lematic analytes in clinical chemistry (10), and no accepted reference method for total bilirubin determination is currently available. Thus, the widely used DPD procedure was chosen as the comparison method in this study. Quality specifications in terms of precision and accuracy met CLIA (11) as well as European regulatory requirements (12).

In the patient samples, the measured bilirubin values covered the physiologically relevant range (85–400 μmol/L) within which decisions on phototherapy or exchange transfusion have to be made (2,5). In general, there was a good agreement between bilirubin concentrations measured in plasma by the DPD method and those obtained from whole blood by multiple-wavelength photometry. The slope of the regression line was close to 1, and no significant systematic bias between the two methods was observed. There was, however, a rather wide spread of data around the regression line. Thus, correlation was considerably weaker than desirable for equivalent methods. On the basis of the within- and between-subject biologic variability of bilirubin [CV_{within} = 25.6\% and CV_{between} = 30.5\%, respectively (13)], the desirable imprecision (CV) and bias can be calculated to be <13\% and <10\%, respectively. Thus, the desirable total error should be <31.1\% (13). In our study, these quality requirements were met in 86\% of cases.

In conclusion, determination of total bilirubin from whole blood by multiple-wavelength photometry on a blood gas analyzer is a promising method for point-of-care testing. It combines the advantages of small sample volume and fast turnaround time. The manufacturer is currently working on an update of the measurement algorithm.

We thank all the nurses and doctors from the participating ward for their efforts and support. We gratefully appreciate the help of Ingrid Fussing and Anne Svegaard at Radiometer Medical in organizing the study. The study was supported in part by a grant from Radiometer Medical (Copenhagen, Denmark).

Addendum: Since completion of this study, the manufacturer has optimized the algorithm because of a spectral difference between the laboratory blood samples supplemented with “synthetic” bilirubin used to calibrate the ABL and “real” blood samples. Radiometer Medical has implemented this algorithm in the software version 3.61, released in January 2001.

References


Effect of Hemolyzed Plasma on the Batch Measurement of Nitrate by Nitric Oxide Chemiluminescence, Ryon M. Bateman,1,2 Christopher G. Ellis,1,2 Michael D. Sharpe,3 Sanjay Mehta,4 and David J. Freeman5* (1 Vascular Biology Program, Lawson Health Research Institute, London Health Sciences Centre, London, Ontario, N6B 1B8 Canada; Departments of 2 Medical Biophysics, 3 Anaesthesia, 2 Respirology, and 5 Pharmacology-Toxicology, University of Western Ontario, London, Ontario, N6A 5C1 Canada; * address correspondence to this author at: Department of Medicine and Pharmacology-Toxicology, University of Western Ontario, London, Ontario, N6A 5C1 Canada; fax 519-663-3789, e-mail dfreeman@uwo.ca).

Nitric oxide (NO) is a potent vasodilator and regulator of vascular tone, a neurotransmitter, and a cytotoxic agent (1,2). In aqueous aerobic environments, the primary decomposition product of NO is nitrite (NO\textsubscript{2}–) (3), with further oxidation to nitrate (NO\textsubscript{3}–) being dependent on the presence of additional oxidizing species such as oxyhemoproteins (3). Collectively, these NO oxidation products are referred to as NO\textsubscript{x}–. Several analytical techniques have been used to quantify NO\textsubscript{x}– species, including spectrophotometric assays based on the Griess reaction (4,5) and enzymatic reduction of NO\textsubscript{3}– (6), gas chromatography–mass spectrometry (7), chromatographic flow systems (8), and chemiluminescence (9–11). NO\textsubscript{x}– may be important in sepsis (12–16). Of possible analytical importance is intravascular hemolysis during sepsis (17–19). The effect of hemolysis on NO\textsubscript{x}– analysis is unknown. Other potential causes of intravascular hemolysis include drug-induced hemolytic anemia, hemolytic transfusion reactions, and artificial heart valves (20), and hemolysis can also occur during blood collection (21).
and inappropriate blood storage. The objectives of our study were (a) to determine whether hemolysis interferes with the determination of plasma NO₃⁻ by NO chemiluminescence batch methodology and (b) to determine whether the interference could be eliminated by sample pretreatment.

