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Comparison of exhaled nitric oxide analysers

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Summary Currently no published data are available concerning the comparability of different types of NO analysers, making inter-laboratory comparisons difficult. In two sets of experiments we compared 4 and 5 NO analysers, respectively, from 3 different manufacturers using different calibration regimes: calibration with (1) a separate recommended calibration gas for each analyser, (2) a single low concentration for all (394 ppb), and (3) a single high concentration (12.8 ppm). We measured three subjects with known low (L), moderate (M) and high (H) bronchial exhaled nitric oxide concentrations as well as standard gases (SG). In the first set of experiments, calibration regime 1 resulted in the largest differences between analysers (coefficient of variation (CV) for L, M, H, SG: 0.42, 0.22, 0.20, 0.14). The lowest CV between analysers was observed after calibration 2 (0.34, 0.19, 0.12, 0.02). Very similar results were obtained in the second set of comparisons. Thus, differences between analysers existed, but were mainly due to differences in recommended calibration gases/procedures. Only a small part was explainable by deviations from target flow. These differences need to be taken into account when comparing data between laboratories or replacing the calibration gas of an analyser, as well as for the establishment and interpretation of normal values.

Introduction

The measurement of exhaled nitric oxide (eNO) is increasingly used in clinical settings to monitor airway inflammation and to support the diagnosis of diseases such as asthma or primary cilia dyskinesia.[1–3] Though the distribution of eNO values clearly differs between airway diseases, normal values do not exist. They are, however, increasingly demanded, though it is not known, for example, how comparable data obtained with different analysers actually are.

It was therefore the aim of this study to compare NO analysers from different manufacturers under different setup and calibration regimes. At present,

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NO analysers from 4 main manufacturers seem to be most commonly used. Despite the fact, however, that they all use the chemiluminescence method for detection, there are a number of factors influencing the readings, such as the basic setup, measuring chamber and sampling tube design, the way expiratory flow is controlled or measured, sensitivity to ambient conditions, or differences in calibration. To account for these factors as much as possible we compared NO analysers from 3 different manufacturers under the same or similar ambient conditions, checked flow rate by the use of a bell spirometer, and studied the effect of calibration by comparing the analysers using different calibration regimes. Comparisons were performed by measurements in 3 subjects (1 healthy, 1 rhinitic, 1 mild asthmatic) with known stable eNO values, as well as by NO standard gases. In a first set of measurements we compared 4, and in a second set 5 NO analysers.

Material and methods

Analysers

Two NIOX® (NIOX1, NIOX2) (Aerocrine, Solna, Sweden) analysers used in research mode were constantly running, while two Sievers NOA®280 (NOA1, NOA2) (Boulder, USA) and one ECO medics® (ECOM) (ECO PHYSICS, Duernten, Switzerland) were started at least 3 h prior to calibration and measurements. NIOX analysers were used with the calibration gas recommended by Aerocrine® (198 ppb, accuracy 5%, Hoek Loos Specialty Gases, Amsterdam, The Netherlands). ECO medics recommends and delivers its analyser with a 12.8 ppm standard gas (accuracy not available, certified grade, SIP Analytical Ltd., Kent, UK). This gas was diluted within the analyser about 17-fold, resulting in a calibration with approximately 750 ppb. The NOA1 was calibrated with 394 ppb (accuracy 5%, Messer Griesheim (MG), Krefeld, Germany) and the NOA2 was calibrated with 450 ppb (accuracy not available, Westfalen, Osnabrück, Germany) in the first set of comparisons and with a 417 ppb gas (accuracy 5%, MG) in the second set.

The NIOX analysers had a built-in NO trap for zero point calibration, while an external zero gas (SIP Analytical Ltd.) was used for the ECOM and a zero filter (Sievers, FMI, Seeheim, Germany) for both NOA1 and NOA2.

All analysers except the NOA2 were situated in one room to ensure equal ambient conditions; the NOA2 was permanently used for clinical measurements and could not be moved but ambient conditions were similar. The standard gases (SG) used for testing, in addition to subjects, had 411 ppb (accuracy 5%, MG), 394 ppb (MG, also used for calibration), and 476 ppb (accuracy 5%, MG).

