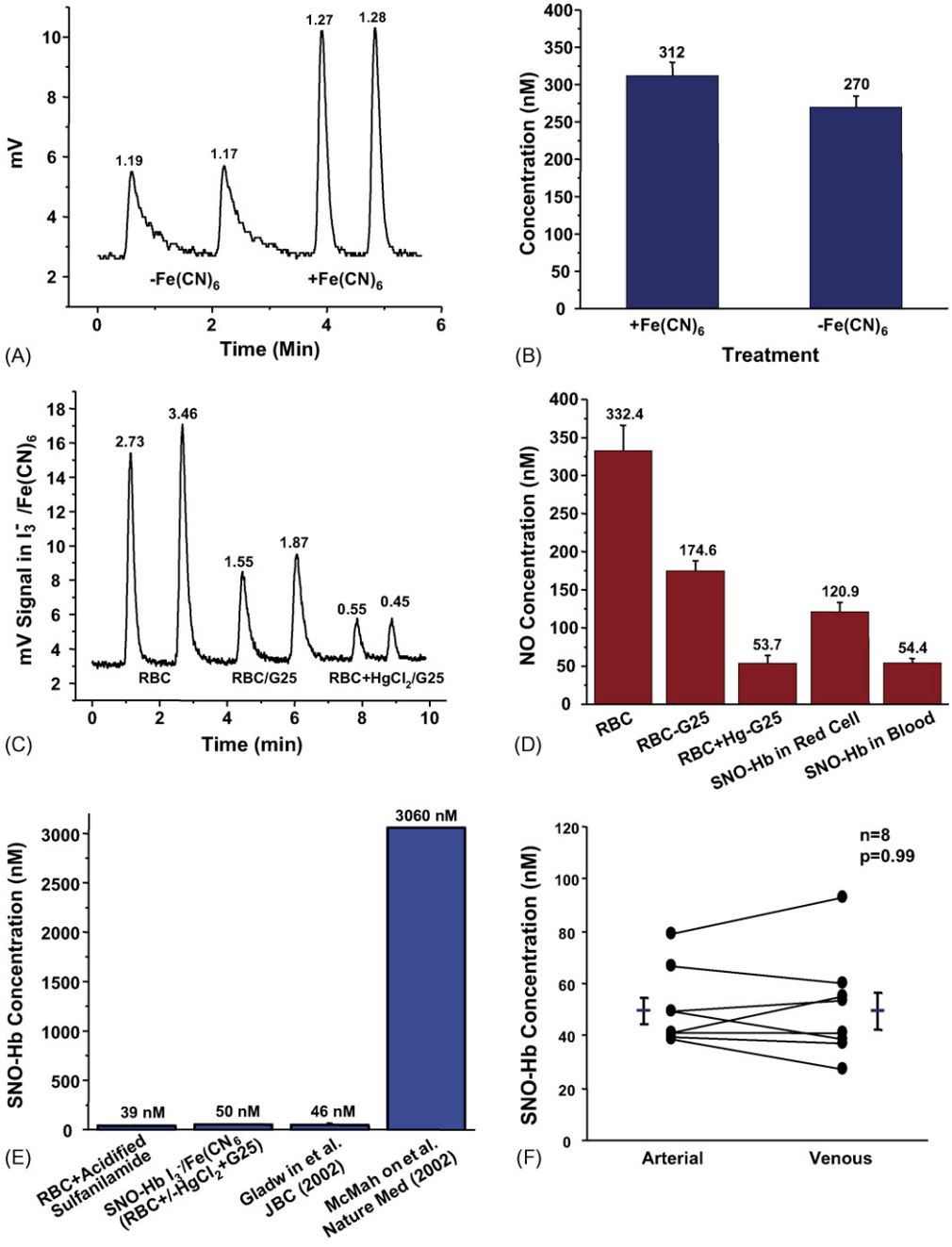


Fig. 5. Critical need for nitrite removal from red blood cells and consensus values for red blood cells SNO. (A and B) Lysed RBCs (1:9, v/v, in 0.5 mM EDTA) were injected into the tri-iodide assay. Panel A shows the duplicate signals of this sample when $K_3Fe(CN)_6$ (25 mM) was included or excluded from the tri-iodide solution. Panel B is the quantification of the data shown in (A). (C and D) Red blood cells were injected into tri-iodide untreated (RBCs), after passage through a G-25 column (RBCs-G25), or after treatment with $HgCl_2$ treatment followed by passage through a G-25 column (RBCs + Hg-G25). Panel (C) depicts the signals generated by the NOA when these samples were injected into tri-iodide, and panel (D) shows the NO concentration measured. (E) Levels of arterial SNO-Hb as measured by four independent methods: (1) immediately treating freshly drawn red cell pellet lysed 1:9 (v/v) in 0.5 mM EDTA with and without $HgCl_2$, and then with 5 wt.% acidified sulfanilamide (9:1, v/v), (2) the modified Rogers et al. assay (method described in text), (3) the levels published by Gladwin et al. [30] using SNO-stabilization solution, and (4) the levels published by McMahon et al. [44] using UV photolysis. (F) Artery-to-vein SNO-Hb gradient in eight normal volunteers as measured using the modified Rogers et al. method. Figures C–F were reproduced with permission from [31].

pose nitrite, making it difficult to distinguish nitrite signals from those of *S*-nitrosothiol compounds, suggesting an explanation for overestimated *S*-nitrosothiol yields by groups using the UV photolysis methodology [10,27,44,45]. The ability of the photolytic cleavage assay to release NO from other NO-species further demonstrates its lack of specificity. In fact, even nitrate may be reduced to NO in the presence of low mM-concentrations of reduced thiols following high energy UV photolysis, misrepresenting nitrate as *S*-nitrosothiol [46]. In this case, $HgCl_2$ treatment binds the thiols and blocks this chemistry, incorrectly suggesting that the NO species is a mercury-unstable (degrades in presence of $HgCl_2$) *S*-nitrosothiol [46].

