Video Article

Fast and Accurate Exhaled Breath Ammonia Measurement

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Abstract

This exhaled breath ammonia method uses a fast and highly sensitive spectroscopic method known as quartz enhanced photoacoustic spectroscopy (QEPAS) that uses a quantum cascade based laser. The monitor is coupled to a sampler that measures mouth pressure and carbon dioxide. The system is temperature controlled and specifically designed to address the reactivity of this compound. The sampler provides immediate feedback to the subject and the technician on the quality of the breath effort. Together with the quick response time of the monitor, this system is capable of accurately measuring exhaled breath ammonia representative of deep lung systemic levels.

Because the system is easy to use and produces real time results, it has enabled experiments to identify factors that influence measurements. For example, mouth rinse and oral pH reproducibly and significantly affect results and therefore must be controlled. Temperature and mode of breathing are other examples. As our understanding of these factors evolves, error is reduced, and clinical studies become more meaningful. This system is very reliable and individual measurements are inexpensive.

The sampler is relatively inexpensive and quite portable, but the monitor is neither. This limits options for some clinical studies and provides rational for future innovations.

Video Link

The video component of this article can be found at http://www.jove.com/video/51658/

Introduction

Ammonia is a ubiquitous byproduct of protein metabolism[1]. Ammonia measurement can therefore help clinicians assess various disease and wellness states[2]. However, ammonia is difficult to measure accurately, via blood or breath, because it is very reactive. Though commonly used, blood assays have numerous drawbacks, including basic concerns about accuracy[3]. But the major problem with blood assays is the reality that they only ever collected episodically. This is important because ammonia physiology, much like blood glucose and many other metabolic processes, are fluid and ever changing[4]. In contrast, breath assays are fully non-invasive and quick, thereby easily enabling repeated measures. Thus, breath ammonia measurement is attractive because it may address a serious unmet need in a unique way.

Breath collection, however, presents unique concerns. Whereas phlebotomy inherently carries the jeopardy of error in several unpredictable ways (*e.g.*, tourniquet time, sweat contamination, blood cell hemolysis, delay in laboratory measurement, etc[5]), breath measurement researchers must contend with a different group of novel challenges: variability in breathing, contamination with oral mucosal or bacterial ammonia, influence of ambient air and apparatus humidity and temperature, etc[6]. Indeed, it is unwise to underestimate the task in connecting experimental equipment to humans using experimental procedures to discover unknown biology. In part due to these obstacles, breath ammonia has not yet met its potential.

Herein, we present our breath ammonia measurement protocol for fast and accurate results. Our protocol has strength in three areas: the monitor, the interface sampler, and attention to the human influences. The monitor was built by colleagues at Rice University as previously described[7]. The basis of the measurement is a quartz enhanced photoacoustic spectroscopy (QEPAS) technique that employs a piezoelectric quartz tuning fork as an acoustic transducer. Photoacoustic effect occurs when acoustic waves are produced by the absorption of modulated laser radiation by target trace gas species. The trace gas is detected using an acoustic cell that is acoustically resonant to the modulated frequency. An absorption wavelength for ammonia was selected that is free from spectral interferences from interfering species in breath. For the purposes of human exhaled breath measurement, the main features of the monitor include a wide measurement range (from ~50 parts per billion, ppb to at least 5,000 ppb) and speed (1 sec measurements). The speed of the monitor enables time resolution throughout the breath cycle.

The monitor is coupled to a specially designed breath sampler. The sampler consists of a pressure sensor and capnograph. It displays and archives real time measurements of mouth pressure and carbon dioxide as well as the ammonia concentrations determined by the sensor. This

sampler, therefore, enables the technician to evaluate the quality of the breath effort as the breath is collected. This enables us to exceed the recommendations for analyzing breath nitric oxide (Fe_{NO}) proposed by Task Force of the American Thoracic Society/European Respiratory Society (ATS/ERS)[8]. For all breath sampling, a disposable one-way in-line valve was used on the mouth port of the breath sampler.

Because of the speed of the monitor and the quality controls provided by the sampler, we were able to carefully evaluate human influences[9]. Most subjects, for example, initially hyperventilate when instructed to breathe. Other important influences, such as oral pH and mouth rinses, temperatures of the sampler, monitor and all associated tubing, and mode of breathing, were then studied, and are the basis for the illustrative experiments below.

