Video Article

Measuring Nitrite and Nitrate, Metabolites in the Nitric Oxide Pathway, in Biological Materials using the Chemiluminescence Method

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Abstract

Nitric oxide (NO) is one of the main regulator molecules in vascular homeostasis and also a neurotransmitter. Enzymatically produced NO is oxidized into nitrite and nitrate by interactions with various oxy-heme proteins and other still not well known pathways. The reverse process, reduction of nitrite and nitrate into NO had been discovered in mammals in the last decade and it is gaining attention as one of the possible pathways to either prevent or ease a whole range of cardiovascular, metabolic and muscular disorders that are thought to be associated with decreased levels of NO. It is therefore important to estimate the amount of NO and its metabolites in different body compartments — blood, body fluids and the various tissues. Blood, due to its easy accessibility, is the preferred compartment used for estimation of NO metabolites. Due to its short lifetime (few milliseconds) and low sub-nanomolar concentration, direct reliable measurements of blood NO *in vivo* present great technical difficulties. Thus NO availability is usually estimated based on the amount of its oxidation products, nitrite and nitrate. These two metabolites are always measured separately. There are several well established methods to determine their concentrations in biological fluids and tissues. Here we present a protocol for chemiluminescence method (CL), based on spectrophotometrical detection of NO after nitrite or nitrate reduction by tri-iodide or vanadium(III) chloride solutions, respectively. The sensitivity for nitrite and nitrate detection is in low nanomolar range, which sets CL as the most sensitive method currently available to determine changes in NO metabolic pathways. We explain in detail how to prepare samples from biological fluids and tissues in order to preserve original amounts of nitrite and nitrate present at the time of collection and how to determine their respective amounts in samples. Limitations of the CL technique are also explained.

Video Link

The video component of this article can be found at https://www.jove.com/video/54879/

Introduction

Nitrite, and to a less extend nitrate, levels in blood reflect overall state of body NO metabolism. Nitrite concentrations in blood and most organs and tissues are only in high nanomolar or low micromolar range, nitrate is usually present in much higher amounts — in micromolar range. Changes in nitrite levels due to disease progression or changes in dietary habits are quite small and can be only measured using a very sensitive method. Because of their very different levels and different metabolic processes, separate determination of nitrite and nitrate levels is essential. So-called "NO_x determination" where nitrite and nitrate are measured together has very little value.

Several methods for quantifying nitrite in various biological samples have been developed — the most common being the oldest one, based on the Griess reaction that had been originally described in 1879. Even with modern modifications, the sensitivity limit for nitrite attainable by Griess' method is in low micromolar range. Chemiluminescence (CL), combined with tri-iodide reducing solution, is currently considered the most sensitive method, allowing quantification in the low nanomolar range of nitrite concentrations[1-8,10,11]. The same CL method, combined with vanadium(III) chloride reducing solution, can be used for sensitive measurements of nitrate, with precision in the nanomolar range[9].

CL detects free gas NO. Therefore, nitrite, nitrate, R-nitrosothiols (R-SNO), R-nitrosoamines (R-NNO), or metal-NO compounds (later in manuscript referred as "R-(X)-NO"), must be converted into free NO gas in order to quantify their original amounts via CL. Conversion to NO is achieved using several different reducing solutions, depending on the nature of the NO metabolite. After conversion, free NO gas is purged from the reaction vessel by a carrier gas (He, N_2 or Ar) into the reaction chamber of CL analyzer where ozone (O_3) is combined with NO to form nitrogen dioxide (NO_2) in its activated state. With return to the ground state, NO_2* emits in infrared region and emitted photon is detected by photomultiplier (PMT) of CL instrument. The intensity of emitted light is directly proportional to NO concentration in reaction chamber, which allows calculation of the concentration of the original species using proper calibration curves.

In our protocol, we first present CL-based determination of nitrite and nitrate in the most used clinical settings — in blood and plasma, and then we discuss how to determine these ions in tissue samples. We also explain in detail how to preserve the original physiological nitrite concentration in nitrite-reactive environments, such as blood and its compartments, plasma and red blood cells.

Protocol

All protocols including use of animals were approved for use by NIDDK Animal Care and Use Committee and human blood was obtained from NIH Blood bank from healthy donors.

1. Sample Preparation

1. **Preparation of nitrite preserving solution**
    1. Prepare a solution containing 890 mM potassium ferricyanide ($K_3Fe(CN)_6$) and 118 mM NEM (N-ethylmaleimide) in distilled water. Dissolve well until it is clear yellow with no crystals present. Add NP-40 (octyl phenoxylpolyethoxylethanol) in a 1:9 ratio (v/v, NP-40/ solution). Mix gently to avoid foaming and store at 4 °C for about a week).

