Modified Microassay for Serum Nitrite and Nitrate

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Nitric oxide (NO) is produced in mammalian cells by one of three broad classes of nitric oxide synthase (NOS) (10). Large amounts of NO are generated by the cytokine-inducible isoform of NO synthase (iNOS) and have been implicated in cytotoxicity or cytostasis of activated macrophages toward parasites and tumors, in the pathology of septic shock, and in the suppression of cardiac function (10). Thus, assaying NO rapidly and efficiently in various fluids is of critical importance. High-output NO production by cells or its accumulation in biological solutions has been assayed in various ways (1–5, 7–9, 11). The simplest assay methods for relating NO production by cells or its accumulation in various fluids have relied on the reaction of NO in aqueous solutions to form nitrite (NO$_3^-$) and nitrate (NO$_2^-$) (1, 3, 4, 11). In urine and plasma, NO is found primarily as NO$_3^-$, but NO$_2^-$ can be assayed more easily by colorimetric diazotization (Griess reaction; References 1, 3, 4 and 11). Upon reduction with metallic cadmium (Cd) (3, 4, 11), or enzymatically by means of bacterial nitrate reductase (1, 2), NO$_3^-$ is converted to NO$_2^-$ and can then be detected by the Griess reaction.

Quantitation of NO$_2^-$ and NO$_3^-$ in serum using bacterial nitrate reductase followed by the Griess reaction can be expensive and requires standardization of different batches of the enzyme (2). As currently implemented, reduction on Cd is cumbersome and time-consuming for many samples and may involve complex apparatus setup, as is the case with semi-automated nitrate analyzers (3). The method for Cd-mediated reduction of NO$_3^-$ to NO$_2^-$ described by Hegesh and Shiloah (4) and Shi et al. (11) is inexpensive, but requires packing of toxic Cd shavings into columns, thus necessitating sample volumes greater than 100 µL. This report describes a modification of these protocols that is suitable for rapid, quantitative and inexpensive determination of serum NO$_2^-$ and NO$_3^-$ in samples containing as little as 10 µL of serum. Furthermore, toxicity is reduced since granulated Cd instead of Cd shavings is used.

Normal rat serum was obtained from Life Technologies (Gaithersburg, MD, USA). Alternatively, C57BL/6J x SV129 mice (6) were sacrificed and blood obtained by cardiac puncture. Following a 30-min incubation on ice, the blood samples were centrifuged and the supernatant (serum) was removed for further processing. To reduce NO$_3^-$ to NO$_2^-$, Cd filings (0.4–0.7 g/filing; Fluka Chemical, Ronkonkoma, NY, USA), one per sample to be assayed, were placed into 1.5-mL Eppendorf® microcentrifuge tubes (Brinkmann Instruments, Westbury, NY, USA). The filings were washed as follows: water (2× 1 mL); 0.1 M HCl (2× 1 mL); 0.1 M ammonium hydroxide, pH 9.6 (2× 1 mL). Between washes, the samples were vortex mixed and rotated end-over-end for 10 min, and each Cd filing was distributed to a separate microcentrifuge tube. Each serum sample (10–50 µL) was brought up to 200 µL with water, and then 10 µL of 30% (wt/vol) ZnSO$_4$ solution were added. The samples were vortex mixed, incubated at room temperature for 15 min and centrifuged for 5 min. The resulting supernatants were added to the Cd-containing microcentrifuge tubes and incubated at room temperature for overnight, rotating end-over-end. The samples were transferred to fresh microcentrifuge tubes and centrifuged again. The supernatants were subsequently assayed for NO$_2^-$ + NO$_3^-$ content. Nitrite was measured by the Griess assay (3, 4, 11, 12). Briefly, 100 µL Griess reagent (1% sulfanilamide/0.1% naphthylethylenediamine dihydrochloride/2.5% H$_3$PO$_4$; Sigma Chemical, St. Louis, MO, USA) were added to 100 µL of each of the above supernatants. The plates were read using a Vmax$^TM$ microplate reader ( Molecular Devices, Sunnyvale, CA, USA) at 550 nm against a standard curve of NaNO$_2$. The values were corrected for

![Figure 1. Recovery of NO$_2^-$ after deproteination of serum. 190 µL of the indicated concentrations of NaNO$_2$ in water (0, 1.56, 3.13, 6.25, 12.5, 25, 50 and 100 µM) were incubated with 10 µL of normal rabbit serum and then deproteinated (filled circles, solid line). Control samples contained the same concentrations of NaNO$_2$ and ZnSO$_4$, but did not contain serum and were not deproteinated (open squares, dashed line). After centrifugation of all the samples, the supernatants were assayed for NO$_2^-$ by the Griess assay. Each point represents the mean ± standard error of three separate experiments. Lines represent a linear regression analysis of recovered NO$_2^-$ vs. input NO$_2^-$ either with or without added serum.](image-url)
the \( \text{NO}_2^- + \text{NO}_3^- \) content of water, and the recovery of \( \text{NO}_2^- \) was calculated.

