Published in final edited form as:

Nitric Oxide. 2015 January 30; 44: 1–2. doi:10.1016/j.niox.2014.10.003.

Pitfalls in Measuring NO Bioavailability using NOx

Daniel B. Kim-Shapiro^{1,2} and Mark T. Gladwin^{3,4}

¹Department of Physics, Wake Forest University, Winston-Salem, NC 27109

²Translational Science Center, Wake Forest University, Winston-Salem, NC 27109

³Heart, Lung, Blood and Vascular Medicine Institute, University of Pittsburgh, Pittsburgh, PA 15213

⁴Department of Medicine, Division of Pulmonary, Allergy, and Critical Care Medicine, University of Pittsburgh, Pittsburgh, PA 15213

Keywords

Nitrite; Nitrate; Measurement

Given the importance of NO in signaling, measurement of its bioavailability is crucial to understanding many chemical, cellular, and physiological processes. However, direct measurement of steady state NO is often not feasible due to relatively low rates of steady state production and high rates of intravascular and cellular catabolism. Thus, it makes sense to assess NO production capacity using metabolites from reactions involving NO. In fact, commercial products are available that claim to measure total “NO levels”. Many of these measure the sum of nitrite and nitrate using a Griess-based colorimetric assay. While well-appreciated by vascular biologists and chemists, we caution the general scientific community that in most situations the sum of the concentrations of nitrite and nitrate do not accurately represent NO production rates, but in fact measure dietary levels of nitrate, renal clearance of nitrate, and artifactual formation or contamination of nitrite in lab and clinical glassware and additives (heparin solutions, vacutainers, etc). Furthermore, the use of any measure of nitrate or the sum of nitrate and nitrite (regardless of the technique employed) to assess NO bioavailability has no scientific basis.

Both nitrate and nitrite can be formed from reactions involving NO. Nitrate is formed through its rapid reaction with oxygenated heme proteins such as hemoglobin ($k = 6-8 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ [1])

© 2014 Elsevier Inc. All rights reserved.

Address correspondence to either: Dr. Kim-Shapiro (shapiro@wfu.edu, 336-758-4993, ¹Department of Physics, Winston-Salem, NC 27109) or Dr. Gladwin (gladwinmt@upmc.edu, 412-624-8725, E1244 BST, 200 Lothrop Street Pittsburgh, PA 15261).

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Nitrite is formed by autoxidation of NO

with a rate constant of $k = 9 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ [2]. At low NO concentrations, autoxidation is very slow. In plasma nitrite forms via oxidation reactions with ceruloplasmin [3]. Nitrite can also be formed by reaction with ferric hemes as a step in what is known as reductive nitrosylation. For example, in the case of methemoglobin,

which proceeds with kinetics described by a rate constant of $k = 3 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ [4], but faster kinetics have been reported under certain conditions [5]. These reactions are not the only ones whereby NO form nitrite and nitrate, but they are thought to be the major pathways under physiological conditions. However, a major consideration in using a compound as a marker for NO production is whether that compound is formed by other pathways that do not involve NO as a reactant.

In 1995, the Moncada group concluded that plasma nitrite in fasting human volunteers is mainly derived from endothelial nitric oxide synthase (eNOS) by administering isotope labeled arginine (the substrate for eNOS) and measuring isotope labeled nitrite in plasma [6]. In 2001, the Kelm lab provided strong evidence that plasma nitrite, but not nitrate, reflects NO production from eNOS in humans [7]. They both increased NO production using acetylcholine and decreased it using the NOS inhibitor N^G -monomethyl-L-arginine and observed a 71% increase and 60% decrease in plasma nitrite respectively along with expected changes in forearm blood flow [7]. Importantly, no changes in plasma nitrate were observed. The Kelm lab confirmed these results in other mammals, finding that about 70% of plasma nitrite is derived from eNOS. The inability of plasma nitrate to reflect production of NO is attributed to the fact that nitrate concentrations are largely influenced by dietary intake, liver metabolism, and nitrate formation by gastric bacteria [7]. One must use care in the measurement of plasma nitrite as a surrogate measure of steady state NO production rates. Nitrite is consumed via reactions with oxy- and deoxyhemoglobin [8] and so plasma must be rapidly centrifuged within 5-minutes of collection or the levels begin to drop. As mentioned above, nitrite frequently contaminates clinical and laboratory glassware. We use concentrated nitrite-free heparin in plastic syringes and falcon plastic tubes, which we have tested to not contain nitrite. Testing of labware and clinical vacutainers for contaminating nitrite is recommended.