We purchased helium and oxygen from Praxair. Other chemicals were from Sigma-Aldrich. All chemicals were reagent-grade quality. Deionized water was used to prepare all solutions. Blood samples were obtained from healthy human volunteers by venipuncture with heparin as anticoagulant. Aqueous NO₃⁻ calibrators (25 μmol/L) were prepared in deionized water. Digitonin in phosphate-buffered saline (PBS), at final concentrations of 0, 20, 45, and 90 μmol/L, was used to control the degree of whole-blood hemolysis (21). Whole blood was also treated by dilution (1:2 by volume) with lysis buffer (155 mmol/L NH₄Cl, 10 mmol/L KHCO₃, 0.14 mmol/L EDTA, pH 7.2) to obtain complete hemolysis. Whole blood was diluted 1:2 by volume with digitonin solutions (35 °C) and lysis buffer and mixed gently for 5 min. Supernatants were collected by centrifugation (10 min at 1500g and 18 °C) and refrigerated. Hemoglobin (Hb) content was estimated by hemoximetry (OSM2 Hemoximeter; Radiometer) and determined quantitatively (plasma hemoglobin test reagent set; Sigma Diagnostics). The Hb plasma solutions were supplemented with NO₃⁻ (NO₃⁻ / Hb) to produce a final concentration of 25 μmol/L. Solutions were left at room temperature for 10 min to allow sample equilibration.

In a separate series of tests, Hb was removed from the mildly hemolyzed sample (20 μmol/L digitonin) and the completely hemolyzed sample (lysis buffer) by precipitation with ethanol (1:2 by volume). Samples were mixed vigorously for 1 min and left standing at room temperature for 15 min before supernatants were collected by centrifugation (2 min at 5000g and 4 °C). Background NO₃⁻ (endogenous NO₂⁻ / NO₃⁻ ) was subtracted from NO chemiluminescence signals. Digitonin at 1000 μmol/L had no effect on the NO₃⁻ calibration signal.

A NO chemiluminescence analyzer (NOA) system (Sievers 270B, NO Chemiluminescence Analyzer; Sievers Instruments) was used to detect NO₃⁻. NO₃⁻ was chemically reduced under reflux conditions to NOgas in the Radical Purger™ by hot (92 °C, circulating hot water bath) vanadium(III) (3.5 mL of 0.05 mol/L VCl₃ in 0.8 mol/L HCl, pH 0.48) and subsequently stripped and carried to the NOA reaction chamber (under reduced pressure; Edwards Pump) by helium. The internal reaction chamber pressure was adjusted to 800 Pa. Within the reaction vessel, NOgas reacted with ozone to generate oxygen and the excited state NO₂⁺ species, which decayed to give a weak infrared (>600 nm) chemiluminescence signal that was detected and amplified by a photon multiplier tube (22, 23). Output signals in mV were recorded on a strip recorder (Chromatopak C-R1A; Shimadzu) with the areas of the NO peaks (mV·s) being electronically integrated from baseline to baseline.

To test the effects of hemolysis on NO₃⁻ detection, a batch protocol was used: a 25 μmol/L aqueous NO₃⁻ calibrator was injected five times to establish a baseline NO signal (Pre-Hb) and was immediately followed by 10 repeated injections of a NO₃⁻-supplemented hemolyzed plasma sample (NO₃⁻ / Hb) and then by 5 injections of the aqueous NO₃⁻ calibrator (Post-Hb). Ten-microliter injections (gas-tight syringe; Hamilton Company) were used for all calibrators and samples. The influence of Hb accumulation in the purge vessel on NO₃⁻ determination was calculated by the difference in NO signal between the Pre-Hb and Post-Hb aqueous NO₃⁻ calibrators. Duplicate batch runs of each treatment were performed.

All values are reported as mean ± SE unless otherwise stated. For all tests of significance, P < 0.05 was considered statistically significant. A t-test (Sigma Stat 2.0; Jandel Scientific) was used to assess the influence of the Hb on the difference between Pre-Hb and Post-Hb NO₃⁻ calibrators. CVs were based on the combined results of aqueous NO₃⁻ calibrators from duplicate batch experiments (n = 10).

The effect of different degrees of hemolysis on NO₃⁻ determination is shown in Fig. 1, A–E and G. As Hb accumulated in the purge vessel to concentrations >1000 μg (Fig. 1, C, D, and G), the NO chemiluminescence response of the hemolyzed plasma samples decreased sequentially with repeated injections. The effect of injecting hemolyzed plasma samples on the subsequent determination of aqueous NO₃⁻ calibrators was assessed by comparing the NO responses of the calibrators before (Pre-Hb) and after (Post-Hb) injections of hemolyzed plasma. The results are summarized in Table 1. As the degree of hemolysis increased from 2.9% to 30%, the difference between the values obtained for the Pre-Hb and Post-Hb aqueous NO₃⁻ calibrators increased from 5.2% (P = 0.047) to 19% (P < 0.001), respectively, showing that sample hemolysis inhibited not only the plasma NO₃⁻ signal, but also that of the calibrator by a carryover effect.