5. Copper(I)/cysteine chemiluminescent assay–measurement of *S*-nitrosothiols

An alternative to reduction by tri-iodide is the reduction of RSNO by a solution of copper(I) and cysteine (Cu(I)/Cys) which can be coupled to chemiluminescent detection of NO. This particular assay exploits the liberation of NO from RSNO in the presence of Cu^+. At a neutral pH, the Cu(I)/Cys solution has the ability to reduce the *S*-nitroso group, but lacks the reducing power to reduce nitrite or nitrate to NO, providing assay specificity [25].

The Cu(I)/Cys solution (1 mM L-cysteine; 100 μM cuprous chloride in 400 ml distilled water) should be prepared on the day of use and is adjusted to pH 6.5 by the addition of sodium hydroxide. The solution (7 ml) is placed in a water-jacketed purge vessel that is maintained at 50 °C using a circulating water bath. The top of the purge vessel (above the level of the Cu(I)/Cys solution) should be cooled to 4 °C to limit the migration of water vapor, and this whole system is connected in line with the chemiluminescence detector. When injecting samples containing hemoglobin, it is important to pretreat the samples with an excess molar ratio of $K_3Fe(CN)_6$ (6 M $K_3Fe(CN)_6$–1 M heme) to oxidize ferrous heme to its ferric state in order to prevent autocapture. This is followed by passage of the sample through a pre-washed Sephadex G25 column to eliminate excess $K_3Fe(CN)_6$ [31]. The Cu(I)/Cys solution should be replaced following each injection to prevent both the exhaustion of the reducing agent and excessive foaming [37]. The addition of antifoam agents (0.1 vol.% Antifoam B Emulsion) to the purge vessel provides slight relief with smaller injection sizes.

