Clinical Chemistry 43:4
657–662 (1997)

Automation and Analytical Techniques

Effects of reducing reagents and temperature on conversion of nitrite and nitrate to nitric oxide and detection of NO by chemiluminescence

Fan Yang, Eric Troncy, Martin Francœur, Bernard Vinet,[1] Patrick Vinay,[2] Guy Czaika, and Gilbert Blaise*

To measure the concentration of nitrites and nitrates by chemiluminescence, we examined the efficiency of five reducing agents [V(III), Mo(VI) + Fe(II), NaI, Ti(III), and Cr(III)] to reduce nitrite (NO_2^-) and (or) nitrate (NO_3^-) to nitric oxide (NO). The effect of each reducing agent on the conversion of different amounts of NO_2^- and (or) NO_3^- (100–500 pmol, representing concentrations of 0.4 to 2 μmolar) to NO was determined at 20 °C for NO_2^- and at 80 °C for NO_3^-. The effect of temperature from 20 to 90 °C on the conversion of a fixed amount of NO_2^- or NO_3^- (400 pmol or 1.6 μmolar) to NO was also determined. These five reducing agents are similarly efficient for the conversion of NO_2^- to NO at 20 °C. V(III) and Mo(VI) + Fe(II) can completely reduce NO_3^- to NO at 80 °C. NaI and Cr(III) were unable to convert NO_3^- to NO. Increased temperature facilitated the conversion of NO_3^- to NO, rather than that of NO_2^- to NO. We evaluated the recovery of NO_2^- and NO_3^- from plasmas of pig and of dog. Recovery from plasma of both animals was reproducible and near quantitative.

INDEXING TERMS: endothelium-derived relaxing factor • nitric oxide synthase • free radical • vasodilation • inflammation • thrombosis • immunology • neurotransmission

Nitric oxide (NO) is a free radical that reacts rapidly with several molecules in vitro or in vivo to form mainly nitrite (NO_2^-) and nitrate (NO_3^-). Interest in NO, NO_2^-, and NO_3^- measurements has increased exponentially with the discovery that NO or a chemically related compound plays a major role in vasodilation, inflammation, thrombosis, immunology, and neurotransmission *[1]*. Measurements of NO_2^- and NO_3^- are also important in clinical chemistry as markers of nitric oxide synthase activity *[2]*.

Measurement of NO concentration in biological systems is a challenging analytical problem *[3, 4]*. The chemiluminescence detector-based method for trace NO_2^- and (or) NO_3^- in aqueous samples was first reported by Cox *[5]* and was later widely applied *[6–8]*. This earliest and most commonly applied method is based on the conversion of NO_2^- to NO at room temperature by an acetic acid–sodium iodide (NaI) mixture. Ammonium molybdate [Mo(VI)] with ferrous ammonium sulfate [Fe(II)] in hot, 50% concentrated sulfuric acid was used for the reduction of both NO_2^- and NO_3^- to NO. NO_3^- was then determined as the difference of results obtained by the two methods. Vanadium (II) [V(II)] was mentioned as a possible reducing agent in the initial work by Cox *[5]*, who reported that it reduces NO_3^- to NO. Later work by Braman and Hendrix *[9]* indicated that it is V(III), not V(II), that reduces NO_3^- to NO. Stronger reducing agents such as chromium (II) [Cr(II)] and titanium (III) [Ti(III)] could also reduce NO_3^- to NO *[10, 11]*. However, a systematic evaluation of different reducing agents and temperature conditions for the conversion of NO_2^- and NO_3^- to NO has not been performed. We compared the efficiency of V(III), Mo(VI) + Fe(II), NaI, Ti(III), and Cr(III) at different temperatures (20, 30, . . . 90 °C) for the conversion of NO_2^- and (or) NO_3^- to NO. We also evaluated the recovery of NO_2^- and NO_3^- from plasmas of pig and of dog.