Finally, and perhaps most significantly, it must be emphasized that multiple highly experienced groups are measuring breath ammonia using entirely different sensors and measurement procedures. These may have important advantages and validity. A complete comparison is beyond the scope of the present work[10,11,12].

Protocol

1. Preparation of Instruments

1. Turn on the external power supply to the ammonia optical sensor platform, laser diode controller, a custom built control electronics unit (CEU), the breath sampler, air pump, and laptop.
2. Check that both the exhaust and cooling fans of the ammonia sensor are operating. NOTE: One is located in the rear of the sensor, the second is found within the sensor, which is easily accessible.
3. Ensure the acoustic detection module and needle valve temperature are at 38.0 °C by checking the digital display located on the side of the ammonia sensor box. Wait approximately 35 min from the time the sensor is provided power for the temperature to stabilize.
4. Set inlet tubing and mouthpiece temperature to 55 °C by clicking on an icon name “ChangeT” found on the desktop of the breath sampler. The temperature can then be changed by clicking the up or down arrow followed by clicking “Update Temp”. Click “Exit” to return to the desktop. Allow the system at least 5 min for the temperature to stabilize. Maintaining the temperature of the sampler and tubing at 55 °C minimizes the surface loss of ammonia.
5. Open the software on the laptop that controls the ammonia sensor. The program can be accessed within a folder named “NH_3 Breath Sensor Program” on the desktop. Within this folder, the user must select the icon “main labview software”. This folder contains several applications, but the user must select “Mainsequence.vi” to access the desired interface. Select “run” in the top left corner of the screen. NOTE: This begins the line locking calibration sequence. The laser of the QEPAS monitor operates on an optimal current, which is selected during an automated line-locking procedure. This process will take approximately 25 min.
6. Create a new session on the sampler such that each subject session has its own file that is saved to an attached flash drive. NOTE: This is performed by opening the program “Breath Sampler” found on the desktop of the sampler. There is space to identify the session appropriately. All data generated during the session will be saved in the flash drive under this identifier. The date of the experiment is typically used as part of the file name. The inlet and mouthpiece temperature must be adjusted before entering the breath-sampling program.
7. Insert a new disposable mouthpiece into the inlet pipe. Wear disposable gloves as to avoid contaminating mouthpiece with ammonia from fingers.

2. Breath Sample Collection

NOTE: The relevant Institutional Review Board (Ethics Board) must approve any study that involves human subjects. There are many factors that can drastically influence breath ammonia. These factors can alter breath ammonia measurements by directly affecting systemic ammonia levels or by affecting the journey of breath metabolites from the lungs to the instrumentation.

1. Ensure subjects arrive to the lab in a fasting state, having consumed no food for approximately 12 hr upon arrival and that they have refrained from exercise the morning prior to testing.
2. Ensure no substance has been introduced into the mouth for at least 1 hr prior to data collection. Ensure that subjects brush their teeth greater than 1 hr before testing.
3. Seat the subject in front of the ammonia sensor. Instruct the subject to hold the inlet pipe, and make sure they do not touch the mouthpiece to avoid ammonia contamination.
4. Click “Start” on the sampler interface. Have the subject exhale into the mouthpiece for as long as they can, or until the operator deems the sample to be sufficient. NOTE: This is a single full exhalation lasting at least 10 sec. Mouth pressure is measured in real-time as a surrogate for flow rate. A color-coded pressure gauge helps the subject produce and maintain the desired exhalation pressure of 10 cm of water, which represents an exhalation flow rate of 50 ml/sec. This flow rate was selected since it has been adopted by the ATS/ERS for the protocol to determine Fe_{NO}. This exhalation flow rate is achievable by children and adults. Similarly three reproducible exhalations should be obtained that differ by less than 10%.
5. Click “Stop” on the sampler interface when the breath sample is completed.

3. Breath Sample Measurement

1. Once a breath sample has been analyzed, the laboratory technician operating the monitor then has the ability to analyze any segment of that breath profile. The portion of the breath that is of interest is the phase III segment. This is characterized by a “plateau” in the concentration of carbon dioxide and is found in the middle to late stage of the breath.
2. Select the phase III portion of the sample by dragging the vertical lines on the sampler interface to begin when carbon dioxide plateaus and stop right before the pressure drop of the breath. See **Figure 1** for clarification.
3. Save the data to the flash drive by clicking “Store” on the sampler touch screen interface.