Expiratory flow rate

The NIOX had an internal flow restrictor maintaining a flow of 50 mL/s as long as subjects kept sufficient pressure. For both NOA we used a custom-made system including a number of resistors and a computer program to achieve a visual feedback for the subjects to expire at a pressure of 20 mbar. For all resistors, non-linear flow–pressure curves had been assessed using a calibrated bell spirometer (VOLUTEST, Mijnhardt, Zeist, The Netherlands) as flow integrator, so that deviations from the target pressure/flow could be taken into account. This setup was used to correct for deviations from target flow of eNO in each subject, using a 2-parameter transfer model of flow dependence ($NO=conc_{airway\ wall}*(1-\exp(-\text{transfer factor}/\text{flow rate})$, e.g. Jörres[1]).

The ECOM also used a flow resistance. The actual flow rate was measured by an ultrasonic sensor, which was calibrated according to recommendations prior to measurements. The accuracy of flows through all systems (2 NIOX flow restrictors, 1 NOA resistor, ECOM flow sensor) was checked using a calibrated bell spirometer by integrating flow over time at 20 mbar.

Calibration regimes

Handling of calibration gases was performed according to the manufacturers' instructions. We carefully rinsed pressure valves after attachment to bottles and avoided sudden pressure changes to minimise the risk of contamination with air, which can result in the slow decay of the NO in calibration flasks.

Calibration 1: Each analyser was calibrated prior to comparisons with the gas either provided by or in a concentration recommended by the respective manufacturer as described above.

Calibration 2: All analysers were calibrated with the same calibration gas (394 ppb, MG). This gas with this concentration was not recommended by Aerocrine, but could be used in research mode. The ECOM needed a concentration >1 ppm for calibration; therefore, this calibration required a special method provided by the manufacturer.

Calibration 3: Analogous to calibration 2, but a 12.8 ppm gas (SIP) was used. Due to internal

dilution this resulted in a calibration with approximately 750 ppb in the ECOM. Both NOA analysers were calibrated by indicating that this calibration should be used for measurements in both the ppb and ppm range.

Study design

One healthy female with known low eNO value (mean over study period: 11 ppb), one male subject with rhinitis tested out of season (32 ppb) and one male subject with a history of mild asthma in stable condition without medication (50 ppb) were chosen for this study. Subjects were experienced in performing NO measurements.

The first set of measurements (comparison 1) was performed on 3 days within a 4 day period to avoid major changes of NO values (NOA1/NOA2, NIOX1, ECOM). On each consecutive day one of the 3 calibration regimes was used. To check for a drift of analysers, we additionally performed the measurements of calibration regime 2 at midday and in the afternoon.

On each day, NO measurements of the 3 subjects and standard gases were done in random order. Each subject performed at least 3 measurements and NO levels were determined from mean values of valid plateaus according to the published guidelines.[4] The flow rate indicated by the respective analyser was always within 5% of 50 mL/s. Subjects inhaled NO-free air from the NIOX and ECOM built-in NO scrubbers, while NOA measurements were performed by inhaling ambient air. Ambient air NO concentrations as detected by all analysers were always below 3 ppb and NOA values did not differ when NO-free air (supplied by the ECOM NO-scrubber) was inhaled. Standard gases were measured by leading the gas through an open tube under low pressure, with the input nozzle of the analyser actively sampling from this flow. A custom-made adapter was used for the NIOX and sampling was started manually.

The second set of experiments (comparison 2) was performed about 8 weeks after the first one, to verify the differences observed in comparison 1 and because an additional NIOX analyser was available at this time. Measurements were performed as described above in the same 3 subjects using calibration regimes 1 and 2. Based on the results of comparison 1 we refrained from repeating calibration regime 3. Calibration gases from comparison 1 were used again, except for the NOA2. In calibration regime 1, both NIOX analysers were calibrated with 198 ppb. All measurements of comparison 2 were repeated the next day.

Data analysis

To quantify the differences between analysers in an illustrative way, standard deviations (SD) and coefficients of variation (CV) were evaluated instead of Bland–Altman-like plots which did not seem to offer an advantage. Friedmans ANOVA was used to compare CV and SD between the different calibration regimes, and the Wilcoxon matched-pairs signed-ranks test for comparisons between single conditions. A P value of <0.05 was considered statistically significant.

