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Review

Methodologies for the Sensitive and Specific Measurement of *S*-nitrosothiols, Iron-nitrosyls, and Nitrite in Biological Samples

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INTRODUCTION

Nitric oxide (NO) is a soluble gas that is synthesized by the nitric oxide synthase enzyme systems in endothelial, neuronal, and immune cells and is a critical endogenous vasodilator.[1–3] While it is generally believed that endothelium-derived NO is the primary regulator of NO-mediated basal blood flow,[4,5] recent studies have focused on the role of intravascular NO-derived molecules that could stabilize NO bioactivity and contribute to blood flow and oxygen delivery. These species include plasma *S*-nitrosothiols,[6–10] nitrite[4,11] and NO hemoglobin adducts. NO reacts reversibly with hemoglobin to form an NO-heme adduct, iron-nitrosyl-hemoglobin ($HbFe^{II}NO$), and can nitrosate a surface thiol on cysteine-93 of the β-globin chain to form *S*-nitrosohemoglobin (SNO-Hb). A model has been proposed that NO can be delivered by SNO-Hb in an oxygen-dependent manner, providing the red cell with oxygen sensor and NO donor properties.[12–14] This model rises from the observation that gradients of SNO-Hb exist from artery to vein with levels in oxygenated blood ranging from 300 nM to 2.5 μM and levels in venous blood that are not measurable.[12]

However, the evidence for a dynamic vascular cycle of SNO-Hb is brought into question by widely varying reports for the basal levels of intracellular SNO-Hb in arterial and venous blood.[12,15,8,9] We have employed highly sensitive methodologies that reduce the S–NO bond from hemoglobin in solutions of I_3^-, releasing NO gas from the cysteine for ozone-based chemiluminescent detection, and have found the levels of SNO-Hb *in vivo* to be approximately 50 nM with no artery-to-vein gradient.[16,8,4,17] These findings are consistent with a reappraisal of the levels of SNO-albumin in plasma by numerous laboratories[4,18,10,5,19] and with the observation that SNO-Hb is unstable in the presence of mM concentrations of glutathione[20–23] and in the reducing red blood cell environment.[17] Resolution of this controversy will require active investigation by numerous laboratories and the application of robust sensitive, specific and reproducible methodologies. The goal of this review is to present clear and detailed methodologies for measurement of plasma nitrite and nitrate and for the detection of nanomolar quantities of plasma *S*-nitrosothiols, plasma iron-nitrosyl complexes, and red blood cell SNO-Hb and iron-nitrosyl-hemoglobin. These methodologies have been developed in numerous laboratories and

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modified for specific protein targets. We hope this approach will set the stage for further validation and consensus on methodologies, and lead to a clearer understanding of the physiological relevance of these intravascular NO modified proteins *in vivo* and their role in pharmacology and pathophysiology. Intense recent interest in the role of *S*-nitrosation of proteins for signal transduction presents additional opportunities for the application of these methodologies to other protein targets.

THE PROBLEM

There are a number of challenges involved in the quantification of NO modified proteins in biological systems, such as their degradation in the presence of thiols and metals, their very low levels *in vivo*, the ubiquitous contamination of nitrite, and the presence of mixtures of iron-nitrosyls and *S*-nitrosothiols in plasma and blood. Because protein *S*-nitrosothiols are sensitive to reduction by low molecular weight thiols and contaminating copper, special preparations of samples is necessary. For example, *S*-nitrosoalbumin is rapidly decomposed in plasma unless *N*-ethylmaleimide (NEM) is added to bind all free thiols.[18,10,24] The use of EDTA as a copper chelator may actually promote *S*-nitrosothiol degradation in plasma while DTPA effectively stabilizes it (Tanos-Santos and Gladwin, manuscript in preparation). SNO-Hb rapidly degrades in red blood cell lysates and under denaturing conditions but can be stabilized by oxidation with ferricyanide.[17]

The low levels of SNO-Hb and plasma *S*-nitrosothiols *in vivo* prohibit the use of classic assays such as the Griess-Saville; results obtained using these methods are suspect and must be validated with alternative methodologies. Assays based on the UV-light induced photolysis or chemical reduction of *S*-nitrosothiols to liberate NO for detection in a chemiluminescent nitric oxide analyzer provide the required sensitivity but are not immediately specific for *S*-nitrosothiols. In addition to liberating NO gas from both *S*-nitrosothiols and iron-nitrosyls, both systems reduce nitrite to NO, requiring extensive treatment of samples through molecular sizing columns. However, hemoglobin and albumin possesses anion binding sites that may retain nitrite, raising concerns that the measured NO levels may be overestimated as a result of conversion of hemoglobin and albumin bound nitrite to NO. In this manuscript we will present methodologies that have been developed and extensively validated that use sequential reactions to selectively eliminate iron-nitrosyl, *S*-nitrosothiol, or nitrite for the specific and sensitive measurement of these compounds using tri-iodide reduction and ozone-based chemiluminescent NO detection.