2. **Collection and preparation of blood samples**
    1. Collect blood using at least a 20 G needle to avoid hemolysis with heparin to prevent coagulation (5 U/ml). For the whole blood sample, mix blood with nitrite preserving solution immediately in a 1:4 ratio (v/v solution/blood).
    2. For plasma and red blood cells samples, centrifuge the collected blood for 5 min at 4,000 x g at 4 °C to separate red blood cells (RBC) and plasma. Take the supernatant (plasma) and mix it with nitrite preserving solution as described above for determination of nitrite levels in plasma.
    3. Carefully remove remaining plasma and buffy coat from the sample and pipette RBC sample from the bottom of the tube to avoid contamination by other types of blood cells using a cut-off pipette tip, and transfer it into a tube containing nitrite preserving solution in the same ratio as above.
    4. Mix each sample (plasma and RBC) with cold methanol at a ratio 1:1 or 1:2 (v/v sample/methanol) and centrifuge them at 13,000 x g at 4 °C for 15 min to precipitate proteins. Take the supernatant and use it for nitrite measurement or freeze prepared samples, if needed, on dry ice and store at -80 °C.
    Note: Nitrite rapidly reacts with oxyhemoglobin (oxyHb) in blood forming nitrate. Since oxyHb is always present in large excess over nitrite, this reaction leads to almost complete annihilation of native blood nitrite with a time frame of ~ 10 min. In order to preserve most of endogenous blood nitrite, nitrite-preserving solution is added to the whole blood samples immediately after the blood draw. The solution will rapidly lyse red blood cells and oxidize oxyHb into methemoglobin (metHb), an inactive form of hemoglobin that does not chemically react with nitrite.

3. **Preparation of samples from other types of body fluid**
    1. After sample collection into appropriate container, mix well with nitrite preserving solution, deproteinate and measure immediately or freeze and store at -80 °C.

4. **Collection and preparation of tissue samples**
    1. Collect tissues from animals perfused with heparinized saline solution (10 U heparin/ml). Excise about 1 g of desired tissue and homogenize either using a manual homogenizer or an automated homogenizer.
    2. Add known amount of nitrite preserving solution as needed to achieve smooth homogenate.
    3. Once tissue is homogenized, precipitate proteins using cold methanol (1:1 or 1:2 ratio v/v sample/methanol) as described above, then centrifuge samples at 13,000 x g at 4 °C for 15 min.
    4. Collect supernatant and measure nitrite levels. If needed, freeze samples at any stage of preparation on dry ice and store at -80 °C.

2. Preparation of Reducing Solutions

1. **Tri-iodide (I_3) reducing solution for nitrite and R-(X)-NO species determination**[1,3,4]
    1. Prepare a solution of 301 mM KI together with 138 mM I_2 solution in water. Mix with glacial acetic acid in 2:7 ratio (v/v solution/acid) on a magnetic stirrer for ~ 30 min until all crystals are dissolved. Preferably keep in dark bottle, as iodide is light sensitive, and use within one week from preparation.
    Note: This solution will reduce nitrite into NO and it will also release NO from R-SNO, R-NNO and Fe-NO functional groups (R-(X)-NO), but nitrate will not be affected. The signal from the above NO-based functional groups can be separated from true nitrite signal by treatment of half of the sample with acidified sulfanilamide (AS) and comparing AS-treated and untreated signal. AS irreversibly forms diazonium cation with nitrite and this complex cannot be reduced by I_3 solution. For AS treatment, prepare 290 mM solution of sulfanilamide in 1 M HCl and add it to an aliquot of sample in ratio 1:9 (v/v AS/sample). Measure the CL signal in AS-treated and untreated parts of the sample. Calculate the true nitrite signal as a difference of AS-treated and untreated part of sample. Nitrite can be also measured by using a selective nitrite-reducing solution of ascorbic / acetic acid mixture as described in part 2.2. However, due to the usually small amount of R-(X)-NO species present, measurements using AS-untreated samples are in most cases a very good approximation of total nitrite content.

