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 relatively large amounts of NO in biological fluids have relied on the reaction of NO in aqueous solutions to form nitrite (NO_2^-) and nitrate (NO_3^-) (1,3,4,9,11). 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_3PO_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™ microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 550 nm against a standard curve of $NaNO_2$. The values were corrected for

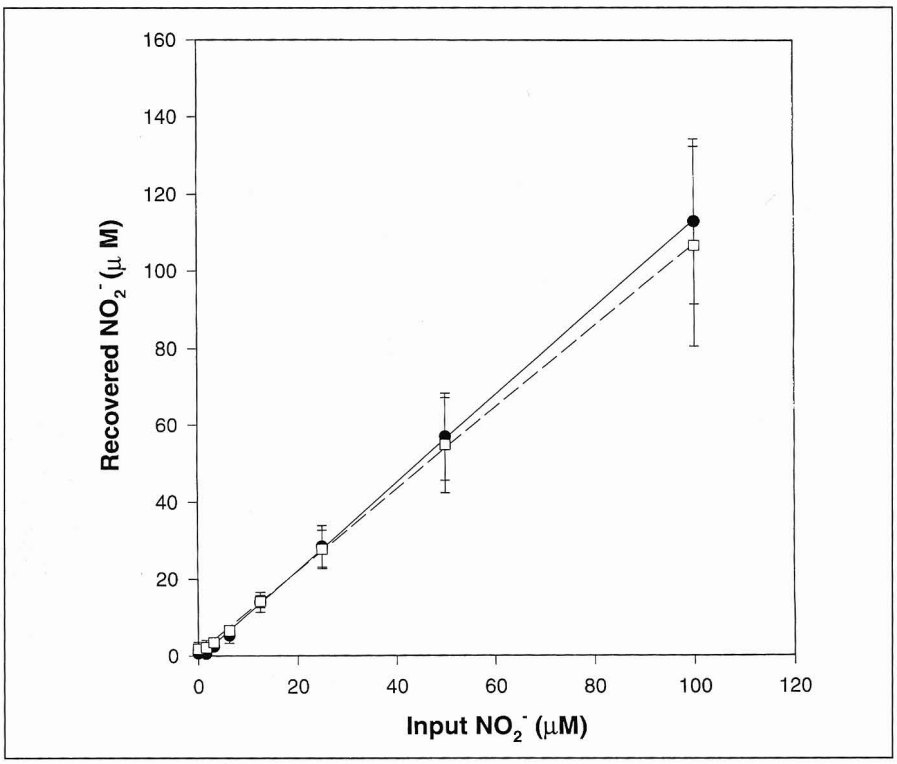

Figure 1. Recovery of NO_2^- after deproteination of serum. 190 µL of the indicated concentrations of $NaNO_2$ in water (0, 1.56, 3.13, 6.25, 12.5, 25, 50 and 100 µM) were incubated with 10 µL of normal rabbit serum and then deproteinated (filled circles, solid line). Control samples contained the same 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.

the NO_2^- + NO_3^- content of water, and the recovery of NO_2^- was calculated.

Previous reports have stated that NO in serum and urine is present predominantly as NO_3^-, not as NO_2^- (3). In accordance with this observation, no 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 NO_2^- added exogenously to serum. Standard concentrations of NO_2^- (1–100 μM) were assayed after the addition of water alone or after serum and $ZnSO_4$, the deproteinating agent. Detection of NO_2^- without serum and deproteination occurred with an efficiency of 113%, as calculated from the slope of a regression line of input NO_2^- vs. recovered NO_2^- over a concentration range of 1–100 μM NO_2^- (n = 21), while the detection of NO_2^- following addition of serum and deproteination occurred with an efficiency of 105% (n = 21) over the same range of NO_2^- concentrations (Figure 1). The conversion of NO_3^- to NO_2^- mediated by Cd occurred with an efficiency of 104% (mean ± SEM; n = 16), taken across all concentrations tested (3–100 μM; 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 NO_2^- occurred with approximately the same efficiency whether NO_2^- was added to serum or whether NO_3^- was added and then reduced to NO_2^- by Cd. Using the method described herein, I determined the concentration of NO_2^- + NO_3^- in C57BL/6J x SV129 mouse serum to be 51 ± 10 μM (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 μM [n = 5]). Syngeneic mice lacking the gene for transforming growth factor-β1, which profoundly suppresses iNOS expression in vitro (12), had fourfold elevated levels of serum NO_2^- + NO_3^-. This increase in systemic NO production could be inhibited by N^G-monomethyl-L-arginine,