When mildly and completely hemolyzed samples were precipitated with ethanol (Fig. 1, F and H), there was no evidence of sequential decreases in NO₃⁻ / Hb signal, nor was there a significant difference in the NO signal of the aqueous calibrator before and after exposure to precipitated samples (Table 1). Recovery of the NO signal was nearly quantitative from the hemolyzed plasma samples, 112% and 115% with 20 μmol/L digitonin and lysis buffer, respectively.

The NO chemiluminescence technique, based on the chemical reduction of NO₂⁺ species to NO and subsequent reaction with ozone, has several advantages over other methods. These advantages include increased accuracy and precision, and reduced susceptibility to sample interference from colored species, suspended materials (24), and nitro-organic compounds (10). Braman and Hendrix (10) showed that reduction of NO₂⁻ and NO₃⁻ by vanadium(III) to NO was both temperature and pH dependent. Yang et al. (25) confirmed the suitability of vanadium(III) as the reductant of choice and indicated that a temperature of 80–90 °C should be used to reduce
Fig. 1. NO chemiluminescence batch profiles.
Repeated injections (10 μL) of Pre-Hb and Post-Hb aqueous NO_3^- calibrators (● and △) were followed by repeated injections of the NO_3^--supplemented hemolyzed plasma samples (NO_3^- / Hb; ○ and □) and a second series of aqueous NO_3^- calibrators. Whole-blood hemolysis was induced by digitonin (0, 20, 45, and 90 μmol/L) and lysis buffer. Panels A–D show the effect of Hb accumulation on NO_3^- determination. Panels E–H compare the effect of mildly hemolyzed untreated (20 μmol/L digitonin) and completely hemolyzed (lysis buffer) samples with ethanol-treated samples. ■, accumulated Hb. Values on x axis indicate number of injections. Vertical dashed lines demarcate NO_3^- calibrators from hemolyzed samples.
### Table 1. Effect of sample hemolysis and ethanol treatment on measurement of NO\textsubscript{3}\,- calibrators.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Digitonin, ( \mu \text{mol/L} )</th>
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</tr>
</thead>
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<tr>
<td></td>
<td>20</td>
<td>20,EtOH\textsuperscript{b}</td>
</tr>
<tr>
<td>Pre-Hb NO\textsubscript{3},- calibrators</td>
<td>( (\text{2.8}) )</td>
<td>( (\text{2.7}) )</td>
</tr>
<tr>
<td></td>
<td>( (3.5) )</td>
<td>( (3.0) )</td>
</tr>
<tr>
<td>Post-Hb NO\textsubscript{3},- calibrators</td>
<td>( (6.0) )</td>
<td>( (5.8) )</td>
</tr>
<tr>
<td></td>
<td>( (2.0) )</td>
<td>( (2.0) )</td>
</tr>
<tr>
<td>Difference, %</td>
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<td></td>
</tr>
<tr>
<td>0.0</td>
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</tr>
</tbody>
</table>

\textsuperscript{a} To assess the effect of sample hemolysis on NO\textsubscript{3}\,- detection, chemiluminescence values from \( n = 10 \) NO\textsubscript{3}\,- calibrators was compared with chemiluminescence values from pre-Hb (no hemolysis) on NO\textsubscript{3}\,- calibrators. Because \( n = 10 \), no significant differences were detected. We wondered whether sample hemolysis ranging from 2.9\% to 30\% hemolysis would have been equivalent to a sample with \( \approx 10\% \) hemolysis. This degree of hemolysis, according to Table 1, would still have produced a significant decrease in the signal produced by the Post-Hb aqueous NO\textsubscript{3}\,- calibrators. Because no free iron nor glutathione is present in NO\textsubscript{3}\,- treatment, no deterioration of plasma NO\textsubscript{3}\,- signal or increase in NO\textsubscript{3}\,- signal can be expected.

\textsuperscript{b} EtOH, ethanol; NS, not significant.