The Cu(I)/Cys detection method is linear ($R^2 = 1.0$) over a range of 10 nM to 1 mM and can accurately detect amounts as low as 10 pmoles of RSNO (GSNO, Cys-SNO, HSA-SNO). Pretreatment of these samples with $HgCl_2$ eliminates the signal consistent with the detection of RSNO, and the method does not detect nitrite, nitrate, or 3-nitrotyrosine [25]. Furthermore, Huang et al. demonstrated that the assay produces similar yields to the tri-iodide-based chemiluminescence when measuring SNO-Hb standard samples [41]. Preparation of purified RSNO standards has been described previously [30,31], and several RSNOs, such as GSNO, can be purchased commercially (e.g. from Sigma). The concentration of each RSNO standard can be determined spectrophotometrically using the proper molar absorptivity at the appropriate wavelength (e.g. 800 M^{-1} cm^{-1} for GSNO at 334 nm, 855 M^{-1} cm^{-1} at 338 nm for Cys-SNO).

However, the Cu(I)/Cys technique is not without its limitations. Although the assay provides a competent measure of most purified RSNOs with reportedly quantitative recovery for GSNO [25], Cys-SNO, HSA-SNO, and SNO-Hb [31], a host of problems arise when using it to detect RSNO in human red blood cell sample. As with any chemiluminescence-based method used to detect SNO-Hb, there is considerable foaming to negotiate even with the use of commercially available antifoaming agents. Also, because of the relatively small amount of RSNO in the red blood cells, large injection volumes (0.5–1.0 ml) are required to achieve a measurable chemiluminescent signal. However, with the Cu(I)/Cys method in particular, the signal peaks are very wide, owing to the relatively low-reducing power of the solution, and because the Cu(I)/Cys reagent does not oxidize the heme, there is a major problem of heme capture of NO to form *Fe*-nitrosyls [31]. These effects greatly reduce sensitivity and can be overcome by pretreatment with $K_3Fe(CN)_6$ to block the heme [31] or by reaction in-line with purging CO gas to block the heme [47]. The latter method presents new challenges such as CO scavenging (a CO detector should be used as this gas is colorless and odorless) and an exothermic reaction between CO and the commercially available scrubber on the NOA ozone generator (this reaction will melt the scrubber and represents a burn/fire risk so great care is required [by personal communication from D. Kim-Shapiro, Wake Forest University]). It should also be pointed out that this CO-based Cu(I)/Cys assay releases NO from *Fe*-nitrosyls, likely by displacement by the purging CO gas, and thus requires treatment with and without $HgCl_2$ in order to distinguish RSNOs [47]. Doctor et al. have found their CO modification of the Cu(I)/Cys assay to be specific for RSNO and sensitive to 2–5 pmol. When measuring SNO-Hb the assay was linear with a coefficient of variation of 5.5% for sequential 20 nM SNO-Hb injections [47].

Table 4
S-Nitrosothiol detection by the tri-iodide (I_3^-) vs. the Cu(I)/Cys method

S-NO species	I_3^-	Cu(I)/Cys
Cys-SNO (5 μM)	11.3 ± 0.5	11.4 ± 0.3
GSNO (10 μM)	23.6 ± 1.3	20.2 ± 0.9
SNO-HSA (1.5 μM)	3.3 ± 0.1	3.4 ± 0.03
SNO-Hb (15 μM)	30.4 ± 2.1	31.43 ± 2.0

Comparison of NO yields (area under the curve) from the reduction of purified Cys-SNO, GSNO, SNO-albumin, and SNO-Hb standards using two ozone-based chemiluminescent methods: (1) treatment with acidified sulfanilamide and injection into the tri-iodide solution, and (2) injection into the Cu(I)/Cys solution (including pretreatment with an excess molar ratio of $K_3Fe(CN)_6$ followed by passage through a Sephadex G25 column in the case of SNO-Hb). RSNO concentrations, standardized by spectrophotometry, were in the (0.5–10 μM) range.