Laboratory of Anesthesia, Departments of Anesthesia, [1] Biochemistry, and [2] Medicine, Hôpital Notre-Dame, Université de Montréal, 1560 Sherbrooke St. East, Montréal, QC, Canada H2L 4M1.

*Author for correspondence. Fax 514-896-4754; e-mail blaiseg@ere.umontreal.ca.

Received August 8, 1996; revised November 21, 1996; accepted November 21, 1996.

Materials and Methods

The NO concentration in samples was detected by chemiluminescence with a Sievers 270B NO analyzer (Sievers Instruments, Boulder, CO). The detector is based on the reaction of NO with ozone (O_3) to give nitrogen dioxide in an excited state (NO_2*) plus molecular oxygen. The excited state of NO_2* decays to give a weak infrared chemiluminescence above 600 nm. A microreaction purge vessel coupled with a condenser and heating jackets permitted introduction of the sample directly into the reduction solution via a gas-tight syringe (Hamilton, Reno, NV). The condenser jacket temperature was controlled by a continuous flow of cold water while the temperature of the heating jacket was controlled by a continuous flow of warm water regulated by a Haake constant-temperature circulating bath Model D1-L (Fisher Scientific, Montréal, QC, Canada). Nitrogen, at a rate of 100 mL/min, was used as the carrier gas of NO to the NO analyzer. The flow into the reaction chamber of the NO analyzer could be adjusted with a needle valve placed between the filter (Nupro Co., Willoughby, OH) and the NO analyzer. A MacLab data acquisition system (ADInstruments Pty, Castle Hill, NSW, Australia) was used to collect and report the data as area under the curve response from baseline to baseline *[9]* (Fig. 1). We did not detect any NO_2^- or NO_3^- in the blanks used in this study.

REAGENTS

NO gas and nitrogen were purchased from Air Liquide (Canada), Montréal, QC, Canada. Vanadium trichloride, and sodium nitrite and nitrate were purchased from Aldrich Chemical Co. (Milwaukee, WI). Chromium trichloride, titanium trichloride, ferrous ammonium sulfate, ammonium molybdate, sodium iodide, and other reagents were purchased from Fisher Scientific. All were reagent-grade quality and used without further purification. High-purity distilled water was used in the preparation of all solutions.

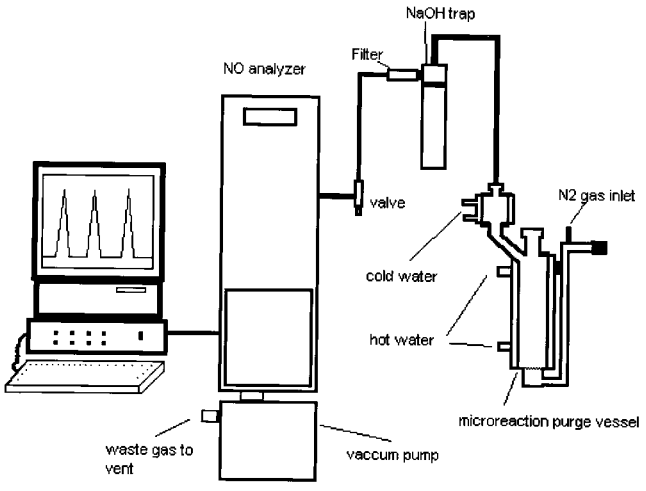

Fig. 1. Schematic of the analysis system.