THE I_3^- OZONE-BASED CHEMILUMINESCENT ASSAY

We have previously reported that I_3^-, synthesized by addition of sodium or potassium iodine (100 mg) and iodide (65 mg) to acetic acid (7 ml) and water (2 ml), will rapidly release NO gas from both *S*-nitrosohemoglobin and $HbFe^{II}NO$. I_3^- will also reduce nitrite (but not nitrate) to NO gas. The released NO gas is carried from the solution in a stream of helium into a chemiluminescent nitric oxide analyzer which can detect from 0.3 to 1 pmole NO gas (depending on the sensitivity of the particular machine). This methology for the measurement of *S*-nitrosothiols was first described by Samouilov and Zweier[25] and involves the following redox cycle:

1. $I_2 + I^-(\text{from HI}) \rightarrow I_3^-$
2. $2RS{-}NO + I_3^- \rightarrow 3I^- + RS^\bullet + 2NO^+$
3. $2RS^\bullet \rightarrow RSSR$
4. $2NO^+ + 2I^- \rightarrow 2NO^\bullet + I_2$

A similar assay has been validated for the measurement of plasma *S*-nitrosothiols by Marley and colleagues using a mixture of copper(I)/iodine/iodide to generate I_3^-.[10] These I_3^- based assays compare favorably with the well-established Griess-Saville assay over a wide range of concentrations for both *S*-nitrosohemoglobin and *S*-nitrosoalbumin, but extend the sensitivity from a limit of 250–500 nM, for the Griess-Saville, down to 1–5 nM.[17]

The I_3^- Reagent

A stock solution of 180 ml of I_3^- reagent is prepared using the following proportions:

1. Two grams of potassium iodide and 1.3 g of iodine are dissolved in 40 ml of distilled water.
2. One hundred and forty milliliters of acetic acid is then added and mixed thoroughly for at least 30 min.
3. The solution should be dark brown in color.
4. This stock solution of I_3^- reagent is prepared fresh each day.
5. Note that the stock solution can be prepared at different volumes, and directly in the purge vessel, as long as the proportions are maintained.

Ozone-based Chemiluminescent Assays

Helium gas is bubbled through the I_3^- reagent in a glass purge vessel. While many laboratories heat the solution to 60°C, we find this is not necessary for the assays described. The vessel is linked to a trap containing 15 ml of 1N NaOH and is then connected to the chemiluminescent nitric oxide analyzer

(Sievers, Model 280 NO analyzer, Boulder, CO). The nitric oxide analyzer can detect levels of NO gas as low as 0.3 pmole. With the system closed and the reagent of choice in the reaction chamber of the purge vessel, the cell pressure, as indicated on the NO analyzer, should be slightly higher than the cell pressure when the system is open (purge vessel uncapped). Using the flow restrictor on the purge vessel, adjust the flow of helium into the purge vessel to ensure that there is adequate gas flow into the NO analyzer. Schematics of the system have been previously published and are available from the manufacturer.[25]

MEASUREMENT OF PLASMA *S*-NITROSOTHIOLS, NITRITE, AND IRON-NITROSYL COMPLEXES

As mentioned earlier, the I_3^- reagent will react with both *S*-nitrosothiols and nitrite (NO_2^-) to stoichiometrically release NO gas that is released in the purge vessel and detected by the nitric oxide analyzer. While KI and acetic acid (without iodine) will form HI and selectively reduce nitrite, the reaction produces I_2 and then I_3^-, ultimately reducing *S*-nitrosothiols as well. For this reason, we measure both nitrite and *S*-nitrosothiols in the I_3^- reagent. *S*-nitrosothiols are distinguished from nitrite by pre-treating samples with 5% acidified sulfanilamide. Acidified sulfanilamide reacts with nitrite to form a diazonium complex that is not reduced to NO in the I_3^- chemiluminescent assay (and thus does not have a signal).[10] The plasma *S*-nitrosothiols can be further distinguished from plasma iron-nitrosyl complexes by reaction of the sample, prior to addition of acidified sulfanilamide, with and without mercuric chloride (which reduces S–NO to nitrite). Figure 1 shows a flow diagram of the procedure. Figure 2 illustrates the reactions described using standards of nitrite, *S*-nitrosoglutathione, nitrite with *S*-nitrosoglutathione, and iron-nitrosyl-hemoglobin. Ferricyanide can be used to eliminate iron-nitrosyls and is shown in Fig. 2.

Detailed Steps (Fig. 1):

1. Assemble the chemiluminescent assay apparatus as described above.
2. Place 9 ml of I_3^- reagent in purge vessel.
3. Allow the system to reach a steady baseline.
4. Collect 10 ml whole blood into vacutainers containing heparin (no EDTA) and 400 μl of 200 mM NEM (8 mM final concentration).
5. Spin whole blood in a centrifuge at 750*g* for 5 min at 4°C.
6. Remove plasma.
7. Aliquot three 270 μl fractions of plasma solution into light-protected eppendorf tubes, two of which contain 30 μl of PBS and one with 30 μl of 50 mM $HgCl_2$.
8. Vortex and allow mixture to react for 2 min at room temperature.
9. Add 30 μl of 5% acid sulfanilamide to one sample with $HgCl_2$ and to one sample without $HgCl_2$. Add 30 μl PBS to a third sample. Vortex and allow the samples to incubate at room temperature for 3 min.
10. Inject 330 μl samples into purge vessel containing I_3^- reductant, in-line with the NO analyzer, to release nitric oxide for chemiluminescent detection. Allow the mV peaks to return to baseline prior to subsequent injections.
11. Change reagent after one injection (more samples can be injected but foaming will occur and the rate of NO release begins to slow, resulting in wider peaks).
12. Account for dilutional adjustments by multiplying the concentration of detected NO by 1.22 (330/270 μl).
13. NO concentrations from the samples containing acidified sulfanilamide and $HgCl_2$ represent plasma iron-nitrosyl levels only.
14. Subtract iron-nitrosyl levels (with $HgCl_2$) from the sample containing acidified sulfanilamide but not treated with $HgCl_2$ to get *S*-nitrosothiol levels.
15. The peak without mercury or acidified sulfanilamide treatment represents nitrite (technically this value requires subtraction of the other two measurements but it will be two orders of magnitude higher and the subtraction will be inconsequential).