Previous reports have stated that NO in serum and urine is present predominantly as \( \text{NO}_3^- \), not as \( \text{NO}_2^- \) (3). In accordance with this observation, no \( \text{NO}_2^- \) was detected in normal rat serum samples prior to reduction by Cd (data not shown). The next parameter tested was whether addition of serum and deproteination affected the recovery of \( \text{NO}_2^- \) added exogenously to serum. Standard concentrations of \( \text{NO}_2^- \) (1–100 \( \mu \text{M} \)) were assayed after the addition of water alone or after serum and \( \text{ZnSO}_4 \), the deproteinating agent. Detection of \( \text{NO}_2^- \) without serum and deproteination occurred with an efficiency of 113%, as calculated from the slope of a regression line of input \( \text{NO}_2^- \) vs. recovered \( \text{NO}_2^- \) over a concentration range of 1–100 \( \mu \text{M} \) \( \text{NO}_2^- \) (\( n = 21 \)), while the detection of \( \text{NO}_2^- \) following addition of serum and deproteination occurred with an efficiency of 105% (\( n = 21 \)) over the same range of \( \text{NO}_2^- \) concentrations (Figure 1). The conversion of \( \text{NO}_3^- \) to \( \text{NO}_2^- \) mediated by Cd occurred with an efficiency of 104% (mean ± SEM; \( n = 16 \)), taken across all concentrations tested (3–100 \( \mu \text{M} \); Figure 2). This occurred regardless of the exact weight of the Cd filings used (0.4–0.7 g; data not shown). Thus, the detection of \( \text{NO}_2^- \) occurred with approximately the same efficiency whether \( \text{NO}_2^- \) was added to serum or whether \( \text{NO}_3^- \) was added and then reduced to \( \text{NO}_2^- \) by Cd. Using the method described herein, I determined the concentration of \( \text{NO}_2^- + \text{NO}_3^- \) in C57BL/6J x SV129 mouse serum to be \( 51 ± 10 \mu \text{M} \) (\( n = 30 \)). When C57BL/6J x SV129 mouse serum was assayed by a previously described fluorometric method (9; courtesy of Dr. Jane Connor, Searle-Monsanto, St. Louis, MO, USA), the results were essentially identical (49 ± 7 \( \mu \text{M} \) [\( n = 5 \)]. Syngeneic mice lacking the gene for transforming growth factor-\( \beta \), which profoundly suppresses iNOS expression in vitro (12), had fourfold elevated levels of serum \( \text{NO}_2^- + \text{NO}_3^- \). This increase in systemic NO production could be inhibited by \( \text{NO}_2^- \)-monomethyl-L-arginine.

![Figure 2. Conversion of \( \text{NO}_3^- \) to \( \text{NO}_2^- \). 200 \( \mu \text{L} \) of indicated concentrations of \( \text{NaNO}_3 \) in water (0, 1.56, 3.13, 6.25, 12.5, 25, 50 and 100 \( \mu \text{M} \)) were subjected to reduction by Cd. After centrifugation, supernatants were assayed for \( \text{NO}_2^- \) by the Griess assay. Each point represents mean ± standard error of three separate experiments. Lines represent a linear regression analysis of recovered \( \text{NO}_2^- \) vs. input \( \text{NO}_3^- \).](image-url)
an inhibitor of NOS activity (13).

This modification of the Cd-mediated reduction of NO₃⁻ to NO₂⁻ is accurate, reproducible and also rapid, as many samples can be assayed simultaneously. It does not require sophisticated apparatus or relatively large sample volumes. Finally, since granulated Cd instead of fine Cd shavings are used, there is a lower likelihood of inhaling small particles of Cd and thus a potentially lower toxicity to the researcher.

As the study of NO and its physiologic and pathophysiologic effects develops, a quick, easy and inexpensive assay for serum NO levels is needed. The assay described herein should facilitate these studies described herein should facilitate these studies.

REFERENCES


11. Shi, Y., H. Li, C. Shen, J. Wang, S. Qin, R.