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Measurement of Plasma Nitrite by Chemiluminescence

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Abstract

Studies have demonstrated that plasma nitrite (NO_2^-) reflects endothelial nitric oxide (NO) production. In addition, NO_2^- itself has been shown to have biological activities associated with its reduction to NO in the blood and tissues. Therefore, determination of plasma NO_2^- has been proposed as a prognostic marker for cardiovascular diseases. Typical concentrations of NO_2^- in the plasma are in the nanomolar range and determination of this NO_2^- poses a challenge both in terms of sensitivity and specificity. For instance, a highly sensitive, chemiluminescence method that is based on the reduction of NO_2^- by potassium iodide and iodine is being used to determine the nitrite in biological fluids. This method also measures other nitric oxide species such as S-nitrosothiols and N-nitrosamines. We, therefore, developed an alternative method based on the reduction of NO_2^- by ascorbic acid in strongly acidic media. As part of the methodology, glacial acetic acid and ascorbic acid are introduced into the purge vessel of the NO analyzer. Samples containing NO_2^- are injected into the purge vessel and the chemiluminescence signals generated as a result of the formation of NO are then measured. We find that under these conditions NO_2^- is stoichiometrically reduced to NO. Other traditional NO-generating species, such as, S-nitrosothiols, N-nitrosamines, nitrated lipids and nitrated proteins did not interfere in the determination of plasma NO_2^- . Using the present method, plasma NO_2^- in fasting human subjects has been determined to be in the range of 56 to 210 nM (mean $\pm$ SD = 110 ± 36 nM; n = 8).

Keywords

Plasma; Nitrite; nitric oxide; Endothelial nitric oxide synthase; Chemiluminescence; Ascorbic acid

1. Introduction

Nitric oxide (NO) synthesized by endothelial nitric oxide synthase (eNOS) diffuses randomly into smooth muscle cells and blood. The life span of NO in blood milieu is less than 0.2 ms. The reaction of NO with blood components results in the formation of nitrite (NO_2^-), nitrate (NO_3^-), S-nitrosothiols (RSNOs), iron nitrosyls, N-nitrosamines, nitrated lipids and nitro-proteins (1). Related to the role of NO in regulating vascular tone and blood flow, decreased NO synthesis in the endothelium is a strong risk factor for the development

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¹Nitrite contamination is very common in solvents, reagents, glassware, and especially blood collecting tubes. It is important to check this nitrite contamination by injecting the test solutions into glacial acetic acid/ascorbic acid reagent system (above) before using them. Appearances of any signals indicate the nitrite contamination.

⁴Always use fresh high quality water (Ultra pure water with resistivity $\geq 18.2 \text{ M}\Omega\text{-cm}$) to prepare the solutions and to rinse the syringes and NOA purge system.

⁵Always cap the reagent bottles tightly; otherwise atmospheric nitric oxide species dissolves in liquids over time.

¹⁰When high protein samples (plasma) are injecting, leave a sufficient gap between the injections until the gas bubbles are regenerated in the purge vessel.

of atherosclerosis, vascular thrombosis, reperfusion injury, and hypertension (reviewed in refs 2 and 3). The measurement of predominant species of NO_2^- and NO_3^- in plasma are used to reveal the NO synthesis in vascular endothelial cells (4). The reliability of measurement of NO_3^- to assess the eNOS activity has been a subject of debate as the levels of NO_3^- are also influenced by several eNOS-independent mechanisms such as diet, intestinal bacteria, renal function, and breathing of atmospheric NO and NO_2 gases. In addition, due to the presence of high concentrations of NO_3^- in plasma ($\sim 25\mu\text{M}$), acute changes of eNOS activity is not easy to determine. Recent studies have demonstrated the possibility of measuring of nanomolar concentrations of plasma NO_2^- alone and the scope to evaluate the activity of eNOS (5–7). Further, some studies have documented that NO_2^- can serve as an intravascular NO storage molecule (8–11). Despite all the biological significance this compound has, a serious limitation faced by workers in the area has been the lack of an accurate, sensitive, and reproducible method for the determination of plasma NO_2^- .

Currently, for the determination of NO_2^- in human biological fluids the methods employed rely on mass spectroscopy, colorimetry, HPLC and flow injection analysis (4). One of the most widely used methods for the determination of NO_2^- is the colorimetric method using the Griess reagent (12). This method, however, lacks sensitivity for the determination of NO_2^- in physiological fluids, which are likely to be less than $1\mu\text{M}$. Among others, the ozone based chemiluminescence method is highly sensitive and nanomolar concentrations of NO can be determined by this method. This method relies on the reaction of NO with ozone and the measurement and quantification of the energy released while returning from an excited state (NO_2^*) to the ground state.