Purified *S*-nitrosothiols (prepared as previously outlined in [30]) such as HSA-SNO and GSNO can be readily measured using the Cu(I)/Cys assay and yield concentrations consistent with those attained using the tri-iodide method (Table 4). The Cu(I)/Cys assay can also produce comparable SNO-Hb measurements when appropriate pretreatment techniques (i.e. $K_3Fe(CN)_6$) are applied to limit NO-heme interaction or CO gas is employed [41,47]. When NO is released from the cleavage of SNO-Hb, it immediately binds to ferrous heme groups in the Cu(I)/Cys assay, resulting in underestimated yields [31]. However, pretreatment with $K_3Fe(CN)_6$ prevents both NO autocapture and SNO-Hb underestimation by converting heme to methemoglobin, which does not react with NO. It should be noted that this assay is still less sensitive than the tri-iodide assay, unless performed with CO gas as described by Doctor et al. [47].

6. Pitfalls and shortcomings

6.1. Nitrite contamination

Considering the frequent need to measure NO in the nanomolar range when dealing with biological samples, the 137 ± 45 nM nitrite contamination [32] found in tap water is highly problematic. Further, because of the ubiquitous nature of nitrite, the use of nitrite-free water is imperative not only when diluting standards and samples, but also when rinsing glassware and syringes. We recommend using distilled and filtered Millipore water or glass bottled Molecular Biology Grade Water (BioFluids, Biosource International) as both contain undetectable levels of nitrite contamination [32]. The importance of using fresh filtered or glass-bottled water is underscored by the fact that NO from the air can react with oxygen in water to form nitrite. The use of nitrite-free water in solutions such as the preservation solution and to dissolve EDTA is also important in order to eliminate nitrite contamination in these solutions.

6.2. NOA peak integration

In our experience, the Sievers NOA packaged software has difficulty integrating low-mV peaks (0.3–5 pmol NO) and produces some error when measuring the area under the curve [28]. To correct this problem, we export raw data from the Sievers program into a second program, Origin 7.0 (Microcal Software, Inc., Northampton, MA). Using Origin, peaks can be measured with greater precision and increased accuracy.

6.3. Foaming

As mentioned above, one of the difficulties encountered with the Cu(I)/Cys assay is foaming following injection of biological samples, and particularly protein-containing red blood cells. This underscores one of the significant advantages of the tri-iodide method—the relatively minimal foaming associated with the assay. If foaming is encountered, and causes overflow into the NaOH-trap, a silica-based antifoam agent such as Sigma's Antifoam B Emulsion may be used in limited quantities (0.1 vol.%).

6.4. Machine variability

We have also observed that the sensitivity of NOA instruments can vary significantly between machines [28]. When operating with multiple Sievers brand NOA, we recommend testing a set of nitrite standards on each machine to determine which has the greatest sensitivity. Machines with greater sensitivity should be used when measuring samples of low concentration. If attempting to measure low nM-concentrations of NO-modified proteins (i.e. RSNOs, *N*-nitrosoamines), there may be an advantage to using the Eco Physics device, which has greater sensitivity than the Sievers system.

7. Concluding remarks

Reductive chemiluminescence provides a sensitive and specific method to measure NO-species, such as nitrite and SNO-Hb, if performed with the proper methodology. Reductive chemiluminescence is a central tool necessary for an expanded understanding the relative roles of nitrite and *S*-nitrosothiols as potential storage sites of NO in biological systems. While consensus on the levels and roles of NO metabolites in human physiology and pathophysiology and the operational techniques used to measure them has yet to be reached, we hope that the careful exposition of the many methodologies employed will help advance this field.