As shown in Fig. 2, all of these assays can be performed with standards, cell media, cell lysates, and with isolated protein experiments. The injection volume and reagent concentrations can be modified as dictated by the experiment. In general, the large 300 μl injection volumes are only needed for the detection of low nM NO concentrations. In Fig. 2 only 20 μl of sample was injected.

MEASUREMENT OF RED BLOOD CELL *S*-NITROSOTHIOLS AND *S*-NITROSOHEMOGLOBIN

SNO-Hb is unstable in the reductive red blood cell environment and rapidly decays in a temperature and redox dependent fashion, independent of oxygen affinity.[17] In order to stabilize the SNO-Hb for measurement, the red blood cell must be rapidly oxidized with ferricyanide. We have devised

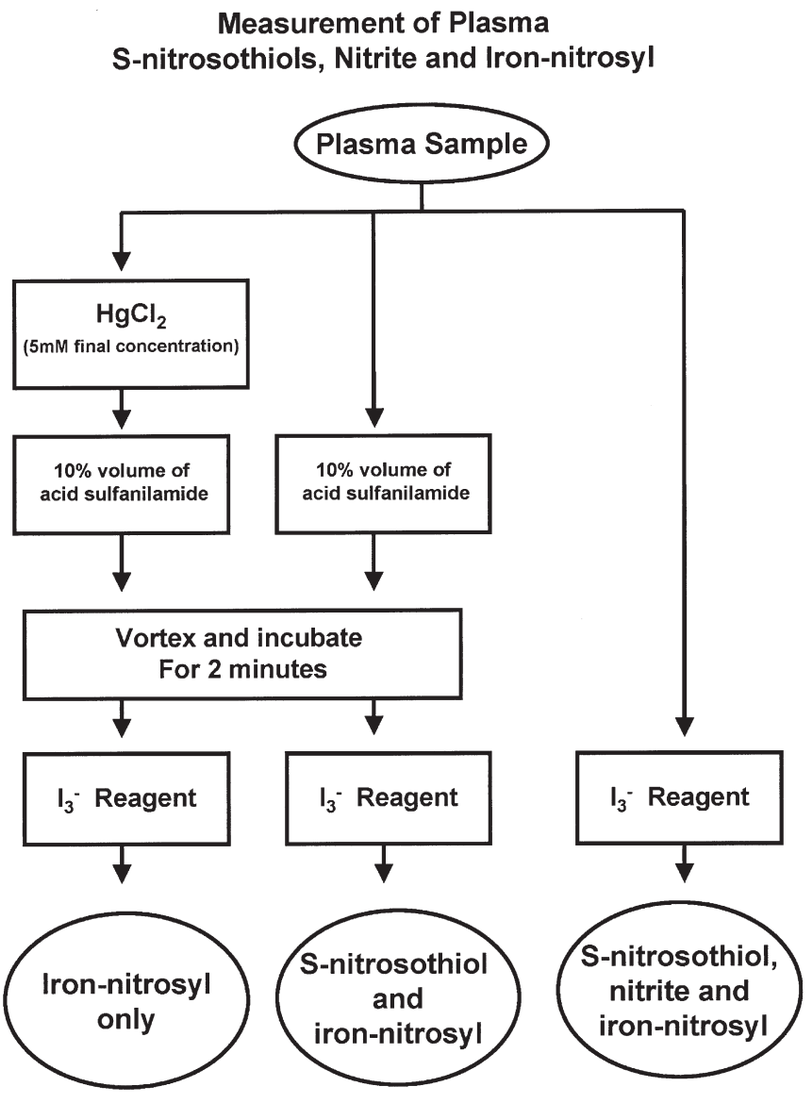

FIGURE 1 Flow diagram for the measurement of *S*-nitrosothiols, nitrite and iron-nitrosyl complexes in plasma or buffer.

a methodology to rapidly lyse the red cells and solubilize the membranes with 1% NP40, stabilize the SNO-Hb by oxidation with ferricyanide, eliminate artifactual *S*-nitrosation by reaction of free thiols with NEM and chelate contaminating metals with DTPA. Following treatment with this "SNO-Hb stabilization solution" the reaction mixture is passed through a Sephadex G25 sizing column to remove low molecular weight *S*-nitrosothiols and the hemoglobin fraction is then treated with and without mercuric chloride to identify SNO-Hb and then all the samples are treated with acidified sulfanilamide to eliminate contaminating nitrite. This assay is sensitive to 5 nM SNO-Hb in whole blood (0.00005% SNO per heme) and specific for *S*-nitrosated hemoglobin (Fig. 3A–C)