RECOVERY FROM AQUEOUS SOLUTION

V(III), Mo(VI) + Fe(II), NaI, Ti(III), and Cr(II) have been reported as reducing agents for the conversion of NO_2^- and (or) NO_3^- to NO *[5, 6, 8–13]*. Except for Cr(II), for which we substituted Cr(III) with its three valences, all of these reducing agents were prepared as reported. Cr(III) was prepared in the same conditions as the other two reducing agents with three valences. Samples of inorganic NO_2^- ($NaNO_2$) or NO_3^- ($NaNO_3$) in 250 μL of water (100, 200, . . . 500 pmol) were injected into the microreaction purge vessel containing 5 mL of reducing agent solution, and the quantity of produced NO was measured after conversion by each reducing agent at 20 °C for NO_2^- and at 80 °C for NO_3^-. The effect of temperature on the conversion of 400 pmol of NO_2^- and (or) NO_3^- (in a volume of 250 μL) to NO with each reducing agent was determined by changing the temperature scale from 20, 30, . . . to 90 °C. The chemiluminescence analyzer was calibrated by injecting known amounts of NO gas (100, 200, . . . 500 pmol) through the microreaction purge vessel heated at different temperatures in the absence of reducing agent solution. Data were collected as area under the curve response from baseline to baseline and divided by the mean standard response of NO gas for each concentration. From this we obtained a recovery factor (R) expressed in percentage. Serial measurements can be performed for each NO_2^- or NO_3^- concentration without changing the reducing agent solution in the microreaction purge vessel, since the volume of added samples is very small compared with the volume of the reducing agent solution.

RECOVERY FROM PLASMA

We added known amounts (100, 200, . . . 500 pmol) of inorganic NO_2^- or NO_3^- in 0.1 mL of pig and dog plasma and then measured baseline amounts of NO_2^- and (or) NO_3^- to evaluate their recovery. Excessive foaming in the microreaction purge vessel caused by plasma proteins interfered with the reduction process. Therefore, all determinations in plasma samples were performed after deproteinization. Plasmas were diluted 10-fold with distilled water and deproteinized by addition of ½0th volume of zinc sulfate to a final concentration of 15 g/L. After centrifugation at 1000*g* for 15 min, 0.1 mL of supernatant was applied to the microreaction purge vessel containing NaI solution at 30 °C for the conversion of NO_2^- to NO, or V(III) solution at 80 °C for the conversion of NO_3^- + NO_2^- to NO. Samples of NO_2^- or NO_3^- added in plasma were compared with those prepared in distilled water in the same condition. This method only detects NO_2^- and NO_3^- that can readily pass into the gas phase to react with the O_3 in the microreaction purge vessel. Thus, any of the NO formed in vivo that would react with thiol groups in low-molecular-mass compounds is not detected here.

STATISTICAL ANALYSIS

Unless otherwise stated, all data are expressed as the mean ± SE of R. Global mean indicates the mean of all values of R calculated for each of the five agents with all concentrations of inorganic NO_2^- or NO_3^- used. The statistical analyses were done with the SAS statistical analysis program. The significance level was set at 0.05. The analysis of variance (GLM procedure) was used to compare values of global means (P_1) for the five reducing agents. The analysis was repeated for each amount of samples used (100–500 pmol). The post hoc analysis between the five reducing agents was realized by using the Student–Newman–Keuls (SNK) tests. The effect of temperature and agents on R values was analyzed by using the two-way analysis of variance (P_2). The SNK post hoc analysis was used to compare R values for each agent at different temperatures and R values for different agents at the same temperature. The optimal reduction temperature(s) was (were) determined as the temperature(s) with the highest R value significantly different from the other R values. Separate paired *t*-tests were used for dog and pig plasma when the recovery of NO_2^- was compared with that of NO_3^- (P_3).

Results

EFFECT OF DIFFERENT REDUCING AGENTS ON CONVERSION OF NO_2^- TO NO AT 20 °C

Analysis of variance indicated a difference on the global mean of the five agents (P_1 = 0.0001). The post hoc analysis distinguished two groups: One, comprising Mo(VI) + Fe(II) and Ti(III), had higher recovery rates than the second, comprising V(III), NaI, and Cr(III). The same two groups were identified for each amount (100–500 pmol) of nitrite and nitrate. However, the difference between both groups was not statistically significant when the global mean values for the five reducing agents were compared. This analysis is summarized in Table 1.