Results

Tables 1 and 2 summarise the data for both comparisons. Data of comparison 2 in Fig. 1 were normalised by defining the mean concentration over all analysers as 100%. In both sets of experiments the variability (CV) between analysers was significantly different (Friedmans ANOVA, $P<0.05$ each), and the lowest variability between analysers was observed when they were calibrated with the same low concentration gas (394 ppb) (compared to calibration with separate gases $P = 0.04$ in comparison 1 and $P = 0.07$ in comparison 2; Wilcoxon test). In line with this, SD as a measure of variation between analysers yielded similar results (ANOVA, comparison 1: $P = 0.007$, comparison 2: $P = 0.045$; post hoc Wilcoxon: $P = 0.04$ and 0.07, respectively).

After sufficient warm-up of all analysers we found no indication for a major drift of measurements over the day, the overall median (quartiles) of the differences between the two time points being 0.0 (−4.2; 1.5) ppb (3 subjects and 2 standard gases × 4 analysers, $n = 20$). The coefficient of reliability for these repeated comparison between analysers was 0.997.

The mean ± SD nominal flow rate indicated by the analysers was 50.2 ± 1.4, 50.5 ± 1.3 mL/s for the NOA1/NOA2, 50 ± 0 mL/s for both NIOX1/NIOX2, and 49.6 ± 1.7 mL/s for the ECOM. For NOA1/NOA2 and NIOX1/NIOX2 analysers, actual flow rates as determined by the bell spirometer were close to nominal values: NOA1/NOA2: 47.4 ± 0.5 mL/s (+2.5 mL/s sample tube flow=49.9 mL/s); NIOX1: 46.0 ± 0.7 (+4.1 mL/s = 50.1 mL/s); NIOX2: 44.0 ± 1.8 mL/s (+4.1 mL/s = 48.1 mL/s). Using a calibrated flow of 47.9 ± 1.0 mL/s, the ECOM showed 43.4 ± 4.0 mL/s. Based on the data from the NOA1 and the custom-made resistors and software, we found that a deviation from target flow by 4 mL/s would explain changes in NO values of ± 1, ± 2, and ± 3 ppb in subjects L, M, and H.

Table 1 NO values (ppb) in comparison 1 using 4 analysers.

	Calibration	ECOM	NIOX1	NOA1	NOA2	Mean	SD	CV
Subject								
L		5.4	11.4	16.7	10.9	11.1	4.6	0.415
M	1	23.6	33.9	39.6	28.8	31.5	6.9	0.219
H		37.6	56.0	57.7	44.8	49.0	9.5	0.195
Standard gas								
SG 411 ppb		330.0	387.0	440.0	340.0	374.3	50.4	0.135
SG 394 ppb		305.0	377.0	405.0	315.0	350.5	48.3	0.138
Subject								
L		6.5	9.5	11.8	15.2	10.7	3.7	0.343
M	2	22.3	30.5	33.0	35.3	30.3	5.6	0.186
H		44.5	53.8	59.6	54.7	53.2	6.3	0.119
Standard gas								
SG 411 ppb		440.0	425.0	435.0	445.0	436.3	8.5	0.020
SG 394 ppb		405.0	395.0	400.0	410.0	402.5	6.5	0.016
Subject								
L		5.1	4.1	13.0	9.0	7.8	4.0	0.518
M	3	20.4	23.5	34.5	31.3	27.4	6.6	0.240
H		37.4	46.1	55.1	49.9	47.1	7.4	0.158
Standard gas								
SG 411 ppb		330.0	356.0	385.0	370.0	360.3	23.4	0.065
SG 394 ppb		305.0	328.0	355.0	345.0	333.3	21.9	0.066

Calibration 1: each analyser calibrated with separate recommended calibration gas.
Calibration 2: each analyser calibrated with the same low concentration gas (394 ppb) Calibration 3: each analyser calibrated with the same high concentration gas (12.8 ppm, the ECOM dilutes this gas internally to approx. 750 ppb). NO values represent the mean of 3 measurements with valid plateau levels.

Discussion

Our data suggest that differences in measured values between NO analysers of different manufacturers exist, as exemplified in a sample of five analysers from three companies. The major factor responsible for these differences appeared to originate from differences in calibration gases and procedures. Only a small part seemed to be explainable by deviations from the target flow rate of 50 mL/s.

For each analyser tested the repeated NO measurements of stable subjects within 8 weeks yielded very similar results (Table 1 vs. Table 2), in line with the known reproducibility of NO values. This was also true for the two measurements performed on 1 day which were performed to assess instrument drift. It is known that especially the NOA and ECOM require sufficient warm-up time to avoid drifts in measured values over the day.