SNO-Hb Stabilization Solution

This solution is composed of 4 mM ferricyanide ($K_3Fe^{III}(CN)_6$), 10 mM *N*-ethylmaleimide (NEM), 100 μM diethylenetriaminepentaacetic acid (DTPA), and 1% nonidet-P-40 detergent (NP-40) in phosphate buffer solution. As shown in Fig. 4, $K_3Fe^{III}(CN)_6$ is the major ingredient necessary for SNO-Hb stabilization. KCN addition produces cyanomethemoglobin, which further redox inactivates the heme. NEM is added to modify any free thiols to prevent the artifactual production of SNO-Hb. DTPA is used to chelate copper and other trace metals that can contaminate labware and lead to degradation of SNO-Hb. NP-40 is used to solubilize the red blood cell membrane, releasing any *S*-nitrosothiols that may be bound to the red blood cell membrane.

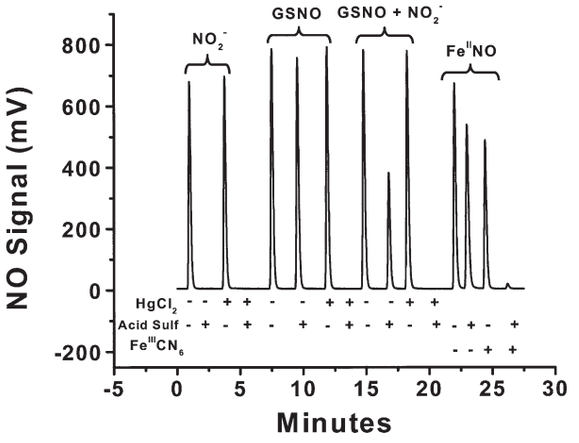

FIGURE 2 Effects of pretreatment with mercuric chloride ($HgCl_2$), acidified sulfanilamide (acid sulf), and/or ferricyanide ($Fe^{III}(CN)_6$) on NO release in I_3^-, reagent from nitrite (NO_2^-), *S*-nitrosoglutatione (GSNO), nitrite and *S*-nitrosoglutatione, and iron-nitrosyl-hemoglobin ($Fe^{II}NO$). Eighty two micromolar or μM nitrite, 77 μM *S*-nitrosoglutathione and a mixture of 38.5 μM *S*-nitrosoglutathione and 41 μM nitrite, were reacted with and without $HgCl_2$ and with and without acidified sulfanilamide. Additionally, 82 μM iron-nitrosyl-hemoglobin was reacted with and without potassium ferricyanide and with and without acidified sulfanilamide. This experiment demonstrates the elimination of nitrite by acidified sulfanilamide, the conversion of *S*-nitrosothiol to nitrite by mercuric chloride and the conversion of iron-nitrosyl-hemoglobin to NO (then nitrite) by ferricyanide.

The stock solutions of each component are prepared as shown in Table I.

Lysis of Red Blood Cell Pellets in SNO-Hb Stabilization Solution

Blood samples can be collected using a butterfly needle set-up and vacutainer system to maintain the physiological oxygen tension of the sample. The blood is centrifuged at 750*g* for 5 min and the plasma rapidly removed. Red blood cells of 100 μl are pipetted from the bottom of the pellet and lysed in the stabilization solution as described in Table II.

Measurement of SNO-Hb

Following treatment with this "SNO-Hb stabilization solution" the reaction mixture is passed through a Sephadex G25 sizing column to remove low molecular weight *S*-nitrosothiols and the hemoglobin fraction is treated with and without mercuric chloride, which transforms SNO-Hb to nitrite to specifically identifiy SNO-Hb. Then all the samples are treated with acidified sulfanilamide to eliminate contaminating nitrite. The difference in NO detected in the I_3^- chemiluminescent assay between the mercuric chloride treated and untreated samples corresponds to the concentration of SNO-Hb in the sample. The stock solutions of each component are prepared as shown in Table III.