EFFECT OF TEMPERATURE ON CONVERSION OF 400 PMOL OF NO_2^- TO NO

Temperature had no effect on the detection of NO gas by chemiluminescence (data not shown; P_1 = 0.63). Recovery of NO from NO_2^- (P_2 = 0.01) by the five reducing agents was affected by temperature (Fig. 2). For NaI, the lowest R value (89.1%) was obtained at 20 °C (SNK P <0.05) and no significant difference was found between the other temperature values. For other agents, the optimal reduction temperature was Mo(VI) + Fe(II) 50–60 °C, V(III) 60–80 °C, Cr(III) 20–70 °C, and Ti(III) 20–60 °C. The comparison between agents showed that the R values with NaI and Mo(VI) + Fe(II) were higher than the R values of the three other agents at 50–60 °C (SNK P <0.05).

EFFECT OF DIFFERENT REDUCING AGENTS ON CONVERSION OF NO_3^- TO NO AT 80 °C

The recovery of NO from different amounts of NO_3^- (100–500 pmol) was <1.7% when using NaI and Cr(III) as reducing agents. Often, no conversion was detected. The global mean of the R value for Ti(III) was 82.3% ± 1.8%, whereas those for V(III) and Mo(VI) + Fe(II) were respectively 105.8% ± 1.6% and 101.1% ± 2.8%. The R values obtained for each amount of NO_3^- are summarized in Table 2.

EFFECT OF TEMPERATURE ON CONVERSION OF 400 PMOL OF NO_3^- TO NO

Temperature affected the recovery of NO from NO_3^- by V(III), Mo(VI) + Fe(II), and Ti(III) (P_2 = 0.0001; Fig. 3). The optimal reduction temperature was 80–90 °C for V(III) and 70–90 °C for Mo(VI)+Fe(II) and Ti(III). Temperature did not affect the low recovery (<1.7% at any temperature tested) of NO from NO_3^- by NaI or Cr(III).

RECOVERY FROM PLASMA

For pig plasma, the recovery of NO_2^- was 96.4% ± 1.9% (n = 20) and the recovery of NO_3^- was 104.3% ± 4.9% (n = 20) over the entire concentration range tested (100–500 pmol). There was no significant difference between the recovery of NO_2^- and NO_3^- (P_3 = 0.21). For dog plasma, the recovery of NO_2^- was 89.6% ± 2.0% (n = 20) and the recovery of NO_3^- was 107.4% ± 2.4% (n = 20) over the entire concentration range tested (100–500 pmol). The difference between the recovery of NO_2^- and NO_3^- was significant (P_3 = 0.0008).

Table 1. Effect of different reducing agents on conversion of NO_2^- to NO at 20 °C.

NO_2^-, pmol	V(III) (n = 5)	Mo(VI) + Fe(II) (n = 4)	NaI (n = 4)	Ti(III) (n = 5)	Cr(III) (n = 5)
100	92.3% ± 1.9%	94.7% ± 1.7%	89.2% ± 1.2%	96.5% ± 1.3%	86.2% ± 1.6%
200	91.6% ± 0.7%	94.8% ± 0.7%	92.2% ± 0.8%	94.4% ± 0.7%	91.2% ± 1.0%
300	92.9% ± 1.2%	97.3% ± 0.7%	93.4% ± 0.9%	96.4% ± 0.6%	93.1% ± 1.1%
400	93.3% ± 1.5%	98.2% ± 0.6%	95.1% ± 0.9%	97.5% ± 0.7%	94.6% ± 1.1%
500	93.9% ± 1.8%	98.8% ± 0.9%	97.1% ± 0.8%	98.3% ± 0.8%	95.5% ± 1.0%
Global mean	92.8% ± 0.6%	96.7% ± 0.5%	93.4% ± 0.5%	96.6% ± 0.4%	93.5% ± 0.6%

n = number of separate experiments (triplicate measurements of each sample).

Recoveries are expressed as relative percentages calculated from the area under the response curve from baseline to baseline of NO gas as 100.0% recovery (mean ± SE).

