Original Research

Stability and Reproducibility of the Measurement of Plasma Nitrate in Large Epidemiologic Studies

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Inorganic nitrate has emerged as a therapeutic agent for cardiovascular disease; however, nitrate can also metabolize to carcinogenic nitrosamines under pathologic conditions. Few large epidemiologic studies have examined circulating levels of nitrate in relation to cardiovascular disease and cancer. Data on the validity of nitrate measurement in blood samples collected in typical epidemiologic settings are needed before nitrate can be evaluated as an exposure in large epidemiologic studies.

We measured plasma levels of nitrate in three pilot studies to evaluate its laboratory variability, stability with delayed processing, and reproducibility over time among women from the Nurses' Health Study and healthy female volunteers.

Laboratory variability of nitrate levels was fairly low, with a coefficient variation (CV) of 7%. Plasma nitrate levels in samples stored as whole blood on ice for up to 48 hrs before processing were very stable; the overall intra-class correlation (ICC) from 0 to 48 hours was 0.89 (95%CI, 0.70-0.97). The within-person reproducibility over a one-year period was modest, with an ICC of 0.49 (95% CI, 0.33- 0.94).

Our results indicate that measurement of nitrate in plasma is reliable and stable in blood samples with delayed processing up to 48 hours. Within-person reproducibility was modest but data from this study can be used for measurement error correction in subsequent analyses. The measurement of nitrate cannot be widely used in epidemiologic research without the documentation of its stability and reproducibility.

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INTRODUCTION

Nitrate (NO_3^-) and nitrite (NO_2^-) are naturally occurring ions that contain nitrogen and oxygen. In the past few decades, nitrate and nitrite are considered to be potentially harmful; however, nitrate and nitrite have received enormous attention in the past few years due to its therapeutic potential. Nitrate can be reduced to nitrite and then to nitric oxide (NO); as shown in **Figure 1**, this nitrate-nitrite-NO pathway has emerged as a new therapeutic pathway for coronary heart disease.[1,2] Nitrate supplements have recently been shown to reduce blood pressure and inhibit platelet aggregation, prevent myocardial infarction, stroke, improve endothelial cell function[3] and stomach ulcer in clinical intervention studies and animal models.[4-7] Because nitrate-derived nitrite can also react with amine to form carcinogenic nitrosamine in addition to converting to nitric oxide,[8] there are still concerns as to whether increased intake of nitrate or using nitrate supplementation can increase risk of cancer.

Until now, there are several prospective studies examining the role of nitrate intake relation to risk of different types of cancer, the results were not consistent.[9,10] However, some researchers failed to recognize that nitrate intake cannot reflect physiological levels of nitrate, which come from both endogenous and exogenous sources. The endogenous NO can be oxidized to nitrite and then to nitrate as shown in **Figure 1**. Many exogenous factors contribute to the circulating levels of nitrate. For instance, vegetable intake account for more than 70% of daily nitrate exposure in humans.[11] Nitrate in drinking water and the preservative of nitrate in processed meat also contribute to circulating nitrate levels. Therefore, assessing circulating levels of nitrate in relation to risk of cancer in large prospective epidemiologic studies may better evaluate the role of nitrate in the development of cancer. Unfortunately, these types of studies are currently lacking. Stability and reproducibility measurement of nitrate in samples collected in typical epidemiologic settings are needed to be evaluated before we apply this marker to large epidemiologic studies.

In blood or plasma, the half life of nitrate, nitrite, and NO vary significantly, with 5-8 hours for nitrate, 1-5 minutes for nitrite, and 1-2 miliseconds for NO.[12] Thus, plasma/serum

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nitrate can be potentially used as a biomarker for epidemiologic studies; whereas nitrite and NO are more difficult to measure. Although nitrate is generally considered to be stable, it is essential to systematically document the stability of the measurement of plasma nitrate in blood samples collected in different anticoagulant tubes and processed at different time after blood draw. Furthermore, only one blood sample per participant is available in most epidemiologic studies although exposure over a longer-term is often of interest. Thus, evaluating whether measurements of nitrate are reproducible within individuals over time will provide valuable information for epidemiologists to assess whether a single measure of nitrate is an adequate measure of longer-term exposure.

