A Method for the Standardized Offline Collection of Exhaled Nitric Oxide*

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Study objectives: Exhaled nitric oxide (ENO) is a noninvasive marker of airway inflammation. The purpose of this study was to compare a standardized offline ENO measurement apparatus with a validated on-line method.

Design: Asthmatic volunteers (n = 21) had ENO measured by the two following methods: (1) inhalation to total lung capacity (TLC) followed by exhalation at a constant flow (45 mL/s) against a high resistance, while monitoring nitric oxide (NO) and pressure on-line; and (2) inhalation to TLC and exhalation into mylar balloons via an apparatus that included the same resistance and flow rate as used in the on-line method. We also examined NO stability in mylar balloons over 48 h.

Measurements and results: ENO values (given as geometric mean in parts per billion [ppb]; 95% confidence intervals) differed between the on-line method (69.6; 42.6 to 113.8) and the offline method (49.5; 30.9 to 79.3), indicating that the offline method gave lower ENO measures than the on-line method (p < 0.001). Furthermore, this difference between measures increased with increasing mean values. The intraclass correlation coefficient (0.931), however, showed excellent correlation between the on-line and offline methods. Within-subject repeatability, as assessed by the coefficient of repeatability (CR), was good for both the on-line and offline methods (CR, 1.09 and 1.17, respectively). Geometric mean NO concentrations (95% confidence limits) in mylar balloons containing exhalate increased from a baseline of 55.8 ppb (36.9, 84.4) to 64.5 ppb (45.6, 91.1) and 69.5 ppb (51.4, 94.0) at 24 h and 48 h, respectively.

Conclusions: The offline method gave reproducible ENO values that were consistently smaller than, but showed good correlation with, values obtained with on-line ENO collection. This method is suitable for offline collection, but the measured values are not interchangeable with those obtained by on-line measurement. *(CHEST 1999; 116:754–759)*

Key words: endogenous; exhaled; nitric; offline; oxide

Abbreviations: CI = confidence interval; CR = coefficient of repeatability; ENO = exhaled nitric oxide; ICC = intraclass correlation coefficient; NO = nitric oxide; ppb = parts per billion; TLC = total lung capacity

Exhaled nitric oxide (ENO) is proposed as a surrogate marker of airway inflammation in asthma and other airway diseases. ENO is high in untreated asthma and falls rapidly after treatment with inhaled corticosteroids. Nasal nitric oxide (NO), which has high concentrations relative to the lower respiratory tract, can be excluded by exhalation against resistance, which closes the velum during expiration. Additionally, ENO levels are markedly flow dependent, requiring a constant expiratory flow rate for measurement.

On-line measurement of ENO refers to exhalations with real-time display of the single breath NO profile and mouth pressure. This allows the operator to examine the pressure and NO profiles and to select exhalations with good pressure control, absent early NO peaks (characteristic of nasal contamination), and...
reproducible NO plateaus. The on-line measurement, however, requires testing in the laboratory in most cases.

The ability to measure ENO offline could convey several advantages. While NO analysis equipment may be present in a hospital, testing requires that the patient come to the laboratory, which may be difficult or impossible for the patient to accomplish. Alternatively, bringing the analyzer with ancillary equipment (calibration gases, computer, etc.) to the patient is labor intensive and inconvenient. Because the current cost of an NO analyzer prevents this equipment from being widely available, standardized offline measurements would enable ENO to be measured in the emergency room, at bedside, in the clinic, or outside the hospital (e.g., workplace, school, or home). The disadvantages of offline measurement include the following: ambient NO contamination of the exhalate, limited ability to monitor and exclude exhalations with suboptimal technique, and error due to NO instability between collection and measurement.

In this study, we constructed a device for the offline collection of ENO which incorporated expiratory resistance to close the velum, and the same exhalation flow rate (45 mL/s) as the on-line method used in our laboratory. In 21 asthmatic subjects, we measured and compared ENO values on-line and offline using this apparatus. In addition, NO stability in mylar balloons was tested over 48 h.

**Materials and Methods**

**Subjects**

We recruited 21 stable subjects with a diagnosis of asthma. Five subjects were taking inhaled corticosteroid medications. We excluded any subject with unstable disease. The protocol was approved by the institutional review board of the hospital, and all subjects signed an informed consent form.