In both calibration regimes the pattern of differences between analysers was very similar (Fig. 1). The smallest differences between analysers were obtained when comparing standard gases in the range of 400 ppb. The comparison of subjects' NO values resulted in larger CV values, which were inversely related to mean eNO: the lower mean eNO, the higher the CV between analysers. Conversely, comparing SD values between analysers we found the lowest SD in the subject with the lowest NO value (L) and the largest SD when standard gases were compared, however, the ratio between both SD values was smaller than that between corresponding mean values (Tables 1 and 2), resulting in the difference of CV values. This demonstrates that SD values between analysers were neither constant nor proportional to the mean value. Although standard gases were in the concentration range of calibration gases, in contrast to subjects, it is unlikely, that the relatively greater differences between analysers as observed in subjects were due to differences in zero point calibration. Ambient air NO levels were always very low, so that potential differences in the efficacy of NO-scrubbers could be neglected. Furthermore, only a minor part of the increase in variability in subjects compared to standard gases could be attributed to deviations from target flow. The fact that human breath is saturated with water vapor also needs to be taken into account when interpreting the differences between measurements of standard gases and subjects. Therefore differences

Table 2 NO values (ppb) in comparison 2 using 5 analysers.

	Calibration	Day	ECOM	NIOX1	NIOX2	NOA1	NOA2	Mean	SD	CV
Subject										
L			5.3	12.3	10.3	8.4	10.9	9.4	2.7	0.289
M			22.5	36.2	36.0	27.7	30.9	30.7	5.8	0.189
H		1	35.9	62.1	56.7	52.6	51.2	51.7	9.8	0.189
Standard gas										
SG 476 ppb	1		360	385	395	475	450	413.0	47.8	0.116
Subject										
L			6.6	*18.2*	11.5	13.4	11.5	12.2	4.2	0.340
M			20.4	*44.7*	33.8	29.9	32.9	32.3	8.7	0.269
H		2	38.9	*66.5*	56.5	52.3	47.8	52.4	10.2	0.195
Standard gas										
SG 476 ppb			370	nd	410	460	457	424.3	42.8	0.101
Subject										
L			3.5	11.6	10.3	8.8	12.2	9.3	3.5	0.372
M		1	24.3	31.9	31.7	26.6	32.9	29.5	3.8	0.129
H			39.5	51.9	55.2	49.6	59.6	51.2	7.5	0.147
Standard gas										
SG 476 ppb	2		445	475	475	470	473	467.6	12.8	0.027
Subject										
L			10.2	14.2	14.8	11.0	14.0	12.9	2.1	0.163
M		2	28.8	36.6	33.9	30.0	33.8	32.6	3.2	0.097
H			43.8	53.5	51.2	45.8	52.2	49.3	4.2	0.086
Standard gas										
SG 476 ppb			442	465	475	470	465	463.4	12.7	0.027

Calibration 1: each analyser calibrated with separate recommended calibration gas.
Calibration 2: each analyser calibrated with the same low concentration gas (394 ppb).