Using the above approach different NO-related species can be detected after selectively reduced to NO. The potassium iodide and iodine (KI/I_2) based reductive chemiluminescence assay has been used to determine plasma NO_2^- (13–15). This reagent measures NO_2^- in the presence of NO_3^- ; it does not reduce NO_3^- to NO. This reagent, however, is not specific for NO_2^- as it also can react with plasma RSNOs and *N*-nitrosamines (16,17).

Therefore, a direct reductive chemiluminescence method is developed to determine NO_2^- without interference of NO_3^- , RSNOs and *N*-nitrosamines (18). This method involves a reduction of nitrite to NO by ascorbic acid under acidic conditions; the NO released is carried to the detector by inert gas where it reacts with ozone to produce a chemiluminescence signal. The signal intensity obtained is proportional

to the concentration of NO generated (or NO_2^- present in the original sample). This method is used extensively in the author's lab for the determination of plasma NO_2^- .

2. Materials

2.1. Equipment

1. Nitric Oxide Analyzer: Sievers model 280 obtained from GE Analytical Instruments (Boulder, CO) (For detailed information, see ref. 19).
2. Liquid program (NO Analysis version 3.21)
3. Origin program (version 6.1)
4. Oxygen and inert gas (argon or nitrogen)
5. Hamilton syringes: capacity 50, 100, and 250 μL with 5-inch long needles
6. Portable centrifuge (Labnet type Z 150)

2.2. Reagents

1. Water: Ultra pure water with resistivity $\geq 18.2 \text{ M}\Omega\text{-cm}$. Prepared using PureLab Plus or Millipore water-Milli Q-Gard 2 purification pack.
2. Sodium hydroxide, 1 mM: prepared by 1,000-fold dilution of 1 M NaOH (Fisher Scientific) using water.
3. Preparation of NO_2^- standards:
 - A. Stock solution 1 (100 mM): dissolve 0.690 g of sodium nitrite (Sigma-Aldrich) in 100 mL of 1 mM NaOH. The solution is stable at 4 °C for one week.
 - B. Stock solution 2 (1 mM): prepared by 100-fold dilution of stock solution 1 using 1 mM NaOH; stable for 24 h.
 - C. Working solution (1 μM): prepared by 1,000-fold dilution of stock solution 2 with water. Typically, 10 μL of stock solution 2 is diluted to 10 mL with water. Use within 2 or 3 h.
4. Dilute the working standard solution with water serially (1:2, 1:4,) down to a concentration of 7.812 nM (Table 1).
5. Diethylenetriaminepentaacetic acid (DTPA; 10 mM): suspend 39.3 mg DTPA in 5 mL of water. With the contents being constantly stirred, add 1 N NaOH drop wise until the DTPA is completely dissolved. Make up the final volume to 10 mL with water. This solution is stable at room temperature.
6. N-ethylmaleimide (NEM; 100 mM): dissolve 125.1 mg NEM in 100 mL of water; should be prepared fresh reagent every day
7. Ascorbic acid (0.5 mM): dissolve 0.881 g of ascorbic acid in 10 mL of water. Prepare fresh reagent every day.

3. Methods

3.1. Blood Collection

Collect venous blood samples (3 mL each) from subjects who fasted overnight into heparinized vacutainer tubes (see Notes 2 & 3). Nitrite is unstable in the whole blood. Therefore, it is advisable to use a bench-top centrifuge near the bed side of the subject in order to rapidly separate the plasma from red cells. The blood samples are centrifuged immediately (i.e., within 30 s following the collection) for 3 min at 5,000 rpm. Plasma (1 mL) free of hemolysis is transferred into dark colored microtubes that also contain 6.5 mM NEM (100 mM; 6.5 μ L) and 0.1 mM DTPA (10 mM; 10 μ L). NEM and DTPA prevent the destruction of plasma S-nitrosothiols to NO_2^- . The samples are immediately frozen on dry ice and stored at -85 or -150 $^{\circ}\text{C}$ for future analysis.

3.2. Calibration Curve for Nitrite

1. Add 7 mL of glacial acetic acid and 1.0 mL of 0.5 M ascorbic acid into the purge vessel.
2. Antifoam reagent is generally not needed or added to the glacial acetic acid-ascorbic acid reagent.
3. A gas bubbler/sodium hydroxide trap is connected between the purge vessel and the detector.
4. The temperature of the water bath used for circulating water around the purge vessel is set at 37 $^{\circ}\text{C}$. Cold water circulation for condenser is not required.
5. Liquid program version 3.21 is used to acquire data. The reaction mixture is continuously purged with inert gas at constant cell pressure by adjusting the gas flow (see Note 6). The data is acquired every $\frac{1}{4}$ s. Once the baseline is stabilized, a 100 μ L aliquot of the working standard of NO_2^- (concentration range: 7.812 nM - 500 nM) is injected into the purge vessel in duplicates and the chemiluminescence signals are recorded.
6. The data from the liquid program is imported into the Origin 6.1 program (see Note 7) and chemiluminescence signals are regenerated. The area under the curve (AUC) is determined using the method provided with the software (Table 2).
7. Standard Curve: The calibration curve is constructed by plotting the peak area versus concentration of the nitrite (Fig.1A). The slope and intercept of the calibration curve is determined by linear regression technique (Fig.1B).