**NO Measurement**

NO measurement was performed with a rapid-response chemiluminescent analyzer (model 280; Sievers Instruments; Boulder, CO). The analyzer was calibrated daily using air entrained via a NO filter (Sievers Instruments) as a zero gas source, and a NO calibration gas (18 ppm; Scott Specialty Gases; Plumsteadville, PA) for the upper point calibration. The NO analyzer requires about 20 mL of gas to give a reliable reading on mylar balloon samples.

**Study Design**

Each subject performed the study 15 min after receiving albuterol, 180 mcg, from a metered-dose inhaler to prevent the onset of bronchoconstriction as a result of the ENO maneuvers. Each subject had ENO measured by the on-line and offline methods, which were administered in random order.

**Method 1: On-line ENO Measurement Technique**

We used an on-line restricted breath technique that employs exhalation via a high resistance and positive mouth pressure to close the velum, thus excluding nasal NO (Fig 1). Subjects without a nose clamp inhaled to total lung capacity (TLC) via a NO filter, reducing the NO concentration to < 5 ppb, and then they exhaled via a 16-gauge needle while targeting a pressure of 20 mm Hg. This produced an expiratory flow rate of 45 mL/s. The NO and pressure profiles, as displayed on a computer monitor in real time, showed a washout phase and plateau. The plateau ENO level was recorded. Exhalations were repeated and suboptimal maneuvers were rejected until three ENO values that varied by < 7% were obtained.

**Method 2: Controlled-Flow Restricted Offline Technique**

A controlled-flow apparatus (Fig 2) was constructed from a disposable paper mouthpiece, a rubber mouthpiece adapter, and a plastic T tube with a 0 to 30 cm H2O pressure gauge mounted on top (Omega Engineering; Stanford, CT). Two one-way valves ensured unidirectional inspiration of filtered room air containing < 5 ppb NO to enter the side arm of the T tube (Fig 2), and unidirectional exhalation via a 16-gauge needle into a mylar balloon (Ellswood’s, Denver, CO) via a three-way stopcock with a Luer lock. This design ensured that the patient and instrument dead space contained the low NO inspired air and that the exhalation proceeded against the same expiratory resistance at the same flow as the on-line method. The subject without a nose clamp inserted the mouthpiece, inhaled to TLC, and then immediately exhaled for 15 s via the expiratory resistance into the balloon while targeting a pressure of 20 mm Hg on the pressure dial mounted on top of the apparatus. The NO levels in three balloons from three exhalations were analyzed immediately after the completion of the last exhalation.

**The NO Filter**

The NO filter consisted of a chamber containing activated charcoal and potassium permanganate, allowing unilateral direction of flow. The same filter was used for both methods. During the on-line method, the inspired ambient NO concentration after passage through the filter was monitored. Despite variations in ambient NO, the filter always reduced the NO content to < 5 ppb.

**Figure 1.** The apparatus used for on-line measurement of NO. The subject inserts the mouthpiece, inhales to TLC, and then exhalates via an expiratory resistance while targeting a fixed mouthpiece pressure. NO and pressure data are transmitted to a computer for real-time display, data analysis, and storage.
The Stability of NO in Mylar Balloons

After completion of exhalation and NO analysis, three mylar balloons from the offline method for each subject were kept at room temperature and NO was measured at 24 h and 48 h to assess NO stability.

Statistical Methods

The distribution of ENO values was skewed so that data were log transformed. All tests were two-sided and conducted at the 5% significance level. Reproducibility of each method was assessed using the three replicate exhalations by calculating the following: (1) the intraclass correlation coefficient (ICC), which assesses the ratio of between-subject variance to the total variance (within-subject + between-subject variance) and can range from 1 (perfect reproducibility) to 0 (no reproducibility); and (2) the coefficient of repeatability (CR), which calculates the upper boundary by which we would expect the replicates to differ 95% of the time based on the within-subject variance. As data were log transformed, the CR expresses the upper bound of the ratio by which two measures should deviate 95% of the time. Means of the three replicate exhalations were used for all further analyses.