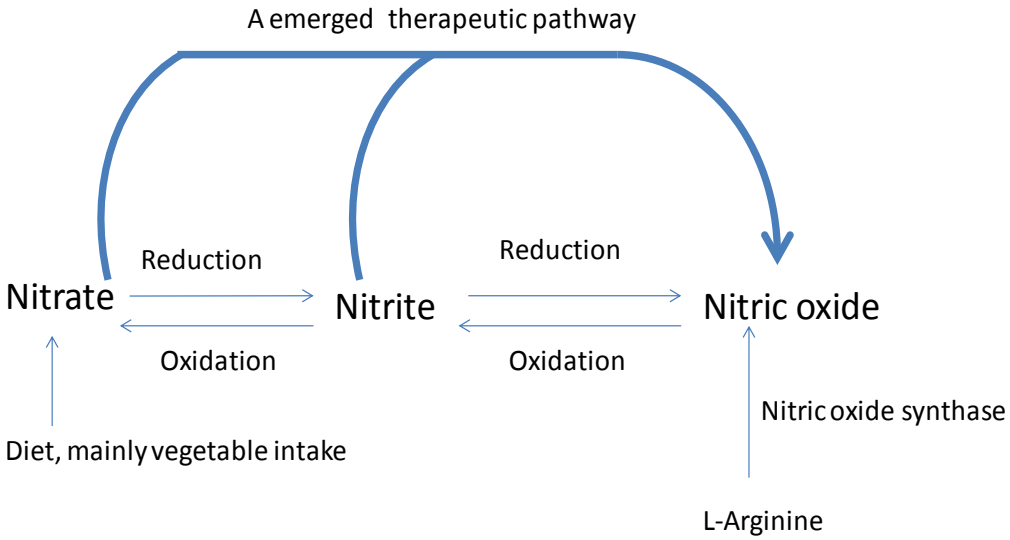

Figure 1. Many intervention studies have used inorganic nitrate or nitrite supplements to treat cardiovascular related disorders using the intrate-intrite-nitric oxide pathway.

METHODS

Nurses' Health Study. The Nurses' Health Study (NHS) began in 1976 when 121,700 nurses returned a mailed questionnaire about their medical history and health behaviors. Updated information has been collected using biennial questionnaires. From 1989 to 1990, blood samples were collected from 32,826 nurses.[13] Briefly, blood samples were collected in sodium heparin tubes and shipped on ice packs to our laboratory via overnight mail. Samples were then processed and separated into plasma, packed red blood cells, and buffy coat, and archived in liquid nitrogen freezers (<130 ^{o}C).

Study population for assessment of assay variability. To evaluate assay variability, the NHS blood lab provided blinded duplicates to assess assay variability. Dr. Wu's lab, the assay lab, was blinded to the identification of which samples were duplicates. We used samples from 12 individual NHS participants as well as quality control (QC) samples from two large plasma pools. Participant and QC samples were aliquoted into two tubes and placed randomly into boxes.

Study population for assessment of the stability of whole blood samples stored on ice for up to 48 hours. Blood samples were collected from six healthy female volunteers (i.e., not NHS participants). For each volunteer, half of the blood was collected in EDTA tubes (three tubes), and the other half was collected in heparin tubes (three tubes). The first of each type of anticoagulant tube was immediately centrifuged at 1530 g for 20 min at 4 °C. After centrifugation, the plasma was removed and aliquots were placed in cryotubes and stored in liquid nitrogen freezers (-130 °C). The second and third tubes were shipped with an ice pack to the laboratory overnight and then processed and frozen 24 h and 48 h, respectively, after blood collection. These processing methods were designed to mimic collection procedures used in large cohort studies, such as the NHS.

Population for within-person reproducibility study. A subset of 225 NHS healthy participants who gave a first blood sample in 1989-1990 provided two additional samples over the following 2 to 3 years. For the current study, we randomly selected a subset of 38 women who provided two blood samples approximately 1 year apart. Given the potential for dietary intake to cause acute changes in nitrate, we restricted this assessment to samples that were drawn at least 8 hours after the last meal. Samples were processed within 24-30 h of collection.