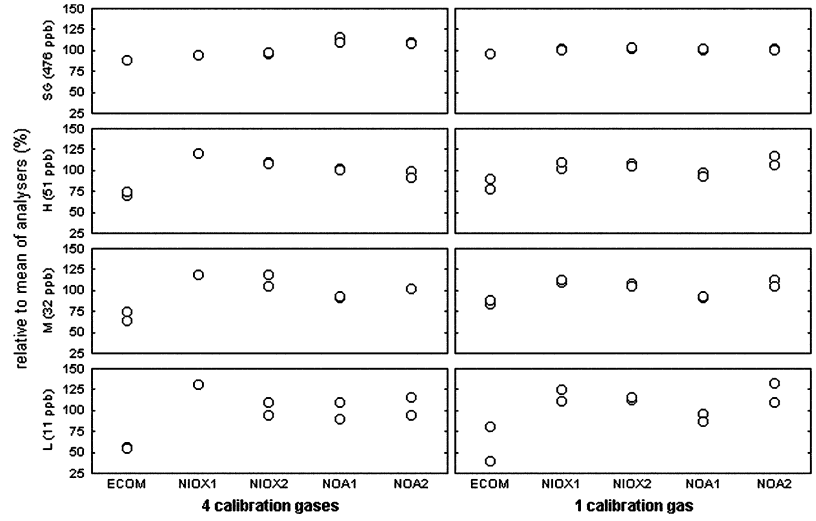

Figure 1 Comparison of 5 NO-analysers. Data were normalized to the overall mean value of all analysers to allow better comparability. NO values of 2 measurements on consecutive days are shown. Left: data after calibration of analysers with separate calibration gases (both NIOX were calibrated with a single gas, 198 ppb; no data available for day 2 for NIOX1 due to technical reasons). Right: data after calibration with a common single gas (394 ppb). Top to bottom: Measurement of standard gas (476 ppb), subject with high (H, overall mean 51 ppb), medium (M, 32 ppb) and low (L, 11 ppb) exhaled NO level.

between analysers regarding the efficacy or method of water vapor removal could have contributed to variability and particularly the differences between measurements in subjects and those of standard gases.

The reduction of differences between NOA1 and NOA2 in comparison 2 was most likely due to the calibration gas used for the NOA2 in comparison 1. Based on the findings obtained with other gases from different manufacturers, there was evidence that the calibration gas had a concentration different from that indicated. It was therefore replaced in comparison 2. This underlines the need to be critical regarding calibration gases and to be able to compare with independent standards, whenever possible. The remaining differences between NOA analysers in comparison 2 could then be attributed only to ambient conditions, as the NOA2 was located in a slightly cooler room, or to physical and electronic factors such as detection tube temperature and vacuum. Similar differences were also observed between NIOX analysers in comparison 2, though they were calibrated with the same gas (Table 2). We would like to mention that on day 2 of comparison 2 (Table 2), NIOX1 did not measure correctly and needed repair. Values for the subjects were much higher compared to those the day before and no value could be obtained for the standard gas. These values were therefore omitted from the analysis.

There was a trend towards lower values detected by the ECOM. The reason for this is not clear but the phenomenon has also been observed by others.[5] Using the bell spirometer as standard, we concluded that in our setup the ECOM measured flow rate too low, which would result in a higher flow rate during measurements of subjects. However, the deviation of about 4 mL/s from the required flow 50 mL/s explained only a small part of the differences. In addition, relatively low values were also observed with the standard gases. The ECOM used a gas mixture of approximately 700–800 ppb for internal calibration, which was higher than concentrations used by the NIOX and NOA. The factors underlying the reduction in measured NO values in the ECOM are unknown to us.

Though the use of the 12.8 ppm gas for calibration in comparison 1 resulted in a lower variability (CV) between analysers, absolute values of measurements were quite different within analysers, except in the ECOM, for which this was the recommended gas. While the NOA could be calibrated with gases in the ppm range, this was not recommended for the NIOX, and the high concentration gas might have led to the relatively large deviations (Table 1). Owing to this, the 12.8 ppm calibration regime was omitted in comparison 2.

We would like to point out that on the basis of our results it is not possible to rate analysers in performance or reliability or to conclude that one analyser was measuring correctly and another not. We did not have the means to chemically analyse calibration gases, and a reference sample is currently not commercially available. Thus one has to rely on data provided by gas suppliers and the "true" NO values of gases as well as exhaled air are not known.

From our results one might conclude that analysers from different manufacturers show a sufficient comparability for practical purposes, provided proper calibration is performed. However, analysers in different laboratories will inevitably use different calibration gases and/or procedures. Therefore the variability observed in this study needs to be considered. In a subject with a mean exhaled NO level of about 50 ppb we observed a maximum deviation of >20 ppb between analyser setups (Table 2, calibration 1). In our view, deviations of this magnitude cannot be neglected. Such differences have also to be taken into account in the establishment and interpretation of normal values. In addition, they might affect longitudinal studies whose duration exceeds the shelf-life of calibration gases. For the analysers tested in this study only one concentration gas was needed, in addition to zero air. In case of calibration gas decay (due to improper handling in connecting gas tubings), there is a high risk for incorrect measurements, as this slow decay can proceed unnoticed for a long period of time. Despite the increase in costs, our results therefore suggest the introduction of an independent standard gas, which is used for frequent tests of analyser setup and the validity of calibration. Noteworthy enough, this is already suggested in guidelines.[4] An alternative, currently not available but extremely desirable, would be widely available calibrated small stable reference gas samples to check analysers and to improve comparability.

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