Plots of the difference in the two methods vs their average were examined for patterns of disagreement that were evident as the mean value increased (Bland and Altman). On the original scale, this plot showed a pattern of increasing variability with increasing mean. On the log scale, no apparent pattern of differences was seen with increasing means values, supporting the analysis on the log scale. Baseline log ENO values for the two methods were compared using a paired t test, thus quantifying the systematic bias between methods. The 95% confidence interval (CI) for the bias between methods was calculated, as were the limits of agreement (95% tolerance or prediction intervals). The Limits of Agreement are the amount by which individual measurements with the two methods should differ 95% of the time (Bland and Altman). Because data were log transformed for analysis, both the estimate of bias between measures, its 95% CI, and the limits of agreement express the ratio of the offline method to the on-line method. Additionally, the ICC values were used to quantify correlation. Like the Pearson correlation coefficient, the ICC is diminished by lack of linear agreement; unlike the Pearson correlation coefficient, the ICC is also responsive to bias between methods.

NO stability data at 24 h and 48 h were obtained from the average of three balloons for each subject at each time (offline method). A mixed-effects model was used to test for the mean differences over time while accounting for repeated measurement made on subjects with a compound symmetric error structure. Pairwise comparisons of means at the three times were then made within the mixed-effects model. The geometric mean ENO values and the back-transformed 95% CI at baseline, 24 h, and 48 h are given as summary statistics.

Results

After a brief training period, all subjects were able to comfortably perform the ENO measurements by the on-line and offline methods. The study session took between 30 to 60 min.

Post-hoc analysis revealed that baseline ENO data for six consecutive subjects were noted to be larger for the offline method than for the on-line method. For all other subjects, the offline measures were smaller than the on-line measures. The grouping of these six results was thought to be extremely unlikely from a statistical standpoint. Additionally, from a theoretical standpoint, the offline NO values should be less, as this collects the dead space containing scrubbed ambient air and the washout phase of the exhalation before the plateau region. We hypothesized that there was a technical reason for the finding, and we recalled four of the six subjects who on repeat testing showed the expected relationship (offline NO less than on-line NO). Baseline data for the remaining two subjects were excluded from the analysis comparing the on-line and offline methods.

Reproducibility of ENO Values

Table 1 presents the assessment of within-method reproducibility for the three replicate exhalations on the study day. The ICC for both methods suggested good reproducibility (ICC = 0.998 for the on-line method and ICC = 0.994 for the offline method). The CR showed that the on-line method was highly reproducible.

![Diagram of apparatus used in the offline collection of NO with added expiratory resistance](http://journal.publications.chestnet.org/pdfaccess.ashx?url=/data/journals/chest/21931/)
repeatable (values which differ by < 9% for 95% of the time) and the offline method was reasonably repeatable (replicate values which differ by < 17% for 95% of the time).

Comparison of ENO Between Methods 1 and 2

Summary ENO values (geometric means; back-transformed 95% CI for log scale means) are presented in Table 1, and sample tracings from both methods for one subject are shown in Figure 3. A paired t test indicated significant bias between methods (p < 0.001), with the offline method giving lower NO concentrations than the on-line method by an average of 29% (expressed as a geometric mean fraction of the on-line method, 0.71; 95% CI, 0.66 to 0.77; limits of agreement, 0.50 to 1.00). Both the on-line and the offline methods showed excellent correlation as assessed by ICC (0.931). Estimates of the amount by which an individual’s offline measure may differ from the on-line measure as a proportion of the on-line measure varied (range, 0.50 to 1.00). Thus, an individual offline measure could be expected to vary between 50% and 100% of the on-line measure 95% of the time.

Ambient NO

Ambient NO varied from 4.6 to 50.2 ppb on the study days but did not correlate with ENO as measured by either of the methods.

Stability of NO Samples at 24 and 48 h

ENO concentration in mylar balloons expressed as geometric mean (95% CI) showed a progressive increase in NO from baseline values of 53.6 ppb (44.6 to 91.0) to 63.7 ppb (34.6 to 83.0) at 24 h and 70.2 ppb (52.0 to 94.7) at 48 h. Both the 24 h and 48 h values differed significantly from baseline (p = 0.002 vs p < 0.001, respectively).