Laboratory assessment of nitrate. The original method (Griess assay) to assess plasma nitrate was documented by Wishnok et al., 1996.[14] The main reagents used for this assay are nitrate reductase, lyophilized reduced β-nicotinamide adenine dinucleotide (NADH) and Griess reagents. Griess reagents include sulfanilamide in hydrocholoric acid and N-(1-Naphtyl) ethylenediamine in hydrochloric acid. The main

instrument for detecting nitrate levels is a 96-well plate reader from Molecular Device, called SpectraMax which can detect fluorescence, chemiluminescence, uv, visual at any wavelength with highly sensitivity. This assay directly determines the total nitrite concentration by enzymatic conversion of nitrate to nitrite by nitrate reductase. The reaction is followed by colorimetric detection of nitrite as an azo dye product of the Griess Reaction. The Griess reaction is based on the two-step diazotization reaction in which acidified NO_2^- produces a nitrosating agent, which reacts with sulfanilic acid to produce the diazonium ion. This ion is then coupled to N-(1-Naphtyl) ethylenediamine to form the chromophoric azo-derivative which absorbs light at 540-570 nm. This assay gives the total amount of nitrite and nitrate. However, nitrite is not very stable, and can be quickly oxidized to nitrate after blood collection if the samples are not processed immediately. In the NHS cohort study, the blood samples were processed 24-48 hr after the blood was collected; therefore, the nitrite was already converted to nitrate during these periods. Additionally, using colorimetric detection, the total nitrite and nitrate levels were observed to be in the μM range, whereas the nitrite level was expected to be in nM range. Therefore, the amount of nitrate and nitrite that we measured reflects the nitrate levels. The inter-assay and between-assay variability were less than 7%.

Statistical analyses

Assessment of assay variability. To assess laboratory error, we calculated the coefficients of variation (CV) for nitrate levels for each duplicate (12 donors and 2 QC pools) and the overall CV was calculated by averaging the CVs across the 14 duplicate samples.

Assessment of the stability of plasma nitrate from whole blood samples stored on ice up to 48 hrs. For this aim, we included the 0-, 24-, and 48-h samples from 6 donors (with two types of anticoagulant tubes: 3 EDTA and 3 heparin tubes per participant). We calculated the mean nitrate concentrations in samples processed at each time point and compared differences between 0-24 h and 0-48 h using a paired t-test. We also calculated the intraclass correlation coefficients (ICC) among nitrate concentrations across the three time points, as well as by preservative type. To estimate the between- and within-person variances, we used random effects models, with participan ID as the random variable. ICCs were calculated by dividing the between-person variance by the sum of the within- and between-person variances. To improve the normality of nitrate values, we used the natural log-transformed values in the ICC tcalculations. The delta method was used to calculate the 95%CI interval for the ICCs.[13]

*Assessment of within-person reproducibility over time***.** To assess reproducibility of nitrate, we calculated the Spearman correlation and ICC for nitrate over an approximately one-year period. We adjusted the ICC for the number of fasting hours by including time since last meal as an independent variable in the random effects model. Additional adjustment for age at blood draw, body mass index at blood draw, and time of blood collection did not change the results and thus, thus they were not included in the final model.

RESULTS

Assessment of assay variability. Among these samples, 6 participants had fasted over 8 hrs, 6 had less than 8 hrs and 2 with unknown fasting status. The mean plasma nitrate levels across the 14 samples processed in duplicate ranged from 14.3 to 46.3 μM. The overall CV was 7.4%, indicating fairly low laboratory error.

Assessment of stability of plasma nitrate measured in samples stored as whole blood on ice up to 48 hrs. The fasting status among these subjects was mixed, the hour after last meal before blood draw was not recorded. Delays in processing up to 48 hrs appeared to have minimal influence on the measurement of nitrate (**Table 1**). Nitrate levels in samples processed after 24 and 48 hrs were not significantly different from levels in samples processed immediately (p = 0.2 and 0.5, respectively). The ICC was 0.87 between the 0 and 24 hr samples, and 0.89 between the 0 and 48 hrs samples. The overall ICC across all three time points was 0.89 (0.80 for EDTA tubes and 0.95 for heparin tubes).

Table 1. Stability of whole blood samples stored on ice from 0 to 48 hours before processing (n =12).