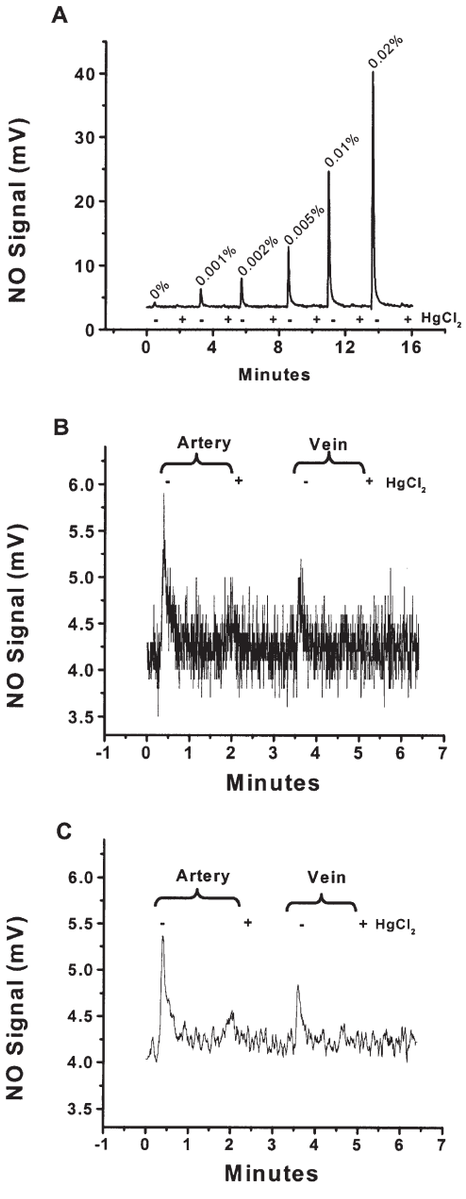

FIGURE 3 *Panel* (*A*) Standards of 0–0.02% SNO-Hb in "preservation solution", passed through a Sephadex G25 column, then reacted with (+) and without (−) mercuric chloride and then acidified sulfanilamide. *Panel* (*B*) Raw data from the nitric oxide analyzer measuring NO (mV signal) release from 300 μl injections of hemoglobin. The samples were drawn from the artery and vein, the red blood cells pelleted and lysed in the SNO-Hb preservation solution, passed through a sizing column and treated with (+) and without (−) $HgCl_2$ and then with acidified sulfanilamide. *Panel* (*C*) We then transferred the raw data from the Sievers program to Origin (Microcal Software, Inc., Northampton, MA). The data was smoothed using the Savitzky-Golay filter method provided with the software (symmetric, 21 point window; polynomial degree = 2) for more accurate quantification of the areas under the curves.

Detailed Steps (Fig. 5):

1. Aliquot 900 μl fractions of stabilization solution into 1.5 ml light-protected eppendorf tubes.

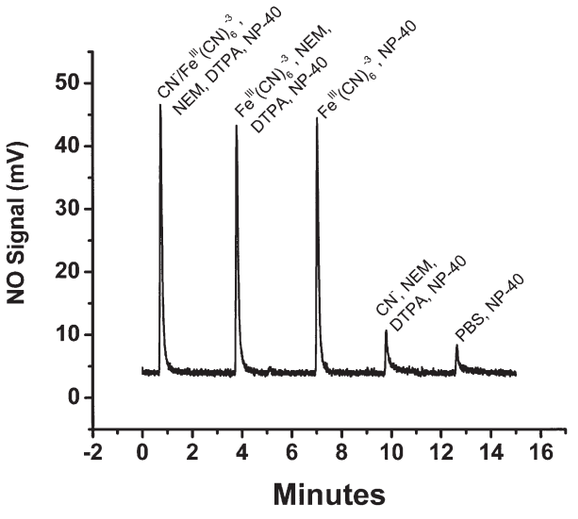

FIGURE 4 SNO-Hb treated with a variety of reagents including potassium cyanide, potassium ferricyanide, *N*-ethylmaleimide, diethylenetriaminepentaacetic acid (DTPA), Nonidet P-40 and PBS. These data demonstrate that SNO-Hb is unstable in red blood cell lysates unless treated specifically with the oxidant ferricyanide. Metal chelators and thiol blocking reagents are not sufficient to stabilize SNO-Hb.

2. Spin whole blood down in a centrifuge at 750*g* for 5 min at 4°C.
3. Remove plasma and buffy coat.
4. Placing the tip of pipette into the bottom of the red blood cell (RBC) pellet (∽ 16–20 mM heme), take 100 µl of RBC pellet into 900 µl of Stabilization Solution. Final concentration of hemoglobin should be ∽ 1.6–2.0 mM heme.
5. Vortex immediately and place in a light-protected environment at room temperature (You may freeze the sample in dry ice at this point).
6. After 5 min, vortex and pipette 500 µl into a stopped 9.5 ml bed volume G-25 Sephadex column (NAP™ 25, Amersham Biosciences, Upsala, Sweden) that has been pre-washed with PBS.
7. Release the stopper and let the column drip until the mixture is completely absorbed.
8. Add 2.5 ml of PBS to column. The sample should stop at the bottom of the column.
9. Collect eluant by adding a final 700 µl volume of PBS to the column (Heme concentration using this column volume will be 0.6–1.0 mM). Place hemoglobin solution on wet ice.
10. Save 50 µl of hemoglobin solution to measure heme concentration with Drabkin's reagent (described below).
11. Aliquot 270 µl fractions of hemoglobin solution into light-protected eppendorf tubes containing either 30 µl of PBS 1X or 30 µl of 50 mM $HgCl_2$.
12. Vortex and allow mixture to react for 2 min at room temperature.
13. Add 30 µl of 5% acid sulfanilamide to both aliquots and mix thoroughly.
14. Inject 300 µl into purge vessel containing the I_3^- reductant, in-line with the NO analyzer, to release NO for chemiluminescent detection.
15. Note that injection volumes depend on the concentration of the sample. Injection volumes can vary from 100 to 300 µl.
16. Account for dilutional adjustments by multiplying the concentration of detected NO by 1.22 (330/270 µl) or equivalent dilution factor.
17. Measure the heme concentration using the Drabkin's assay.
18. Calculate percent SNO per heme

$$= \frac{\text{mM NO concentration determined using } I_3^-}{\text{mM Heme concentration from Step 17}} \times 100$$

TABLE I Stock solutions

Diethylenetriaminepentaacetic acid (10 mM)
• 786 mg DTPA (F.W. 393.3)
• 200 ml water
• Adjust pH to 7.0 with 5N NaOH to bring DTPA into solution
Cyanide/Ferricyanide (200 mM)*
• 330 mg Postassium Ferricyanide (F.W. 329.26)
• 65 mg Postassium Cyanide (F.W. 65)
• 5 ml PBS 1X
N-ethylmaleimide (200 mM)*
• 250 mg *N*-ethylmaleimide (F.W. 125.2)
• 10 ml PBS 1X

* These reagents must be made fresh on the day of use.