Discussion

This study was designed to critically evaluate an apparatus for the offline collection of ENO, while excluding nasal NO and with exhalation at a constant flow rate. Measurements using this apparatus were compared with measurements obtained using a validated restricted breath technique for on-line measurement. Both the on-line and offline methods showed good within-subject reproducibility and correlation. The offline method predictably gave mean ENO values that were significantly lower than the on-line method. The NO concentration in mylar balloons showed a progressive rise at 24 h and 48 h after collection.

The excellent reproducibility of the on-line method is related to prevention of nasal contamination, tight control of expiratory flow, exclusion of ambient NO, and the ability to monitor and discard suboptimal exhalations. Correspondingly, the good within-subject reproducibility of the offline method, as assessed by the ICC and the CR, can also be attributable to exhalation against resistance, which excluded nasal NO, constant expiratory flow rate, and exclusion of ambient NO.

Mean ENO values measured with this device were consistently and significantly lower than the on-line device by approximately 29%. Furthermore, the difference between methods increased with increasing ENO values. This occurrence, limits of agreement, and estimates of bias suggest that the two methods are not interchangeable. The discrepancy between the two methods was expected and can be attributed to the following: (1) dilution of the offline sample by patient, and instrument dead space gas that contained the scrubbed ambient NO gas containing < 5 ppb; and (2) collection of the washout phase of the single breath exhalation that contains lower NO values before achievement of plateau values. An exhalation time of 15 s was used so that a good proportion of the collected gas would have been from the plateau phase (see Figure 3). The increase in the discrepancy between the methods with increasing NO values can be attributed to the following: (1) a longer washout phase with increasing ENO levels; and (2) a greater effect of the disparity between the dead space gas (containing < 5 ppb) when comparing high to low ENO values. In contrast, ENO values interpreted from the plateau levels in the on-line method are not affected by dead space gas or the washout phase.

Paredi et al have reported an offline system that incorporates discardment of the initial portion of the exhalate (dead space) and also the final portion (end-expiratory gas) to avoid nasal contamination of the sample as mouth pressure falls off and the velum opens. They found a good correlation when discarding dead space at a flow rate of 10 L/min only. At lower flow rates, however, their offline method gave greater values than their on-line method; at higher flow rates, their offline values were less than their on-line method values.

Although we could have incorporated into the offline system the means to discard the dead space and washout phase, thus collecting the plateau phase alone, we wished to keep the apparatus simple for patients to use unsupervised. Additionally, the amount of exhalate to be discarded will vary considerably according to the following factors: (1) dead space size; and (2) duration of the washout phase, which is longer with higher ENO levels. One further point is
that the use of three-way valves, which have to be opened and closed, can cause problems if a subject fails to fully open or close the valve, thus allowing changes in the expiratory resistance with altered flow rate. For the sake of ease of operation, we therefore opted not to discard dead space exhalate.
Stored exhalate may show instability due to removal of NO, which is slowly converted to NO$_2$ in the presence of oxygen (quenching), and increase in NO due to reaction of the exhalate with the material of the storage vessel or contamination of the exhalate with ambient NO. We surmise that the significant rise in NO signal may be related to entraining of ambient NO via minute perforations in the mylar balloons, because the repeated use in this study may have imperceptibly damaged these delicate balloons. Since this study, NO stability in new mylar balloons was checked, and it appears to be remarkably stable (personal communication; B. Clay, BSME; Sievers Instruments; Boulder, CO; January 1999).

In conclusion, we have designed and tested a simple apparatus to collect ENO, without nasal or ambient contamination, while exhaling at a constant flow rate. The offline apparatus gave values which were significantly but predictably lower than those obtained with the on-line exhalation, but with good within-subject reproducibility. The offline method correlates well with the on-line method, but the two methods are not interchangeable; both are valid methods for the assessment of ENO. This apparatus is simple and inexpensive to construct, and it will enable ENO measurement and storage in different settings, and a more efficient use of NO analytic facilities. We feel that the simplicity of the system will be ideal for large-scale community or workplace studies, or for use at home. The stability over time of NO in balloons or other storage vehicles needs to be established by individual investigators before performing studies.

**References**