2. **Ascorbic acid / acetic acid (4A) solution for selective determination of nitrite**[2]
    1. Prepare 500 mM ascorbic acid in water. Mix this solution with glacial acetic acid in a ratio 1:7 (v/v, ascorbic acid / acetic acid) to prepare the reaction mixture.
    Note: This solution is specific for nitrite, will not release NO from any R-(X)-NO species or nitrate. However, the solution of ascorbic and acetic acid must be prepared freshly every day before the measurements, as ascorbic acid easily oxidizes in solution. Completion of nitrite reduction depends on ascorbic acid concentration; and at least 50 mM ascorbic acid is recommended for complete reduction of plasma nitrite. However, it is recommended to perform a few pilot experiments with different concentrations of ascorbic acid and nitrite standards in the expected nitrite concentration range prior to final sample measurements. Keep in mind that

ascorbic acid slightly depletes during the measurements, so frequent changes of reaction mixture in the glass reaction chamber are recommended.

3. **Vanadium(III) chloride reducing solution for nitrate determination**[9]
    1. Prepare 51 mM solution of VCl_3 in 1 M HCl. Filter solution through 200 µm filter and store in a dark bottle, use within two weeks.
    Note: This solution will reduce nitrate, nitrite and all R-(X)-NO species, so if comparable amounts of nitrite or other R-(X)-NO species are present together with nitrate, the final nitrate content has to be calculated as the difference between the CL signals obtained with VCl_3 and I_3 reducing solutions.

3. Chemiluminescence (CL) Analyzer Setup and Measurements

1. **Standard solution**
    1. Prepare 1 µM solution of sodium nitrite ($NaNO_2$). The same solution can be used for nitrite and nitrate determinations.

2. **Using NO analyzer**
Note: Currently there are two commercially available NO analyzers that are sensitive enough for biological research purposes — Sievers NOA and Ecophysics CLD 88Y. They both operate on the same principle; the main difference is that CLD 88Y uses oxygen from the room air to make ozone (O_3), while NOA requires an external oxygen tank for this purpose. The procedure below describes the set up for the Sievers NOA.
    1. **Instrument setup**
        1. Perform initial set up of the NO analyzer according to the manufacturer's recommendations as seen on **Figure 1**.
        2. Open O_2 tank connected to the instrument. Choose "analysis" and "enter" from the main menu on the front panel. On the next screen choose "start" and press "enter." Connect the acid trap (containing 1 M NaOH) to the instrument as seen in **Figure 1**.
        3. Wait until photomultiplier cools to temperature below -12 °C and the reaction chamber is evacuated to 6 Torr. It takes about 30 min to reach a stable flat baseline. The baseline needs to stabilize within 1 - 2 mV, its nominal value is less important than its stability.
        Note: If nitrate is measured, turn on the cooling water bath (set at 4 °C, which serves as cold trap for acid and water vapors) and heating water bath (the main reaction chamber, 95 °C). The rate of the reduction of nitrate into NO in VCl_3 solution is temperature-dependent, and it is very slow at room temperature, so substantial increase of temperature is needed to observe the reaction.
        4. Open the He tank and connect the glass reaction chamber to acid trap as seen on **Figure 1**. Fill the reaction chamber with appropriate reducing solution, reduce the bubbling to minimum and connect reaction chamber to the acid trap. Using trial and error, adjust the He bubbling rate to match the instrument suction rate (usually ~ 200 ml/min for Siever's NOA).
        5. Turn on the computer controlling the instrument and start Labview-based Liquid software. To turn on communication between the instrument and the acquisition software, press "enter" when in "data menu" until screen reads "output enabled", then press "clear" to return back to data screen.
        6. Wait for a stable baseline trace on the screen and start injecting samples and nitrite standards into reducing solution through the septum. Always wait to reach a stable baseline after the peak. This can take up to 2 min. The Liquid software has an option to "mark" times of injection and annotate the injection and it will export these comments into a separate file (filename.info) as a useful complement to the data file (filename.data).
        7. Once samples and standards are measured, choose "stop" option in the Liquid software menu — this will write data from a temporary file into a permanent file known as "filename.data". Pressing "abort" option will terminate measurement without writing acquired data into a permanent file.
        8. To terminate experiment, disconnect the machine from the reaction vessel by turning off the stopcock on the top of reaction vessel and disconnect the tubing between acid trap and reaction vessel (see **Figure 1**). Now stop NOA analyzer — press "clear", go to "analysis", put machine on "standby" and "confirm" standby. If used regularly, the instrument can be left in standby mode for several weeks. Turn off supply of oxygen. Then flush the reducing solution from the reaction chamber, disconnect and close the He tank from the glass reaction vessel and finish cleaning reaction vessel.