References

[1] R.F. Furchgott, J.V. Zawadzki, Nature 288 (1980) 373.
[2] L.J. Ignarro, G.M. Buga, R.E. Byrns, K.S. Wood, G. Chaudhuri, J. Pharmacol. Exp. Ther. 246 (1988) 218.
[3] R.M. Palmer, A.G. Ferrige, S. Moncada, Nature 327 (1987) 524.
[4] X. Liu, M.J. Miller, M.S. Joshi, H. Sadowska-Krowicka, D.A. Clark, J.R. Lancaster Jr., J. Biol. Chem. 273 (1998) 18709.
[5] S. Shiva, X. Wang, L.A. Ringwood, X. Xu, S. Yuditskaya, V. Annavajjhala, H. Miyajima, N. Hogg, Z.L. Harris, M.T. Gladwin, Nat. Chem. Biol. 2 (2006) 486.
[6] R. Hataishi, A.C. Rodrigues, T.G. Neilan, J.G. Morgan, E. Buys, S. Sruti, R. Tambouret, D.S. Jassal, M.J. Raher, E. Furutani, F. Ichinose, M.T. Gladwin, A. Rosenzweig, W.M. Zapol, M.H. Picard, K.D. Bloch, M. Scherrer-Crosbie, Am. J. Physiol. Heart Circ. Physiol. 291 (2006) H379.
[7] E. Troncy, M. Francoeur, I. Salazkin, F. Yang, M. Charbonneau, G. Leclerc, P. Vinay, G. Blaise, Br. J. Anaesth. 79 (1997) 631.
[8] Y. Takahashi, H. Kobayashi, N. Tanaka, T. Sato, N. Takizawa, T. Tomita, Am. J. Physiol. 274 (1998) H349.
[9] R.O. Cannon 3rd, A.N. Schechter, J.A. Panza, F.P. Ognibene, M.E. Pease-Fye, M.A. Waclawiw, J.H. Shelhamer, M.T. Gladwin, J. Clin. Invest. 108 (2001) 279.
[10] L. Jia, C. Bonaventura, J. Bonaventura, J.S. Stamler, Nature 380 (1996) 221.
[11] K. Cosby, K.S. Partovi, J.H. Crawford, R.P. Patel, C.D. Reiter, S. Martyr, B.K. Yang, M.A. Waclawiw, G. Zalos, X. Xu, K.T. Huang, H. Shields, D.B. Kim-Shapiro, A.N. Schechter, R.O. Cannon 3rd, M.T. Gladwin, Nat. Med. 9 (2003) 1498.
[12] M.T. Gladwin, A.N. Schechter, D.B. Kim-Shapiro, R.P. Patel, N. Hogg, S. Shiva, R.O. Cannon 3rd, M. Kelm, D.A. Wink, M.G. Espey, E.H. Oldfield, R.M. Pluta, B.A. Freeman, J.R. Lancaster Jr., M. Feelisch, J.O. Lundberg, Nat. Chem. Biol. 1 (2005) 308.
[13] D. Giustarini, A. Milzani, R. Colombo, I. Dalle-Donne, R. Rossi, Trends Pharmacol. Sci. 25 (2004) 311.
[14] D. Tsikas, Free Radic. Res. 39 (2005) 797.
[15] T. Rassaf, N.S. Bryan, R.E. Maloney, V. Specian, M. Kelm, B. Kalyanaraman, J. Rodriguez, M. Feelisch, Nat. Med. 9 (2003) 481.
[16] J.S. Stamler, O. Jaraki, J. Osborne, D.I. Simon, J. Keaney, J. Vita, D. Singel, C.R. Valeri, J. Loscalzo, Proc. Natl. Acad. Sci. U.S.A. 89 (1992) 7674.
[17] A. Meulemans, F. Delsenne, J. Chromatogr. B 660 (1994) 401.
[18] M. Gorenflo, C. Zheng, A. Poge, M. Bettendorf, E. Werle, W. Fiehn, H.E. Ulmer, Clin. Lab. 47 (2001) 441.
[19] D.B. Kim-Shapiro, R.P. Patel, A.N. Schechter, M.T. Gladwin, R.O. Cannon 3rd, N. Hogg, Circ. Res. 95 (2004) e10.
[20] J.S. Stamler, Circ. Res. 94 (2004) 414.
[21] A. Dejam, C.J. Hunter, M.M. Pelletier, L.L. Hsu, R.F. Machado, S. Shiva, G.G. Power, M. Kelm, M.T. Gladwin, A.N. Schechter, Blood 106 (2005) 734.
[22] D. Giustarini, I. Dalle-Donne, R. Colombo, A. Milzani, R. Rossi, Free Radic. Res. 38 (2004) 1235.
[23] S. Archer, FASEB J. 7 (1993) 349.