4. Once the breath data have been stored, the user can select “start” to initiate a new breath sample.

4. Illustrating the Effects of Mouth Rinse and pH on Breath Ammonia

1. Sample 3 breaths to establish the baseline ammonia level. Ensure that breaths are taken at least 5 min apart from each other.
2. Rinse the mouth thoroughly with a 30 ml aliquot of water for 60 sec.
3. Collect a breath sample within 60 sec of the rinse (Section 2). Collect breath samples over the course of the next hour to observe the change in ammonia over time. NOTE: Samples can be taken as frequently as every minute, but longer intervals are typically used.
4. Rinse mouth thoroughly with a 30 ml aliquot of basic solution (sodium bicarbonate in water) for 60 sec and repeat 4.2.1.
5. Rinse mouth thoroughly with a 30 ml aliquot of acidic solution for 60 sec and repeat 4.2.1.

5. Illustrating the Effects of Inlet and Transport Tubing Temperatures on Breath Ammonia

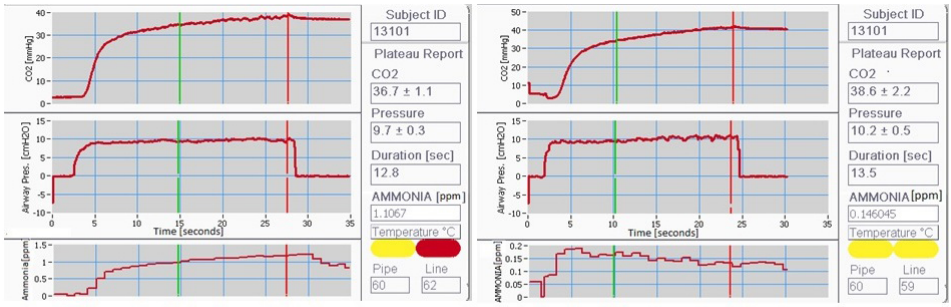
1. Sample three breaths over the course of 15 min with the inlet temperature set to below body temperature, approximately 30 °C.
2. Increase the temperatures of inlet and transport tubing to 55 °C respectively using a desktop icon on the touch screen interface of the breath sampler. Allow the system at least 5 min to reach steady state.
3. Sample 3 breaths, 5 min apart into the heated inlet.

Representative Results

Subjects can be expected to produce a wide range of baseline breath ammonia levels. Healthy individuals may begin the day with a breath ammonia measurement of 100-1,000 ppb. Rinsing the mouth out with any fluid immediately changes the amount of detectable breath ammonia. Neutral and acidic fluids typically cut the amount of observable ammonia by more than half. These levels then return to baseline as the effects of the rinse wears off. The effects of water seem to dissipate within 15 min, while an acid can keep detectable breath ammonia to a minimum for over 2 hr. A basic rinse, such as sodium bicarbonate, will double or triple the amount of detectable breath ammonia before returning to baseline over a 20 min period. Notably, hydrogen peroxide does not seem to affect exhaled breath ammonia more than other rinses; thus, it does not seem that oral bacteria contribute significantly to breath ammonia measurement.

As noted above, the sampler coupled to the ammonia sensor provides continuous data a technician can use to assess the quality of a breath sample. Mouth pressure and carbon dioxide are the two characteristics of an exhalation used to validate a breath sample. Mouth pressure serves as a surrogate to the flow of air from the lungs into the sampler. The technician must make certain that a subject is providing enough flow of alveolar air, which contains the metabolite of interest, into the monitor. Technicians should expect a normal mouth pressure range of 9 to 10.5 cm of water. The exhalation should be quite steady over the course of 10-20 sec, which manifests itself in the standard deviation of mouth pressure. A standard deviation of a quality breath should be under 1 cm of water.