	Anticoagulant	Median (minimum-maximum)			P for paired t test		ICC* (95%CI)		
	tubes	0 hrs	24 hrs	48 hrs	0-24 hrs	0-48 hrs	Overall	0-24 hrs	0-48 hrs
Nitrate (μM)	Combined (n=12)	27.1 (19.8-43.2)	26.0 (20.5-41.5)	26.1 (20.1-42.2)	0.2	0.5	0.89 (0.70-0.97)	0.87 (0.64-0.96)	0.89 (0.68-0.97)
	EDTA tube (n= 6)	30.6 (22.1-43.2)	26.1 (22.1-38.8)	26.1 (20.0-38.4)	0.3	0.2	0.80 (0.48-0.95)	0.73 (0.29-0.95)	0.74 (0.30-0.95)
	Heparin tube (n = 6)	26.0 (19.8-42.2)	24.1 (20.5-41.5)	29.1 (21.1-42.2)	0.3	0.1	0.95 (0.81-0.99)	0.98 (0.92-1.00)	0.95 (0.78-0.99)

*ICC, intra-class correlation

Table 2. Within-person reproducibility of nitrate measurement over one-year period.

			nitrate, mM (median, inter-quartile range)	
	ICC*	**Spearman#**	**1st collection**	**2nd collection**
Overall (n = 38)	0.49 (0.26-0.71)	0.55	47.3 (33.2-56.2)	38.1 (30.0-48.3)

*ICC, Intra-class correlation

#Spearman correlation between first and second blood collection

Assessment of within-person reproducibility over one year.

Characteristics of the 38 sampled women. At the time of the first blood collection, the age range of these women was 51-71 years. Fifty-three percent of women had a body mass index of 25 kg/m^2 or higher. Over the two collections, most samples were collected in the morning, and all samples were collected after fasting for at least 8 hrs.

Reproducibility of nitrate level measurement over time. The mean time between collections was 15 months (range 10-25 months). For the 38 participants, the ICC of nitrate between the two samples was 0.49 (**Table 2**), after adjustment of fasting hrs and 0.48 without adjustment of fasting hrs (not shown in the table).

DISCUSSION

In this assessment of the feasibility of using plasma nitrate as a biomarker in epidemiologic studies, we found fairly low laboratory variability, good stability with delayed processing, and modest within-person reproducibility over time.

We documented fairly low laboratory variability and high stability with delayed processing. Accordingly, a CV% of 7% among duplicate samples and ICC of 0.89 among samples processed after delays of up to 48 hours was reasonable. Though ICCs in EDTA and heparin anticoagulant tubes are both acceptable, the higher ICCs in heparin tubes indicate that heparin may be a better anticoagulant when measuring nitrate. It has been shown in Nagababu et al that EDTA anticoagulant[15] contains higher amount of nitrite than heparin tubes. How EDTA tubes influencing nitrate measurement at different times of blood processing has not been documented previously.

We have found that over a one-year period, the reproducibility of plasma nitrate levels was modest (ICC = 0.49). This ICC is still comparable to the stability of some traditional diagnostic markers such as blood glucose (ICC= 0.52)[16] and pulse (ICC = 0.49).[16] Further, the nitrate levels associated with any disease outcome can be corrected using the ICC obtained in this study, as has been shown previously.[17] Although this study was conducted in women, it may provide valuable information for a cohort of men.

The Griess assay combined with colorimetric detection is more simple and easy to use than combined with gas chromatography-mass spectrometry (GC-MS) and liquid chromatography (LC) detection. However, this assay is not sensitive enough to detect nitrite. Given the typical blood collection feature in large epidemiologic studies, using GC/LC may not be necessary. If blood samples were not processed and treated immediately, nitrite can be oxidized quickly and not be detected. Another limitation is that, in the one-year reproducibility study, we do not have power to adjust confounding factors that influence nitrate levels. These factors will add random variability to the data and most likely attenuate the ICC. However, we measured the fasting plasma levels of nitrate to evaluate one-year reproducibility, therefore plasma nitrate would not be influenced by acute dietary intake but potentially influenced by usual dietary intake and medications such as nitroglycerin that may change circulating nitrate or nitrite. For this reason, a cohort with repeated blood collections over time may be ideal for determining the associations between nitrate levels and disease outcomes.

Measuring circulating levels of nitrate is not only valuable for evaluating nitrate exposure and risk of cancer but also essential for monitoring changes of nitrate in nitrate intervention studies. In summary, our data provide evidence that plasma levels of nitrate can be reliably measured and used for large epidemiologic studies. Our data suggest that fasting plasma levels of nitrate are reasonably reproducible within subjects over time, and is associated with low laboratory error.

CONFLKICT OF INTEREST

None.

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