Measurement of Heme Concentration

Drabkin's Solution—see Table IV.

1. Place 50 µl of hemoglobin solution into 950 µl of Drabkin's Solution in a 1 ml cuvette.

TABLE II Mixture of red blood cell pellet with SNO-Hb stabilization solution

Volume (µl)	Reagent	Final concentration
10	DTPA of 10 mM	100 (µM)
20	Cyanide/Ferricyanide of 200 mM	4 (mM)
50	NEM of 200 mM in PBS	10 (mM)
10	Nonidet P-40 of 100%	1%
810	Phosphate buffered saline (PBS) of 1X, pH 7.4	
100	Volume reserved for red blood cell pellet of ∽ 16–20 mM	∽ 2 (mM)

TABLE III Stock solutions for measuring SNO-Hb

$HgCl_2$ (50 mM)
•67.9 mg of $HgCl_2$ (F.W. 271.52)
•5 ml of PBS 1X
5% Acidified sulfanilamide (290 mM)
•500 mg of Sulfanilamide (F.W. 172.2)
•10 ml of 1N HCl

TABLE IV Drabkin's solution

Dissolve the following reagents in 1000 ml of distilled or deionized water:
•200 mg K_3Fe^{III} $(CN)_6$ (FW 329.26)
•50 mg KCN (FW 65.12, Aldrich Chemicals)
•140 mg KH_2PO_4 (FW 136.09)
•0.5 ml of Sterox SE

Adjust pH to 7.4 if necessary. Protect the solution from any exposure to light by using a brown borosilicate glass bottle or a bottle wrapped in aluminum foil. The color of the solution should be a clear pale yellow.

2. Place 950 μl of Drabkin's Solution into a separate 1 ml cuvette to use as a blank.
3. Mix thoroughly and let sit at room temperature for at least 15 min. Longer times are necessary for higher iron-nitrosyl-hemoglobin concentrations or carboxyhemoglobin.
4. Blank the spectrophotometer with the Drabkin's Solution.
5. Measure the absorbance of the cyanomethemoglobin at the 540 nm wavelength (λ).
6. The concentration of the hemoglobin is determined using the equation below:

$$\text{mM concentration of heme} = \frac{(\text{measured absorb. at } 540\lambda)(\text{dilution})}{(\text{extinction coefficient at } 540\lambda = 11)}$$

Measurement of both Iron-nitrosyl-hemoglobin and *S*-nitrosohemoglobin

The SNO preservation solution described above will destroy iron-nitrosyl-hemoglobin for two reasons.

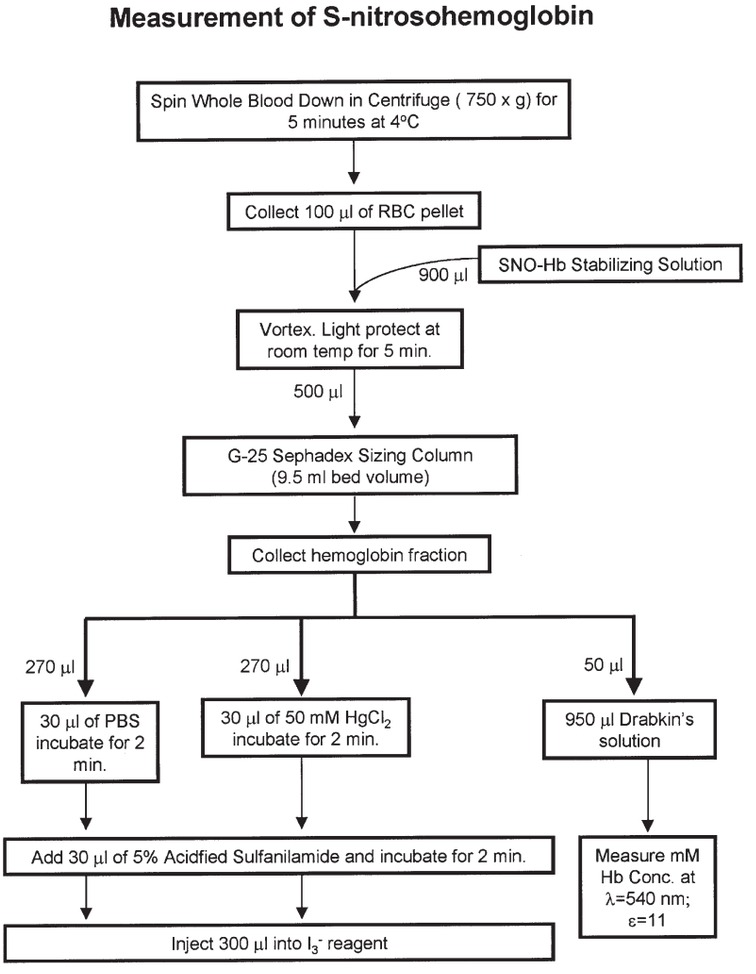

FIGURE 5 Flow diagram for the measurement of *S*-nitrosohemoglobin (SNO-Hb) in erythrocytes.