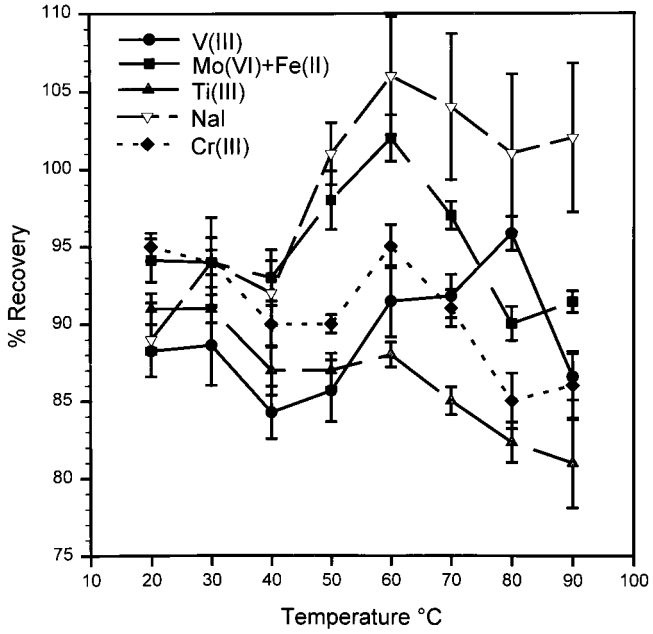

Fig. 2. Effect of temperature on conversion of 400 pmol of NO_2^- to NO by the five reducing agents.

Each point was determined from three separate experiments, and triplicate measurements were made for each sample.

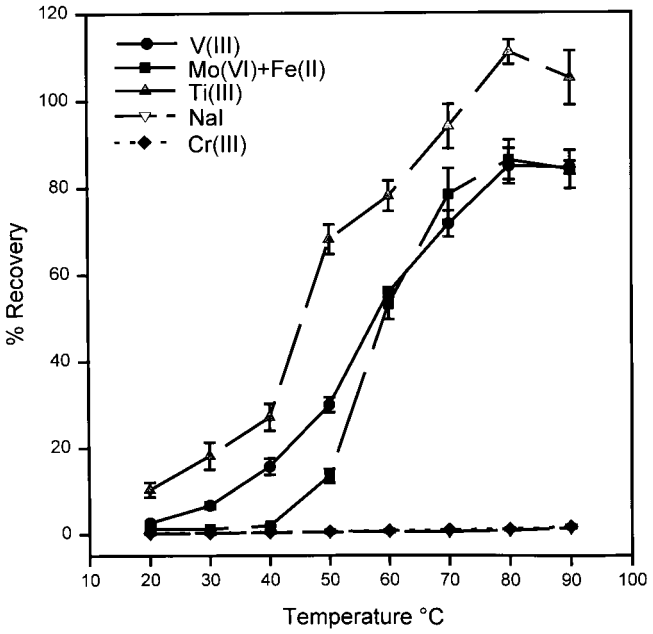

Fig. 3. Effect of temperature on conversion of 400 pmol of NO_3^- to NO by the five reducing agents.

Each point was determined from three separate experiments, and triplicate measurements were made for each sample.

Discussion

NO is a highly reactive messenger molecule that readily diffuses through plasmalemma to exert its biological activity in a variety of cells *[5]*. Several biological actions are attributed to NO via its activation of soluble guanylate cyclase, which catalyzes the transformation of GTP to cGMP. This transformation in turn activates a classical second messenger system that relays signals from the cell exterior to cell interior. Determination of increased NO formation is therefore of the utmost interest *[3, 7, 9]*.