Carbon dioxide measurement is also important as it enables closer evaluation of process of exhalation. During Phase I, exhalation is initiated and the composition of exhaled gas consists of predominantly anatomical dead space air (~21% oxygen, 0.03% carbon dioxide, 78% nitrogen, and 0.5% water) *i.e.*, the air that was breathed in during the inhalation phase of the previous breath cycle. During Phase II, alveolar gas passes into the anatomic dead space and mixes with residual dead space air with the result that the concentration of carbon dioxide increases rapidly. The concentration of carbon dioxide in exhaled breath continues to rise, albeit more slowly, during Phase III exhalation and the peak value (end tidal concentration) corresponds to the concentration of carbon dioxide in venous blood. This gradual rise in carbon dioxide concentration during Phase III is due to mixing of the alveolar gases with the remainder of the dead space air and is due to slow emptying of alveolar sacs. The composition of breath at the end of Phase III is approximately 13% oxygen, 5% carbon dioxide, 78% nitrogen, and 4% water. Carbon dioxide levels during the phase III portion of the breath can range from 30-40 mmHg. Phase III corresponds to where these CO_2 levels plateau (**Figures 1A-D**).



1a. Integrated phase III breath sample

1b. Acidic rinse sample

1c. Low pressure sample

1d. Whole breath integrated sample

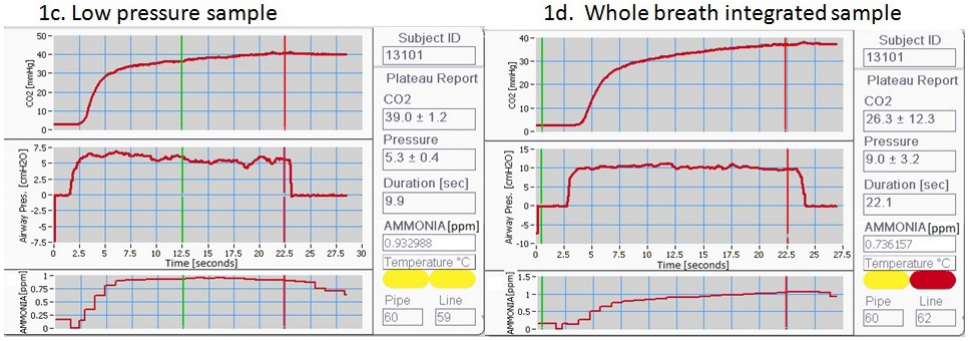


Figure 1. Breath samples that integrate the phase III portion of breath data for various conditions. 1a) A typical breath sample that integrates the phase III portion of the breath data. The green and red vertical lines set the span in which the line is to be analyzed. The first portions of the exhalation are ignored. **1b)** The effect an acidic rinse has on breath ammonia. The conversion of NH_3 to NH_4^+ drastically lowers the amount of detectable ammonia. Decreased flow results in the reduction of alveolar ammonia in the sampled breath as seen in **1c**. The decreased flow does not allow as much alveolar air to be sampled. **1d)** A breath sample that integrates phases I, II, and III. The inclusion of phases I and II in the analysis lowers ammonia and carbon dioxide considerably. This is an inaccurate reflection of systemic ammonia. Please click here to view a larger version of this figure.

Most subjects are able to produce an acceptable breath on their first try. However, some subjects will require a repeat of the breath. Further, as pressure and carbon dioxide are recorded, these can also be considered in data analysis.

Discussion

The benefits of a non-invasive procedure capable of detecting trace metabolites in real time are obvious. However, the field of breath research has struggled to fulfill this potential. Breath measurement is a dynamic process vulnerable to many confounding factors. Our approach has important strengths: specifically, the sensitivity and speed of the Rice QEPAS based ammonia monitor coupled to the breath sampler have enabled us to evaluate and identify breath collection factors germane to accurate measurement. This approach is very reliable: for example, after some preliminary experiments, each of the almost 500 individual breath data points collected for recent experiments was consistent with the expected outcome[9].

Until the various factors affecting exhaled breath ammonia are better understood, it is important to provide thorough and uniform instructions to subjects prior to arrival. At present, we generally ask subjects to fast after midnight for morning collection, brush their teeth greater than 1 hr prior to presentation, and avoid exercise, smoking, or filling automobiles with petroleum fuels. Though we have evaluated various food regimens the evening before data collection (*e.g.*, high fiber v. low fiber), we have not established that diet convincingly impacts baseline data the morning of the test. Morning collections also minimize the impact of apparent diurnal variations, which seem to occur for unknown reasons[13].