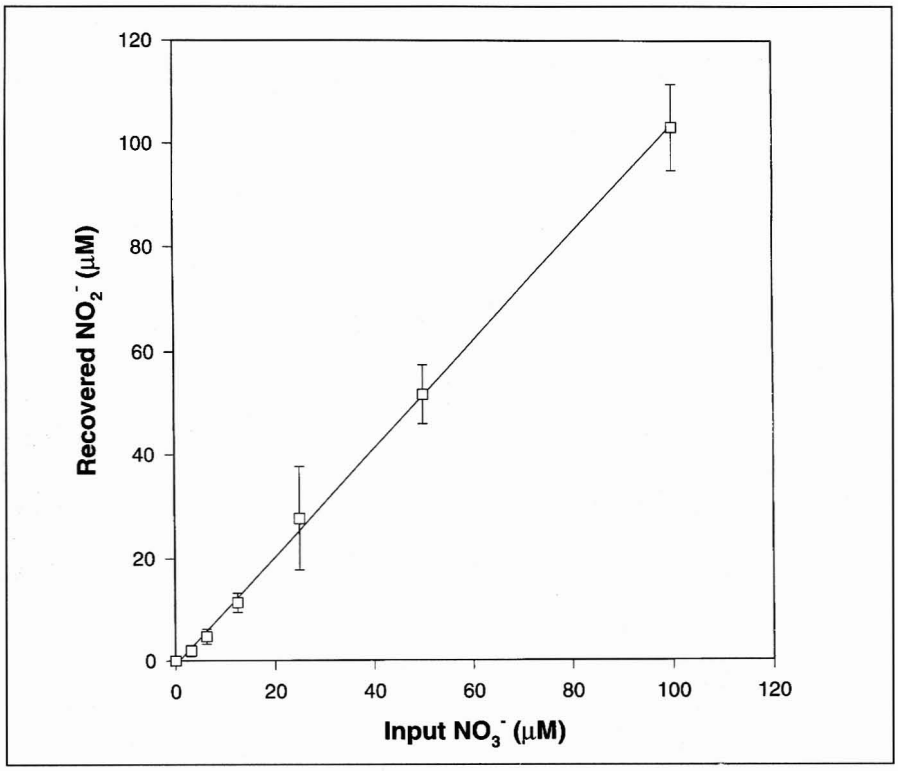

Figure 2. Conversion of NO_3^- to NO_2^-. 200 μL of indicated concentrations of $NaNO_3$ in water (0, 1.56, 3.13, 6.25, 12.5, 25, 50 and 100 μM) were subjected to reduction by Cd. After centrifugation, supernatants were assayed for NO_2^- by the Griess assay. Each point represents mean ± standard error of three separate experiments. Lines represent a linear regression analysis of recovered NO_2^- vs. input NO_3^-.

an inhibitor of NOS activity (13).

This modification of the Cd-mediated reduction of NO_3^- to NO_2^- 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.

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Address correspondence to Yoram Vodovotz, Laboratory of Chemoprevention, National Cancer Institute, Bethesda, MD 20892, USA. Internet: vodovoty@dce41.nci.nih.gov

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Yoram Vodovotz
National Cancer Institute
Bethesda, MD, USA

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Recovering DNA from Agarose Gels with Pumice

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A recent publication (2) and discussions in the Internet newsgroup bionet.molbio.methds-reagnts indicate that the use of glass slurry is still a popular method for purifying DNA from agarose gels. Many of the commercially available glass slurry preparations are based on the description of Vogelstein and Gillespie (4). In this communication, we present a modification of the Vogelstein and Gillespie technique that involves the use of pumice (5), instead of glass, as the DNA-binding agent. We have found the pumice preparation to be an inexpensive substitute for commercially available glass slurries in our instructional laboratories where students use large quantities in their learning experiences. Commercial preparations cost approximately $0.90 to $1.00 per extraction, compared to about $0.04 per extraction for the pumice method.

Two different pumice sources were used with equal success. One source was Baker and Adamson pumice powder (code 2157; Lot No. E175; New York, NY, USA) and the other was Matheson, Coleman, Bell pumice stone, powdered (PX1980 L655; Norwood, OH, USA). A fine pumice suspension was prepared by combining 10 g pumice powder with 100 mL H_2O in a 250-mL beaker and mixing with a magnetic stirrer till homogenous. The suspension was allowed to sit for 30 min to allow larger particles to sediment under unit gravity. The supernatant was carefully decanted to another beaker and allowed to sit for an additional 30 min. The resulting supernatant, containing the fine (suspended) pumice particles, was decanted into two 50-mL polypropylene centrifuge tubes and centrifuged for 5 min at 9000 rpm in an IEC Model B-20 centrifuge (Needham, MA, USA). The supernatant was removed with a pipet and discarded. The sedimented pumice was resuspended in 500 µL H_2O, transferred to a 1.5-mL graduated microcentrifuge tube and centrifuged for 5 min at 14 000 rpm in an Eppendorf® Model