    2. **Standards and sample injections**
        1. Inject 50 µl of 1 µM nitrite solution into reaction mixture using a well-washed Hamilton syringe. Wait for at least 1 - 2 min between injections to achieve good separation of peaks. Repeat injection of 50 µl to get duplicates to each data point. Wash syringe with deionized water after each injection.
        2. To acquire full set of data for standard curve, continue with duplicate injections of 100, 150 µl (additional 200 µl might be needed if high concentrations of nitrite or nitrate are expected) of 1 µM nitrite solution. In each case, take care to wait until the signal drops back to the baseline and then acquire 1 - 2 min of baseline to achieve good separation of peaks.
        Note: Nitrite standards can be measured at any time during experiments. However, it is preferable to acquire the standard curve before data measurement, as it also serves as an independent check of instrument functionality.
        When data are collected, amount of sample injected should lead to well pronounced peaks. However it should be kept in mind that the photomultiplier is linear only up to ~ 800 mV, so none of the peak should be higher than ~ 700 mV. This can be achieved either by decreasing of amount injected sample or diluting the sample with deionized water. Each point should be measured at least in duplicate.

Representative Results

Figure 2 shows representative results collected from standards and five different samples. As shown in this figure, photomultiplier voltage increases immediately after nitrite-containing solution (standards or samples) is injected into reducing solution (injections times are indicated by red arrows below the curve) and returns to the baseline value once all nitrite present in the injected solution was reduced. It is also clear from this figure that accurate volume measurements are necessary to obtain highly reproducible data. We recommend using precision Hamilton syringes to measure injection volumes. The loss of reducing capacity of I_3 (or ascorbic acid or VCl_3) solution is another common source of error. As a general rule, when baseline width of peaks starts to widen considerably, reducing solution in reaction chamber has to be changed. The width of peak depends on the gas flow into the NOA reaction chamber (marked as RC on **Figure 1**), and it slightly varies from one experimental set up to another. We consider the normal width to be around 1 min for nitrite measurements and up to 2 min for nitrate measurements.

In order to relate the signal from the photomultiplier with amount of NO detected, a standard curve is constructed as a plot of the area under the peak and the amount of nitrite (in pmol) injected as seen on **Figure 3A**. To measure the area under the peak any suitable software can be used, such as Origin, Excel or like. The slope of this curve, K (marked in red in **Figure 3B**), gives the amount of pmol of NO causing increase of 1 mV of photomultiplier (PM) signal. The advantage of using the slope of the curve, rather than area under peak that is related to fixed amount of NO, is increased precision. The standard curve is constructed from at least 3 different points, each of them being measured in duplicate and if there is some residual nitrite or nitrate in the water used to prepare the standard solution, using K eliminates the necessity of corrections to this residual amounts. Also, following our initial tests of NOA instrument for its PM linearity, we determined that linear response occurs up to 700 - 800 mV. Therefore, our standard curve is valid for all signals from samples up to this PM range. The linearity range depends on PM and may change with time. It needs to be determined before the instrument is first used and tested as PM ages every couple years.

Data collected from samples are processed in a similar way as data collected for standard curve: First, the area under the peak is determined. Then this area is divided by the slope K of the standard curve, which gives the number of pmol of NO originated from the amount of injected sample.

The necessary adjustments for the dilution of the original sample by nitrite preserving solution, deproteination, or any other necessary dilutions are made. The result is usually expressed as nitrite or nitrate concentration (nM or μM) for biological fluids (blood, urine) or as picomoles / gram of tissue in case of solid samples as shown in **Figure 3B**. Data are then plotted similarly to the example in **Figure 3B**. In the example given in **Figure 3B** we plotted results from 5 individual samples shown in panel A. Here we plot average values from 2 injections and give the SD to show the reproducibility of the individual point measurements.

For samples in the **Figure 3**, S1, S2 and S4 are rat blood and S3 and S5 rat liver. **Figure 3C** shows the final results plot together with SD and expressed in nM (for blood, n = 3) or pmol/mg tissue (for liver, n = 2).