We are unaware of any studies demonstrating that a substantial portion of plasma or tissue nitrate is linked to NOS function. Thus, nitrate should not be used to assess NO production. Moreover, since nitrate is usually present in 100 to 1000 molar fold excess to nitrite, measuring the sum of plasma nitrite and nitrate is not a proper way to measure NO

production either. In addition, we are unaware of studies that support that notion that the nitrite concentration in tissue homogenates (rather than blood or plasma) is indicative of endothelial NOS function. In most cases, plasma nitrite can serve this purpose and it is best to measure nitrite after fasting or, at least, after a low nitrate diet since high dietary nitrate intake can raise plasma nitrite levels several-fold due reduction of nitrate to nitrite by oral bacteria [9].

Acknowledgments

This work was supported by the NIH grants HL058091 and HL09832

References

1. Herold S, Exner M, Nauser T. Kinetic and mechanistic studies of the NO center dot-mediated oxidation of oxymyoglobin and oxyhemoglobin. *Biochemistry*. 2001; 40:3385–3395. [PubMed: 11258960]
2. Ford PC, Wink DA, Stanbury DM. Autoxidation Kinetics of Aqueous Nitric-Oxide. *Febs Letters*. 1993; 326:1–3. [PubMed: 8325356]
3. Shiva S, Wang X, Ringwood LA, Xu XY, Yuditskaya S, Annavajjhala V, Miyajima H, Hogg N, Harris ZL, Gladwin MT. Ceruloplasmin is a NO oxidase and nitrite synthase that determines endocrine NO homeostasis. *Nature Chemical Biology*. 2006; 2:486–493.
4. Hoshino M, Maeda M, Konishi R, Seki H, Ford PC. Studies on the reaction mechanism for reductive nitrosylation of ferrihemoproteins in buffer solutions. *Journal of the American Chemical Society*. 1996; 118:5702–5707.
5. Tejero J, Basu S, Helms C, Hogg N, King SB, Kim-Shapiro DB, Gladwin MT. Low NO Concentration Dependence of Reductive Nitrosylation Reaction of Hemoglobin. *Journal of Biological Chemistry*. 2012; 287:18262–18274. [PubMed: 22493289]
6. Rhodes PM, Leone AM, Francis PL, Struthers AD, Moncada S. The L-Arginine-Nitric Oxide Pathway Is the Major Source of Plasma Nitrite in Fasted Humans. *Biochemical and Biophysical Research Communications*. 1995; 209:590–596. [PubMed: 7794389]
7. Lauer T, Preik M, Rassaf T, Strauer BE, Deussen A, Feelisch M, Kelm M. Plasma nitrite rather than nitrate reflects regional endothelial nitric oxide synthase activity but lacks intrinsic vasodilator action. *Proceedings of the National Academy of Sciences of the United States of America*. 2001; 98:12814–12819. [PubMed: 11606734]
8. Grubina R, Huang Z, Shiva S, Joshi MS, Azarov I, Basu S, Ringwood LA, Jiang A, Hogg N, Kim-Shapiro DB, Gladwin MT. Concerted Nitric Oxide Formation and Release from the Simultaneous Reactions of Nitrite with Deoxy- and Oxyhemoglobin. *J Biol Chem*. 2007; 282:12916–12927. [PubMed: 17322300]
9. Lundberg JO, Weitzberg E, Gladwin MT. The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics. *Nature Reviews Drug Discovery*. 2008; 7:156–167.