Determination of NO in itself is difficult because of its free-radical nature and short half-life. NO reacts rapidly with oxygen to form NO_2^- and with superoxide or with oxyhemoglobin to form NO_3^-. In most cell culture systems *[2]*, NO will be oxidized primarily to NO_2^-, whereas in animal models and human samples, NO is oxidized both to NO_2^- and NO_3^-. Nitrite and nitrate are both stable in frozen plasma for at least 1 year *[2]*. Therefore, determination of the stable end products of the NO radical is most often used to measure its concentration. NO_3^- is the major metabolite of NO in blood *[2]*; thus, determination of NO_2^- alone as a marker for NO concentration is meaningless, even if some previous studies had reported NO_2^- as the major byproduct of NO in blood *[14]*. The availability of a quantitative assay for both NO_2^- and NO_3^- can facilitate further elucidation of some of the physiological, pathophysiological, pharmacological, and therapeutic roles of NO.

Compared with other analytical methods *[2–4]* (e.g., spectrophotometry, electron paramagnetic resonance, gas or liquid chromatography, mass spectrometry), chemiluminescence is highly sensitive, selective, and accurate for NO_2^- and (or) NO_3^-, especially at the low concentrations in complex matrices found in water, food, and biological fluids. The key point in this procedure is to choose the

Table 2. Effect of different reducing agents on conversion of NO_3^- to NO at 80 °C.

NO_3^-, pmol	V(III) (n = 4)	Mo(VI) + Fe(II) (n = 3)	Ti(III) (n = 3)	NaI (n = 3)	Cr(III) (n = 3)
100	88.6% ± 3.0%	73.3% ± 3.1%	77.4% ± 3.5%	ND	ND
200	94.4% ± 2.6%	92.2% ± 5.5%	75.7% ± 4.5%	ND	ND
300	101.9% ± 2.7%	101.5% ± 5.4%	76.1% ± 5.6%	ND	ND
400	109.2% ± 1.5%	106.7% ± 5.7%	84.1% ± 1.3%	0.8% ± 0.1%	1.0% ± 0.2%
500	114.5% ± 0.6%	106.5% ± 4.2%	88.5% ± 3.0%	1.5% ± 0.3%	1.7% ± 0.3%
Global mean	105.8% ± 1.6%	101.1% ± 2.8%	82.3% ± 1.8%	1.5% ± 0.2%	1.6% ± 0.3%

n = number of separate experiments (triplicate measurements of each sample).
Recoveries expressed as percentage based on the area under the response curve from baseline to baseline of NO gas as 100.0% recovery (mean ± SE).
ND = not detectable.

appropriate reducing agent/temperature combination to selectively and completely reduce NO_2^- or NO_3^- to NO.

Several reducing agents have been tested for the reduction of NO_2^- and (or) NO_3^- to NO, such as NaI for the conversion of NO_2^- to NO, and V(III), Mo(VI) + Fe(II), Ti(III), and Cr(II) for the conversion of NO_3^- to NO *[5, 9–13]*. NO_2^- can be reduced to NO by using most reducing agents at room temperature, whereas conversion of NO_3^- to NO requires both a strong reducing agent and high temperature. V(III) and Ti(III) with three valences can reduce most of NO_3^- to NO at high temperature. We compared the efficiency of Cr(III), also with three valences, with V(III) and Ti(III) for the conversion of NO_2^- or NO_3^- to NO.

Our work revealed that the five reducing agents have a similar efficiency for the conversion of NO_2^- to NO at 20 °C, with a slight advantage for Mo(VI) + Fe(II) and Ti(III) over the other three agents. The recovery of NO from NO_2^- was almost complete, compared with the known amount of NO gas standard [lower R value: 86.2% ± 1.6% for Cr(III) for the recovery of 100 pmol of NO_2^-].