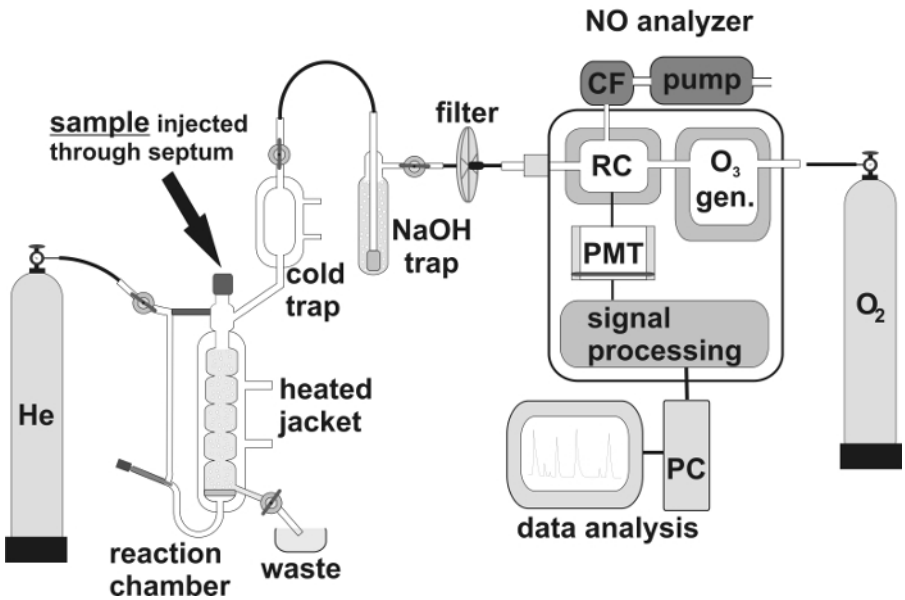

Figure 1: Setup of Sievers NOA Chemiluminescence Instrument. Reaction vessel is filled with I_3 (ascorbic / acetic acid or VCl_3) solution with He carrier gas gently bubbling through. Sample is injected using Hamilton syringe through septum into I_3 solution where NO-related components are reduced to NO gas and carried into NO analyzer. Cold trap, NaOH-filled trap, and filter protect analyzer against humidity and acid vapors. In reaction chamber (RC) NO gas is combined with O_3 (generated in O_3 generator) from oxygen from O_2 tank. Chemiluminescence signal from NO_2^* is detected by photomultiplier tube (PMT) and further amplified and processed. Data acquisition and analysis are carried out on PC. Vacuum pump created low pressure in the reaction chamber (RC) and evacuates toxic NO_2 gas after chemiluminescence measurement through charcoal filter (CF). Please click here to view a larger version of this figure.

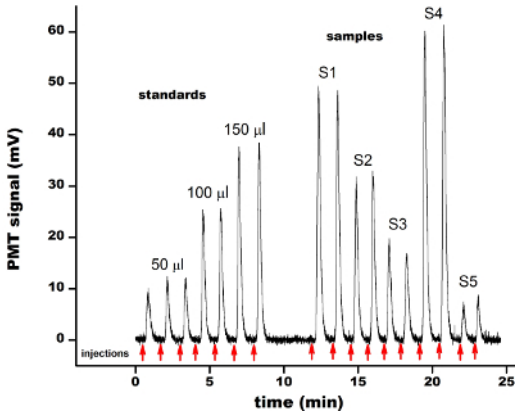

Figure 2: Representative Plot of NO peaks Generated by CL. This graph shows the photomultiplier (PMT) signal as a function of time. Peaks result from injections of various amounts of 1 µM nitrite solution (standards) and 100 µl of samples (S1 - S5) injected in duplicates and triplicate (50 µl of nitrite standard solution). Red arrows under the curve indicate times of injections. Please click here to view a larger version of this figure.