It has been reported that plasma protein causes extensive foaming in the purge vessel and that this interferes with the chemical reduction of NO\textsubscript{2}\,- and NO\textsubscript{3}\,- (25). We found that simply diluting the plasma fraction of whole blood threefold in PBS (Fig. 1B) eliminated the requirement for deproteinization before batch processing recommended by Yang et al. (25). Although plasma protein can interfere with NO\textsubscript{3}\,- determinations, we found that the NO chemiluminescence response was compromised by the presence of Hb in the absence of extensive foaming. In fact, foaming was encountered only with the highest accumulations of Hb, i.e., \( >1000 \mu \text{g} \), corresponding to samples with 19\% and 30\% hemolysis, whereas measurement of aqueous NO\textsubscript{3}\,- calibrators was unaffected by much lower degrees of hemolysis (2.9\%–7.0\%) in the absence of foaming (Table 1). This suggests that the Hb itself is capable of interfering with the NO\textsubscript{3}\,- reduction; additional tests showed that neither free iron nor glutathione produced the effect.

When Hb was removed from hemolyzed samples by ethanol precipitation, no deterioration of plasma NO\textsubscript{3}\,- signal or increase between Pre-Hb and Post-Hb aqueous NO\textsubscript{3}\,- calibrators was detected. We wondered whether sample dilution accounted for the improvement in Post-Hb aqueous NO\textsubscript{3}\,- signal. With respect to the completely hemolyzed sample, a threefold dilution would have been equivalent to a sample with \( \approx 10\% \) hemolysis. This degree of hemolysis, according to Table 1, would still have produced a significant decrease in the signal produced by the Post-Hb aqueous NO\textsubscript{3}\,- calibrators. Because this was not the case, we believe that simple dilution of Hb alone cannot account for the improvement in the NO chemiluminescent signal.

According to Braman and Hendrix (10), the chemiluminescent method of detecting NO using vanadium(III) is superior to other methods because many samples can be analyzed sequentially without the necessity of sample prereduction or other preparation. Our results suggest that sample hemolysis ranging from 2.9\% to 30\% (note that 1\%–2\% hemolysis gives a pinkish color to plasma) may compromise the accurate detection of NO\textsubscript{2}\,- species because Hb accumulates in the purge vessel. Ethanol precipitation can be used to clean up the sample with near-quantitative recovery of NO\textsubscript{3}\,- species. Other precipitants were not evaluated in the chemiluminescent system. As NO\textsubscript{3}\,- measurements gain increasing clinical acceptance in patient monitoring, it will be important to recognize which patient samples are hemolyzed so that corrective measures can be taken to ensure accurate analysis.

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The Diabetes Control and Complications Trial (DCCT) and the UK Prospective Diabetes Study (UKPDS), undertaken in people with type 1 and 2 diabetes, respectively (1, 2), established the significance of glycohemoglobin (HbA1c), as in particular hemoglobin A1c (HbA1c), as a prognostic indicator for long-term micro- and macrovascular complications. However, the HbA1c measured during the DCCT and UKPDS represents a Hb fraction characterized by its retention time on cation-exchange HPLC rather than its unique chemical structure (deoxyfructosylhemoglobin). Accordingly, the DCCT HbA1c procedure represents a selective, but not a specific assay method (3).

Because no definitive or reference method exists for quantification of HbA1c (4), the American Diabetes Association (ADA), in collaboration with the Association of Clinical Chemists, implemented the National Glycohemoglobin Standardization Program (NGSP) (5) to standardize HbA1c values determined by methods different from that used in the DCCT. The ADA (6) now states that their recommended HbA1c thresholds, with respect to patient management goals, are valid only for NGSP-certified methods.

The variability of HbA1c measurements depends on both analytical and biological variation. However, because HbA1c concentrations are used for individual patient management, only analytical imprecision and within-person biological variation (5) are relevant. Whereas the NGSP (5) states that within-person HbA1c variance is negligible, previous studies (7, 8) have reported s2 estimates of 0.17–0.79, and investigations measuring gHb (9) or HbA1 (10) have reported values between 0.45 and 1.03. Accordingly, both analytical and within-person variability, but particularly the latter, increase measurement uncertainty and, therefore, the potential for clinical misinterpretation at ADA-specified HbA1c thresholds.

The following definitions are provided to avoid ambiguity with respect to terminology:

Within-person variance (s2): the degree of random fluctuation of values around a person’s homeostatic set-point for a particular biological analyte. For people with diabetes, the HbA1c homeostatic set-point is controlled by dietary and/or pharmacologic treatment, and not by the normal physiologic mechanism. Repeatability (s1): closeness of agreement between successive results obtained with the same method on identical test material and under the same conditions (same operator, same apparatus, same laboratory, and same time).

Reproducibility (s2): closeness of agreement between individual results obtained with the same method on