3.3. Measurement of Plasma Nitrite

1. The plasma is thawed under subdued light just prior to analysis and kept on ice until injected (see Note 8)

²Nitrite contamination in vacutainer tubes should be checked before use. The tubes are mixed well with ultra pure water and then determine the nitrite in water. We found that heparin tubes are almost free of nitrite while EDTA tubes contain high levels of nitrite. One needs to check the nitrite contamination for each supplier of vacutainers.

³Nitrite is rapidly oxidized to nitrate by oxyhemoglobin. Therefore it is necessary to take all precautions to avoid hemolysis while collecting and processing blood samples. Plasma should be free of hemolysis.

⁶Make sure that there is no gas leak into the purge system. Maintain constant instrument's cell pressure by adjusting the inert gas flow. Run the system at least 30 to 60 min for stabilization prior to initiating the sample analysis.

⁷We strongly suggest the use of the Origin 6.1 program for data analysis. It can be purchased from Origin Lab Corporation, One Roundhouse Plaza, Suite 303, Northampton, MA 01060 USA, www.originlab.com.

⁸Thaw the plasma samples just prior to use while protecting from light. Complete the analysis within 1 or 2 h.

2. Using a Hamilton syringe, inject 100 μ L of either fresh or stored plasma into the bottom of the purge vessel that contain 7 mL of glacial acetic acid and 1 mL of 0.5 M ascorbic acid (see Notes 6& 9)
3. Antifoam reagent is not required even for plasma samples.
4. Each sample is injected in triplicate or quadruplicate. The reagents of purge vessel are changed after four injections.
5. Data are transferred to the Origin 6.1 program and the chemiluminescence signals are regenerated as mentioned for the NO_2^- standard curve (Fig. 2A).
6. To eliminate the noise and for baseline correction, the signals are smoothened using adjacent averaging of 20 points. The chemiluminescence signals are regenerated using the smoothened data. The area under the curve is determined as described for the standard curve (Fig. 2B).
7. The plasma nitrite level obtained for 8 human subjects under fasting conditions are illustrated in Fig.3. The following calculation is used.

Calculations

$$\begin{aligned}
 \text{Nitrite (nM)} &= \frac{\text{Area} - \text{Y-intercept}}{\text{Slope}} \\
 &\times \frac{\text{Volume of stds injected (ml)}}{\text{Volume of plasma injected (ml)}} \\
 &\times \text{Dilution factor (NEM and DTPA)} \\
 &= \frac{2.49 - (-0.01651)}{0.0152} \\
 &\times \frac{0.1}{0.1} \times 1.0165 = 167.62 \text{ nM}
 \end{aligned}$$

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⁹Inject the samples at the bottom of the purge vessel using a syringe with a 5-inch long needle.

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Figure 1.

(A). Generation of chemiluminescence signals following the addition of nitrite standard. Nitrite (7.812 –500 nM; 100 μ L) is injected into the purge vessel that contained 7 mL glacial acetic acid and 1 mL of 0.5 M ascorbic acid. (B) Calibration curve for nitrite is obtained by integrating the area under the curve.

Figure 2.

Chemiluminescence signal of plasma nitrite: 100 μ L of human plasma was injected into purge vessel. The contents of reaction mixture in purge vessel are the same as mentioned for Fig. 1. (A) Chemiluminescence signals from raw data, and (B) chemiluminescence signals from smoothened data used for calculating the area. Area of peak 1. 2.33534; Peak 2. 2.50232; Peak 3; 2.64189.

Figure 3.
Plasma nitrite values of 8 human subjects.

Table 1

Preparation of nitrite standards.

Ratio	Volume of 1 μ M nitrite std (mL)	Volume of water (mL)	Total volume (mL)	Final nitrite concentration (nM)
1:2	5	5	10	500
1:4	2.5	7.5	10	250
1:8	1.25	8.75	10	125
1:16	0.625	9.375	10	62.5
1:32	0.3125	9.6875	10	31.25
1:64	0.156	9.844	10	15.625
1:128	0.078	9.922	10	7.812

Table 2

Average area under the curve of each signal.

Nitrite concentration (nM)	Signal area under curve (AUC) (AU)
7.812	0.116
15.625	0.221
31.25	0.4569
62.5	0.897
125	1.912
250	3.76
500	7.59

The area of the blank value (water) 0.011 is subtracted from the area of all the nitrite standards. A.U. Arbitrary Units