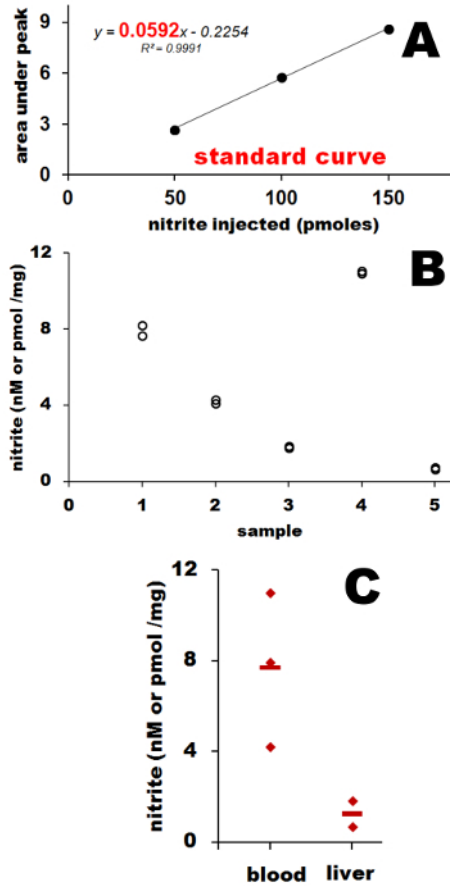

Figure 3: Standard Curve (A) and Representative Results from Rat Blood and Liver (B), Final Results Plot (C). Standard curve (**panel A**) was obtained by injecting 50, 100 and 150 µl of 1 µM nitrite solution in water, measuring the area under peaks. The slope, K (red number) gives nmol of NO required for a 1mV increase in photomultiplier voltage. Original peaks used for standard curve are in **Figure 2** and are marked as 50, 100 and 150 µl. **Figure 2** also shows the original injections for our samples — S1, S2 and S4 (dark gray) from rat blood, S3 and S5 from rat liver tissue, all injected in duplicate. Area under these peaks was measured and, after all corrections for dilutions were made as described in part 3.2.1., results shown in **panel B** for individual samples were calculated as amount of nitrite in pmol/g liver or in nM for blood. To appreciate the reproducibility of the chemiluminescence method, we plotted the averages and standard deviations for each individual sample. **Panel C** shows the final products nitrite and nitrate in rat blood and liver plotted as average of three individual samples for blood and two for liver together with standard deviation. Please click here to view a larger version of this figure.

Discussion

Critical Steps within the Protocol

Aliquots of all solutions (including the water) used to prepare, dilute or otherwise treat original samples have to be saved and checked for possible nitrite or (more often) nitrate contamination. We found that most contamination comes from water and many chemicals used to treat sample (ferricyanide in particular) also contain significant amount of nitrate contamination in some lots that interferes with the endogenous nitrate determination. We therefore check all of our chemicals for nitrite and nitrate contaminants before we use them in regular experiment.

Since samples might be subjected to substantial dilutions several times during treatments, all sample manipulations need to be documented for final concentration calculations. Most mistakes are made in this part of the protocol.

When handling samples for nitrite measurements, quick transfer into nitrite preserving solution is crucial, especially when heme-containing proteins, such as hemoglobin, that reacts with nitrite and oxidizes it into nitrate, are present. Once stabilized, samples can be frozen for prolonged storage before assays.

Amounts of several NO metabolites in biological samples (especially R-X-NO) are very low, sometimes the levels of generated NO are at the background noise level of the NOA analyzer. It is always preferable to prepare samples with as little dilution as possible if such compounds are to be measured.

Limitations of the Technique

With careful sample preparation and injections, the low limit of sensitivity is close to 20 nM of NO adduct present in the fully prepared sample. The usual biological concentrations of nitrite and nitrate do exceed these concentrations, however, the R-(X)-NO amounts might fall close to this range.

CL's high sensitivity demands careful sample preparation and precise volume measurements.

Significance of CL with Respect to Alternative Methods of NO Metabolites Measurements

Chemiluminescence (CL) is a very sensitive method to detect NO, nitrite, nitrate and R-X-NO. Currently, CL is considered the gold standard in determination of NO and its metabolites.

Other common alternative to determine nitrite is Griess reaction (GR). GR is a convenient and inexpensive colorimetric method based on diazotization reaction described by Peter Griess in 1879. Analysis of nitrate requires prior chemical or enzymatic reduction of nitrate to nitrite. Best current commercially available kits have sensitivity around 100 nM for nitrite, sensitivity of most kits allowing to determine nitrate and nitrate is in low µM range. When using GR to determine nitrite and nitrate in sample, two steps are required; first, determine the nitrite amount in first aliquot of sample, then use second aliquot to reduce nitrate into nitrite and measure total nitrite and nitrate (sometimes referred as NOx) content in sample. True nitrate value is the difference of both measurements. Better alternative to this two steps protocol is prior separation of nitrite and nitrate by chromatography. However, this considerably decreases the sensitivity and increases the analysis time.

Future Applications

With growing evidence about the significance of NO pathway in biological system, we foresee the more frequent use of nitrite or nitrate or other NO metabolites as biomarkers of cardiovascular health. Increased evidence also suggests that these molecules could be important in exercise medicine and their levels might be changed in people with diabetes, obesity and metabolic syndrome.

Disclosures

Dr. Alan Schechter is listed as a co-inventor on several patents issued to the National Institutes of Health for the use of nitrite salts for the treatment of cardiovascular diseases. He receives royalties based on NIH licensing of these patents for clinical development but no other compensation.

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