V(III) and Mo(VI)+Fe(II) were equally efficient in converting NO_3^- to NO at 80 °C, and recovery of NO was nearly complete. However, recovery with Ti(III) was lower. NaI and Cr(III) were unable to reduce NO_3^- to NO, as only trace amounts of NO were recovered from NO_3^- regardless of the amount of NO_3^- and the temperature used. NaI and Cr(III) can thus be considered selective for reducing NO_2^- to NO. To our knowledge, this is the first report to show that Cr(III) can selectively reduce NO_2^- to NO. Enzymatic reduction of NO_3^- by using an immobilized *Escherichia coli* nitrate reductase column *[15]* converts ~30% of NO_3^- to NO_2^-. Another assay based on the coupled oxidation of NADPH during the enzymatic conversion of NO_3^- to NO_2^- by *Aspergillus* nitrate reductase only yields ~64% of serum NO_3^- to NO_2^- and is unsatisfactory for NO_3^- analysis in serum samples. Furthermore, though many reports claim a possible recovery of 100%, commercial nitrate reductases are rather expensive *[16]*. Powerful chemical reducing agents such as V(III), Mo(VI) + Fe(II), and to a lesser degree Ti(III) are more efficient than nitrate reductases for converting NO_3^- to NO_2^- in biological samples.

The temperature affected the conversion of both NO_2^- and NO_3^- to NO, as the reduction process is facilitated and more rapid at higher temperatures. This is particularly true for the conversion of NO_3^- to NO. However, increasing temperature had several technical drawbacks on the conversion of NO_2^- and NO_3^- to NO. The first is that the measurement of NO by chemiluminescence is influenced by humidity *[3, 4]*. As temperature increased, the heat evaporated more water, which quenched NO_2^* produced by the O_3 reaction. The second problem encountered is that the reducing solution has a tendency to move from the microreaction purge vessel to the condenser and even to the NO analyzer itself at high temperatures. We therefore propose that 60 °C would be the really appropriate temperature for converting NO_2^- to NO by these five reducing agents. In the case of NaI, the most efficient NO_2^- reducing agent at any temperature, increasing the temperature had the undesirable effect of increasing the variability of the results (larger SE) and lowering the reproducibility, because of the above-mentioned reasons. The same situation was observed for the conversion of NO_3^- to NO. It would thus be very useful to find and select a reducing agent that can reduce NO_3^- to NO at low temperatures.

It has been suggested that strong reducing agents, such as V(III), can be used at different temperatures to achieve different goals: at low temperatures for the conversion of NO_2^- to NO, and at high temperatures for the conversion of NO_2^- + NO_3^- to NO. NO_3^- would then be determined by the difference between analysis of the same sample by both assays. Fig. 3 shows that V(III), Mo(VI) + Fe(II), and Ti(III) can also reduce NO_3^- to NO at low temperature, albeit at a low degree. Use of only two different temperatures cycling for a strong reducing agent to selectively reduce NO_2^- and (or) NO_3^- to NO can be a cause of overestimated NO_2^- and underestimated NO_3^- measurements.

Our results indicate that the most accurate procedure is to use NaI or Cr(III) as a reducing agent to selectively convert NO_2^- to NO at low temperatures and then use a strong reducing agent to convert all NO_2^- + NO_3^- to NO at 80 °C or 90 °C.

Proteins contained in most biological samples can cause excessive foaming in the microreaction purge vessel and interfere with the reduction process. Deproteinization is therefore essential for the analysis of such samples. Investigating the recovery of both NO_2^- and NO_3^- in biological samples with particular attention given to the reproducibility of the assay and the occurrence of artifacts is important. In this study, recovery of NO_2^- and NO_3^- in deproteinized plasma was 93.0% ± 1.6% and 105.9% ± 2.7% respectively. Although the recoveries of NO_2^- and NO_3^- were similar and complete for the pig plasma, we discovered a small but significant difference in the case of the dog plasma. The residual protein environment in the dog plasma seemed to interfere more with NO_2^- than with NO_3^-. Though part of the NO formed in vivo may react with thiol groups in low-molecular-mass compounds, this method does not detect these low-molecular-mass nitroso compounds, as NO must be in the gas state to react with the O_3 in the reaction chamber.