[24] J.F. Ewing, D.R. Janero, Free Radic. Biol. Med. 25 (1998) 621.
[25] K. Fang, N.V. Ragsdale, R.M. Carey, T. MacDonald, B. Gaston, Biochem. Biophys. Res. Commun. 252 (1998) 535.
[26] R. Marley, M. Feelisch, S. Holt, K. Moore, Free Radic. Res. 32 (2000) 1.
[27] A. Samouilov, J.L. Zweier, Anal. Biochem. 258 (1998) 322.
[28] B.K. Yang, E.X. Vivas, C.D. Reiter, M.T. Gladwin, Free Radic. Res. 37 (2003) 1.
[29] M. Feelisch, T. Rassaf, S. Mnaimneh, N. Singh, N.S. Bryan, D. Jourd'Heuil, M. Kelm, FASEB J. 16 (2002) 1775.
[30] M.T. Gladwin, X. Wang, C.D. Reiter, B.K. Yang, E.X. Vivas, C. Bonaventura, A.N. Schechter, J. Biol. Chem. 277 (2002) 27818.
[31] X. Wang, N.S. Bryan, P.H. MacArthur, J. Rodriguez, M.T. Gladwin, M. Feelisch, J. Biol. Chem. 281 (2006) 26994.
[32] M.M. Pelletier, P. Kleinbongard, L. Ringwood, R. Hito, C.J. Hunter, A.N. Schechter, M.T. Gladwin, A. Dejam, Free Radic. Biol. Med. 41 (2006) 541.
[33] M.T. Gladwin, J.H. Shelhamer, A.N. Schechter, M.E. Pease-Fye, M.A. Waclawiw, J.A. Panza, F.P. Ognibene, R.O. Cannon 3rd, Proc. Natl. Acad. Sci. U.S.A. 97 (2000) 11482.
[34] N.S. Bryan, T. Rassaf, J. Rodriguez, M. Feelisch, Nitric Oxide 10 (2004) 221.
[35] T. Lauer, M. Preik, T. Rassaf, B.E. Strauer, A. Deussen, M. Feelisch, M. Kelm, Proc. Natl. Acad. Sci. U.S.A. 98 (2001) 12814.
[36] M.T. Gladwin, J.R. Lancaster Jr., B.A. Freeman, A.N. Schechter, Nat. Med. 9 (2003) 496.
[37] S.C. Rogers, A. Khalatbari, P.W. Gapper, M.P. Frenneaux, P.E. James, J. Biol. Chem. 280 (2005) 26720.
[38] R. Rossi, D. Giustarini, A. Milzani, R. Colombo, I. Dalle-Donne, P. Di Simplicio, Circ. Res. 89 (2001) E47.
[39] H.H. Bjorne, J. Petersson, M. Phillipson, E. Weitzberg, L. Holm, J.O. Lundberg, J. Clin. Invest. 113 (2004) 106.
[40] J.H. Crawford, B.K. Chacko, H.M. Pruitt, B. Piknova, N. Hogg, R.P. Patel, Blood 104 (2004) 1375.
[41] K.T. Huang, I. Azarov, S. Basu, J. Huang, D.B. Kim-Shapiro, Blood 107 (2006) 2602.
[42] T. Rassaf, N.S. Bryan, M. Kelm, M. Feelisch, Free Radic. Biol. Med. 33 (2002) 1590.
[43] X. Xu, M. Cho, N.Y. Spencer, N. Patel, Z. Huang, H. Shields, S.B. King, M.T. Gladwin, N. Hogg, D.B. Kim-Shapiro, Proc. Natl. Acad. Sci. U.S.A. 100 (2003) 11303.
[44] T.J. McMahon, R.E. Moon, B.P. Luschinger, M.S. Carraway, A.E. Stone, B.W. Stolp, A.J. Gow, J.R. Pawloski, P. Watke, D.J. Singel, C.A. Piantadosi, J.S. Stamler, Nat. Med. 8 (2002) 711.
[45] J.D. MacMicking, R.J. North, R. LaCourse, J.S. Mudgett, S.K. Shah, C.F. Nathan, Proc. Natl. Acad. Sci. U.S.A. 94 (1997) 5243.