There may be other equally valid or superior methods for exhaled breath ammonia collection. It is possible, for example, that a standard rinse at a set time point prior to breath collection could result in the useful measurement of oral mucosal ammonia which might also reflect systemic levels. Another path might be to abandon the oral cavity and measure nasal ammonia or both[10,14]. This latter approach could obviate the need for the interface sampler. Regardless, any method needs to carefully consider a variety of technical factors relevant to this volatile metabolite including humidity, temperature, pH, flow, as well as oropharyngeal biology.

Naturally, assumptions and beliefs about measurement method have critical bearing on data analysis. We believe that pressure and carbon dioxide are key aspect of quality control. However, it is not certain, for example, whether ammonia should be reported as measured, or carbon dioxide-adjusted, with units as ppb or picomole. As more experience and confidence are gained in the various technical sources of error, the complex considerations incumbent in data analysis come into greater focus and will propel better understanding of this very challenging biology. (**Figure 2**: Random and Non-Random Error. Measuring True Biological Variability Versus **A**. Biological Epiphenomena or **B**. Abnormal Breathing and Oral/Nasal Factors or **C** and **D**. Equipment Performance.)

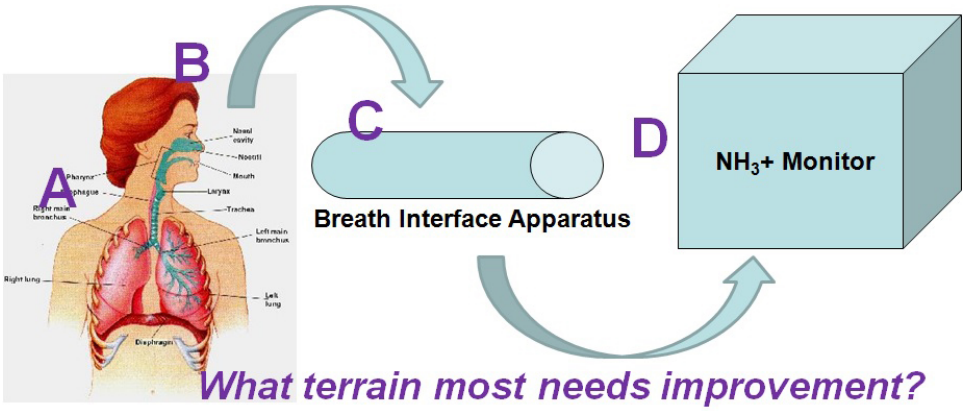


Figure 2. Diagram of patient to breath interface to an ammonia monitor. A) Patient to be tested and is also the most unpredictable portion of the breath collection process. Patients' eating, exercise, and smoking habits can have large impacts on data collection. **B)** Breath exhalations are variable from person to person, so a method to standardize breaths is important. Providing a visual cue to provide desirable flow of air from the lungs works well to keep exhalations uniform. **C)** Breath interface apparatus is closely related to **B** in that breath consistency is an important factor in comparing subjects. The breath sampler interface allows the user to view various breath sample parameters in real-time. **D)** Ammonia monitors can manifest themselves with different technologies. Quartz enhanced photoacoustic spectroscopy has many inherent benefits believed to be ideal for breath analysis. Please click here to view a larger version of this figure.

Important limitations to the present method must be acknowledged. While the sampler is relatively inexpensive and portable, the ammonia monitor, as presently configured, is neither. As a result, subjects need to come to our dedicated breath research space, as we cannot easily move our equipment to the clinic. This factor, along with the requirement that subject's breath exhale a single long breath, impacts which subjects can be studied (*i.e.* sick patients with liver cirrhosis, a key target population, are often practically excluded). Further, because we have only one monitor, we are limited in the number of subjects that can be realistically studied in a given protocol. In turn, this affects sample size and power.

As noted above, the collection of nitric oxide was standardized by the joint effort of the American Thoracic Society and the European Respiratory Society. There is presently no equivalent agreement for breath ammonia, though multiple groups are making critical contributions to the advancement of breath ammonia measurement. As the literature on trace breath metabolite measurement in general and ammonia in particular continues to evolve[15] there will certainly be many modifications and improvements to come. Monitors that are smaller, more portable, and less expensive are critical for successful multicenter clinical trials.

Disclosures

Dr. Risby serves as a consultant for Loccioni Humancare the manufacturer of the breath sampler.

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