Compared with the same amount of NO gas, V(III), Mo(VI) + Fe(II), NaI, Ti(III), and Cr(III) are similarly efficient reducing agents for the conversion of NO_2^- to NO at 20 °C. V(III) and Mo(VI) + Fe(II) can also completely reduce NO_3^- to NO at high temperatures. However, Cr(III) and NaI were unable to convert NO_3^- to NO. Cr(III) and NaI can specifically reduce NO_2^- to NO. We recommend the use of NaI or Cr(III) at room temperature to selectively and completely reduce NO_2^- and the use of V(III) or Mo(VI) + Fe(II) at 80–90 °C to reduce NO_2^- +

NO_3^- to NO. Recovery of both NO_2^- and NO_3^- in experimental animal plasma was reproducible and near quantitative, albeit to a lesser degree in the case of the dog plasma. These results also highlight the need for a relatively large-scale study with human subjects to establish proper baseline measurements for clinical assays of plasma nitrite and nitrate concentrations.

We estimate that a properly organized clinical laboratory could process ~30 samples/h for NO_2 measurements and ~15 samples/h for the measurement of NO_3 concentrations.

We are grateful to Julie Paré for her technical assistance and Olivier Niquette from ATS Scientific Inc. for the NO_x analyzer support. This work was funded by the Medical Research Council of Canada, and the Nitric Oxide Research Foundation of Quebec.

References

1. Culotta E, Koshland DE Jr. The molecule of the year: NO news is good news. Science 1992;258:1862–5.
2. Moshage H, Kok B, Huizenga JR, Jansen PL. Nitrite and nitrate determinations in plasma: a critical evaluation. Clin Chem 1995; 41:892–6.
3. Archer S. Measurement of nitric oxide in biological models. FASEB J 1993;7:349–60.
4. Kiechle FL, Malinski T. Nitric oxide. Biochemistry, pathophysiology, and detection. Am J Clin Pathol 1993;100:567–75.
5. Cox RD. Determination of nitrate and nitrite at the parts per billion level by chemiluminescence. Anal Chem 1980;5:332–5.
6. Rowland IR, Granli T, Bøckman BC, Key PE, Massey RC. Endogenous N-nitrosation in man assessed by measurement of apparent total N-nitroso compounds in faeces. Carcinogenesis 1991;12: 1395–401.
7. Sen NP, Baddoo PA, Seaman SW. Rapid and sensitive determination of nitrite in foods and biological materials by flow injection or high-performance liquid chromatography with chemiluminescence detection. J Chromatogr 1994;673:77–84.
8. Hirata K-I, Kuroda R, Sakoda T, Katayama M, Inoue N, Suematsu M, et al. Inhibition of endothelial nitric oxide synthase activity by protein kinase C. Hypertension 1995;25:180–5.
9. Braman R, Hendrix SA. Nanogram nitrite and nitrate determination in environmental and biological materials by vanadium(III) reduction with chemiluminescence detection. Anal Chem 1989;61: 2715–8.
10. Lingane JJ, Pecsok RL. Volumetric determination of nitrate ion. Anal Chem 1949;21:622–5.
11. Cresser MC. Nitrate determination by reduction to ammonia and gas-phase ultraviolet absorption spectrometry. Analyst 1977; 102:99–103.
12. Ellis CM, Vogel AI. Studies on hypovanous salts as analytical reagents. Analyst 1956;81:693–703.
13. Hassan SSM, Zaki MTM. Gasometric microdetermination of nitrates by reduction with vanadium(II). Anal Chem 1976;282:138.
14. Horton RA, Ceppi ED, Knowles RG, Tithenadge MA. Inhibition of hepatic gluconeogenesis by nitric oxide: a comparison with endotoxic shock. Biochem J 1994;299:735–9.
15. Gilliam MB, Sherman MP, Griscavage JM, Ignarro LJ. A spectrophotometric assay for nitrate using NADPH oxidation by *Aspergillus* nitrate reductase. Anal Biochem 1993;212:359–65.
16. Senn DR, Carr PW. Determination of nitrate ion at the part per billion level in environmental samples with a continuous flow immobilized enzyme reaction. Anal Chem 1976;48:954–8.