[46] A. Dejam, P. Kleinbongard, T. Rassaf, S. Hamada, P. Gharini, J. Rodriguez, M. Feelisch, M. Kelm, Free Radic. Biol. Med. 35 (2003) 1551.
[47] A. Doctor, R. Platt, M.L. Sheram, A. Eischeid, T. McMahon, T. Maxey, J. Doherty, M. Axelrod, J. Kline, M. Gurka, A. Gow, B. Gaston, Proc. Natl. Acad. Sci. U.S.A. 102 (2005) 5709.
[48] T. Rassaf, M. Preik, P. Kleinbongard, T. Lauer, C. Heiss, B.E. Strauer, M. Feelisch, M. Kelm, J. Clin. Invest. 109 (2002) 1241.
[49] P. Kleinbongard, T. Rassaf, A. Dejam, S. Kerber, M. Kelm, Methods Enzymol. 359 (2002) 158.
[50] D. Tsikas, J. Chromatogr. B 851 (2007) 51.
[51] T. Ishibashi, T. Matsubara, T. Ida, T. Hori, M. Yamazoe, Y. Aizawa, J. Yoshida, M. Nishio, Life Sci. 66 (2000) 173.
[52] P. Kleinbongard, A. Dejam, T. Lauer, T. Rassaf, A. Schindler, O. Picker, T. Scheeren, A. Godecke, J. Schrader, R. Schulz, G. Heusch, G.A. Schaub, N.S. Bryan, M. Feelisch, M. Kelm, Free Radic. Biol. Med. 35 (2003) 790.
[53] D. Tsikas, S. Rossa, J. Sandmann, J.C. Frolich, J. Chromatogr. B 724 (1999) 199.
[54] H. Moshage, B. Kok, J.R. Huizenga, P.L. Jansen, Clin. Chem. 41 (1995) 892.
[55] D. Tsikas, R.H. Boger, S.M. Bode-Boger, F.M. Gutzki, J.C. Frolich, J. Chromatogr. B 661 (1994) 185.
[56] R. Keimer, F.K. Stutzer, D. Tsikas, R. Troost, F.M. Gutzki, J.C. Frolich, J. Cardiovasc. Pharmacol. 41 (2003) 284.
[57] K.V. Sastry, R.P. Moudgal, J. Mohan, J.S. Tyagi, G.S. Rao, Anal. Biochem. 306 (2002) 79.
[58] K.M. Miranda, M.G. Espey, D.A. Wink, Nitric Oxide 5 (2001) 62.
[59] P.C. Pinto, J.L. Lima, M.L. de Sousa Saraiva, Clin. Chim. Acta 337 (2003) 69.
[60] K. Schulz, S. Kerber, M. Kelm, Nitric Oxide 3 (1999) 225.
[61] T. Rassaf, P. Kleinbongard, M. Preik, A. Dejam, P. Gharini, T. Lauer, J. Erckenbrecht, A. Duschin, R. Schulz, G. Heusch, M. Feelisch, M. Kelm, Circ. Res. 91 (2002) 470.
[62] D. Jourd'heuil, K. Hallen, M. Feelisch, M.B. Grisham, Free Radic. Biol. Med. 28 (2000) 409.
[63] D. Tsikas, J. Sandmann, J.C. Frolich, J. Chromatogr. B 772 (2002) 335.
[64] R.K. Goldman, A.A. Vlessis, D.D. Trunkey, Anal. Biochem. 259 (1998) 98.
[65] P. Moriel, I.R. Pereira, M.C. Bertolami, D.S. Abdalla, Free Radic. Biol. Med. 30 (2001) 318.
[66] A.B. Milsom, C.J. Jones, J. Goodfellow, M.P. Frenneaux, J.R. Peters, P.E. James, Diabetologia 45 (2002) 1515.
[67] M. Marzinzig, A.K. Nussler, J. Stadler, E. Marzinzig, W. Barthlen, N.C. Nussler, H.G. Beger, S.M. Morris Jr., U.B. Bruckner, Nitric Oxide 1 (1997) 177.