Cyanide and ferricyanide accelerate NO release from iron-nitrosyl-hemoglobin and about 25–30% of the iron-nitrosyl-hemoglobin is eliminated in solutions of acidified sulfanilamide. Therefore, in order to measure iron-nitrosyl-hemoglobin, a different technique is required. Following centrifugation of whole blood or experimentally treated red blood cells, pelleted red cell samples are lysed in distilled water with 0.5 mM EDTA (1:2 dilution) and 200 µl of the hemoglobin lysate is then added to 600 µl of 0.2 M KCN/0.2 M $K_3Fe^{III}(CN)_6$ in PBS with 0.5 mM EDTA or to 600 µl of PBS with 0.5 mM EDTA and incubated for 35 min. Treatment with a 100-fold molar excess of KCN and $K_3Fe(CN)_6$ selectively removes NO from heme while preserving the *S*-nitrosothiol bond.[8]

After the 35 min incubation, 500 µl of the hemoglobin sample is passed through an extensively washed (2 h wash with 0.5 mM EDTA HPLC grade water to remove column preservatives and nitrite) 9.5 ml bed volume Sephadex G25 column (NAP™ 25, Amersham Biosciences, Upsala, Sweden) to remove nitrite, small thiols, and the KCN/K_3Fe^{III} $(CN)_6$. The hemoglobin concentration of the Sephadex G25 effluent is then measured by conversion to cyanomethemoglobin. Hemoglobin samples (200 µl) are then injected into the purge vessel with I_3^- to release NO for chemiluminescent detection. The quantity of iron-nitrosyl-hemoglobin is therefore the difference in amount of total SNO-Hb plus iron-nitrosyl-hemoglobin, determined without pretreatment of KCN/K_3Fe^{III} $(CN)_6$, and SNO-Hb determined with KCN/ $K_3Fe^{III}(CN)_6$ pretreatment. This value was divided by the concentration of the hemoglobin, and the total multiplied by 100 so as to be expressed as % moles nitrosylated-hemoglobin per moles heme subunit.

Because acidified sulfanilamide can NOT be used in this assay and large injection volumes are required it is critical to remove all nitrite from the columns. This requires extensive column washing with nitrite free water (see Pitfalls below). Cyanide has a signal in the I_3^- assay so complete separation from hemoglobin on the column is required. If difficulty occurs at this step it may be necessary to eliminate cyanide from the experiment and use only ferricyanide, which will require longer incubation times to eliminate all the iron-nitrosyl. The sample treated without cyanide and ferricyanide (containing both iron-nitrosyl and SNO) can be immediately passed through the sizing column and measured while the other sample (with KCN/$K_3Fe^{III}(CN)_6$) incubates. This will ensure minimal decay of the iron-nitrosyl-hemoglobin in the red cell. The SNO-Hb in the other sample will be stable in the KCN/ $K_3Fe^{III}(CN)_6$ solution during the 35 min incubation.[17]

Measurement of Nitrate in Plasma

Nitrate (NO_3^-) is measured using vanadium(III) in 1N hydrochloric acid with the reaction chamber heated to a temperature of 90°C. The vanadium reduces the nitrate to NO gas, which is released in the closed system apparatus of the ozone-based chemiluminescent assay as described above. Previous problems with foaming caused by protein interactions with the reagent are solved with an ethanol precipitation step (Fig. 6).

Vanadium–HCl Reagent

1. Measure 0.4 g of vanadium(III) chloride (F.W. 157.3) (50.9 mM) (Aldrich Chem. Co.) while minimizing exposure to light and air.
2. Add 50 ml of 1N HCl to vanadium(III) chloride and mix thoroughly in a light protected environment.

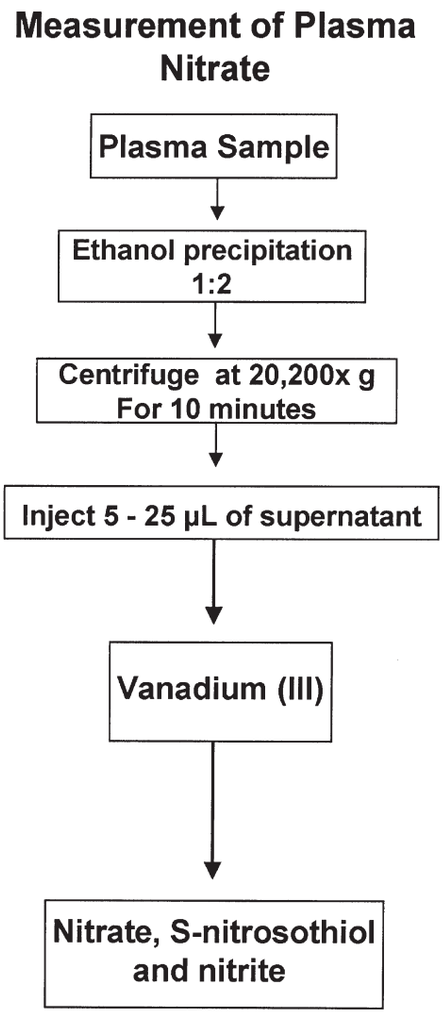

FIGURE 6 Flow diagram for the measurement of nitrate in plasma or buffer using the vanadium assay.

3. Filter the vanadium solution through filter paper (Whatman, Qualitative Circles, England), using a funnel, into a light protected glass bottle.
4. Store the vanadium–HCl solution at 4°C until ready to use.
5. Shelf-life of the solution is 2 weeks.

Detailed Steps (Fig. 6):

1. Connect the purge vessel to circulating water bath (we use the Lauda, Ecoline Immersion thermostat E 100, Germany) and heat the reaction chamber of the purge vessel to 90°C.
2. Connect flowing tap water to the condensation chamber.
3. Assemble the chemiluminescent assay apparatus as described above.
4. Place 5 ml of vanadium–HCl reagent into the purge vessel.
5. The reagent should turn from blue to lime-green in color[†].
6. Let the system run until the NO analyzer reads a steady baseline millivolt signal. This can take up to an hour.
7. Mix the plasma samples with 100% cold ethanol in a 1:2 part ratio for 5 min.
8. Vortex the sample thoroughly and spin at 20,200 g at 4°C for 10 min.
9. Collect the supernatant and inject 5–15 μl into the vanadium–HCl reagent.
10. Multiply the concentration of nitrate acquired by 3 to correct for ethanol dilution. The concentration represents total NOx (nitrate, nitrite and SNO). Subtract the concentration of nitrite and *S*-nitrosothiols for the nitrate value. Note that plasma nitrate concentrations are 20–40 μM and nitrite concentrations 0.2–1 μM and plasma *S*-nitrosothiols 0.002–0.005 μM, so the value obtained by the Vanadium assay is mostly nitrate.

Pitfalls and Problems

Water

In making nitrite standards with water, it was observed that various sources of water contained different levels of nitrite. The lowest nitrite levels we have measured in water used in our laboratory is in glass bottled Molecular Biology Grade Water (Biofluids, Biosource International) and distilled water filtered with a Milli-Q, Millipore system. It is important to use fresh filtered or glass-bottled water because NO in the air can react with oxygen in water to form nitrite.

Standards

Nitrite, *S*-nitrosothiols, and iron-nitrosyl-hemoglobin all stoichiometrically release NO in tri-iodide. Nitrate will do so in vanadium/HCL. Because of this exact stoichiometry we have found that standards of nitrite can be used for the measurement of *S*-nitrosothiols and vice-versa. We prefer standards of nitrite because of the potential decomposition of *S*-nitrosothiols over time. However, when making nitrite standards for very low concentration experiments it is necessary to prepare them in nitrite free water because these nitrite standards cannot be reacted with acidified sulfanilamide to remove nitrite contamination. *S*-nitrosothiol standards such as SNO-Hb and SNO-albumin should be reacted with acidified sulfanilamide to remove contaminating protein-bound nitrite.

Peak Width and Reaction Temperatures

S-nitrosothiols and iron-nitrosyl complexes release their NO more slowly in tri-iodide than solutions of nitrite. This release is even slower for more complex *S*-nitrosothiols such as hemoglobin and albumin. This can be illustrated by serial injections of *S*-nitroso-glutatione (GSNO) incubated at room temperature over several hours. The peak width progressively narrows and the peak height increases as the GSNO degrades to nitrite. However, the integrated area under the curve does not change. If the peak width becomes too large for certain proteins or conditions this can be solved by changing the tri-iodide with each injection or by increasing the reaction vessel temperature to 60°C.

Foaming

One of the significant advantages of the tri-iodide based assays is that there is minimal foaming following injection of protein-containing biological samples. The preparation of a stock tri-iodide solution allows for the rapid and frequent changing of reagent which substantially prevents foaming. If foaming is causing the solution to overflow into the NaOH trap, a silica-based antifoaming agent (antifoam C in a 30 fold dilution, Sigma) can be used.

Integration of Low MV Peaks

We have found that the packaged software available for the Sievers nitric oxide analyzer produces some error when measuring the area under the curve (AUC) for samples with 0.3–5 pmole of NO. We therefore transfer raw data from the Sievers program

[†]Make new vanadium–HCl reagent if this color change does not happen.

to Origin (Microcal Software, Inc., Northampton, MA). The data is smoothed using the Savitzky-Golay filter method provided with the software (symmetric, 21 point window; polynomial degree = 2). Standard HPLC peak integration software can also be used. An example of raw data prior to and after filtering from injections of human red blood cell lysates is shown in Fig. 3B and C.

Machine Sensitivity

We have found that the sensitivities of the ozone-based chemiluminescent nitric oxide analyzers can vary substantially. If you have access to more than one machine, we advise testing the same standard of nitrite in each machine and comparing the mV signals from each. The machine that produces the higher signal can be used for assays requiring greater sensitivity.

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