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9

Analysis of Volatile Organic Compounds in the Breath

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I. History of Breath Tests

Breath tests date from the earliest history of medicine because physicians in ancient times knew that the odor of the breath is altered in some diseases (1). Even today the astute physician uses his or her nose to supplement sight, sound, and touch at the patient's bedside. Some breath aromas are highly characteristic of disease: patients with diabetic ketoacidosis smell like rotting apples, mainly due to acetonemia. Chronic renal failure causes the breath to smell like stale urine, due to increased levels of dimethylamine and trimethylamine in the blood, and advanced liver failure causes the musty stench of "fetor hepaticus." A patient with a lung abscess may smell like a sewer because of the proliferation of anaerobic bacteria, and indulgence in tobacco, alcohol, garlic, or curry each leaves its own distinctive olfactory signature in the breath.

The era of scientific breath testing dawned in 1784, when Antoine Laurent Lavoisier, the father of modern chemistry, demonstrated that carbon dioxide is excreted in the breath of guinea pigs. This was the first evidence that metabolism of foodstuffs is analogous to the burning of fuel, a discovery which laid the basis of modern biochemistry. It was also the origin of the expression "to be a guinea

pig," because Lavoisier went on to confirm this finding in human volunteers. Colorimetric assays were developed during the nineteenth century which made it possible to detect volatile organic compounds (VOCs) present in the breath in millimolar (10^{-3} M) concentrations. The first breath test for ethanol (still the most common application of breath testing) was developed by a British physician, Francis Anstie, in 1874. He found that breath bubbled through chromic acid turned the solution from red-brown to green if alcohol was present. Nebelthau, in Germany, used an alkaline iodine solution to demonstrate that acetone in the breath of diabetics could also induce a change in color. But the modern era of breath testing did not commence until 1971, when Nobel Prize winner Linus Pauling turned his fertile mind to the problem. He used a cold trap, a tube chilled by dry ice, to freeze out breath VOCs. He then heated the sample and injected it into a gas chromatograph, and found that a sample of normal human breath contained hundreds of different VOCs, most of them in picomolar (10^{-12} M) concentrations.

Pauling's historic achievement (amongst his many others) was to provide the first evidence that human breath is a far more complex gas than anyone had previously suspected. He believed that analysis of breath VOCs could open a valuable new window onto human metabolism and illuminate its functions in health and disease. But only in recent years have we begun to achieve this objective (2). Breath testing for endogenous VOCs languished for some years as a mere scientific curiosity for two main reasons. First, it is technically very difficult to analyze breath VOCs present in picomolar concentrations. Second, after overcoming these difficulties, what then? More than 3000 different VOCs have been observed in human breath, and the biochemical significance of most of these compounds is still unknown (3,4). Thus, breath analysis became a solution in search of a problem.

This historical background sets the stage for the three main questions which now dominate the scientific study of breath analysis: How should it be done? What do the results mean? And why should we do it? This chapter will focus on the first of these questions, with some incidental comments upon the second and the third.

II. Classification of Breath Tests

Breath tests are of two kinds: load and no-load. In a load test, the patient consumes a drug or substrate (which may be labeled with a radionuclide), and its metabolites are subsequently measured in the breath. Load tests are used principally in gastroenterology, for the detection of diseases such as *Helicobacter pylori* infection and pancreatic insufficiency. No-load tests are confined to measuring

VOCs in the breath without any prior administration of a drug or substrate. This review will deal only with no-load tests.

III. Breath VOC Analysis: The Major Technical Problems

Technical problems commence at the instant breath exits from the lips, and afflict every step of the analytical process up to and including the final interpretation of the data. There are four main steps in the analytical process:

1. Capture the breath
2. Concentrate the VOCs in the breath
3. Analyze the concentrated VOCs
4. Compensate for VOCs in background air

A. How to Capture Breath

What could be more intuitively easy than capturing breath? One simply asks a donor to blow into a tube which is attached by appropriate plumbing to a collection device. However, there are difficulties in capturing a sample of breath for subsequent analysis.

Resistance to Expiration

It is obviously difficult to breathe out against resistance, especially for the elderly and those with respiratory illness. It is therefore equally obvious that any system for capturing human breath should present the least possible resistance to unimpeded gas flow. Tubing should be wide in bore (e.g., at least 2–3 cm in diameter) and contain little or no obstruction to the free flow of breath, such as valves or water traps. Curiously, there was a long-standing tradition among the pioneer breath researchers (including Lavoisier and Pauling) to design systems with such high internal resistance that only physically fit athletic youths could possibly donate a sample without discomfort.

Infection Control

It is also obvious that the breath collection system should not present an infection hazard to the breath donor. A designer of a breath collection system should always plan for the worst-case scenario: What if a breath donor suffers from pulmonary tuberculosis? Could they contaminate the device with infectious organisms which may potentially be transmitted to subsequent breath donors? The device must employ a combination of disposable components and careful design to minimize this potential problem.

Water Condensation

Breath is saturated with water, which promptly condenses within the tubing of the capture system. Breath VOCs may then partition from the gas phase into the aqueous phase. Since water is very abundant and breath VOCs are present in very low concentrations, this partitioning process may deplete the gas phase of VOCs and result in inaccurate low readings in the subsequent assay. In addition, condensed water may be so abundant as to affect the integrity of the collecting system and might also affect the accuracy of an assay. The author has developed a breath collection apparatus (BCA) with heated tubing which inhibits condensation (3,4). This approach is effective, but it introduces additional complexity and expense.

Chemical Contamination

Breath VOCs are present in picomolar concentrations, and the highly sensitive assays required to detect them may be readily contaminated by VOCs from other sources. These may include volatile adhesives and plasticizers in disposable components. The breath collection apparatus must therefore be constructed from components which are least likely to contribute VOCs, e.g., stainless steel and inactive plastics such as polycarbonate and Teflon. Disposable plastic tubing of the type employed in anesthetic and ventilator systems should be avoided.

Dead-Space Air Dilution

Breath is not a homogeneous gas. At rest, an adult expires approximately 500 mL with each breath, of which the first 150 mL is dead-space air from the upper airways and nasopharynx, and the subsequent 350 mL is alveolar breath from within the lungs. For analytical purposes, dead-space air is useless. Volatile organic compounds in breath and air interchange at the alveolar membrane, so only alveolar breath is of any value for analytical purposes. There are two approaches to this problem, mixed sample collection and alveolar breath collection.

Mixed Sample Collection

A mixture of dead space and alveolar breath is collected (5).

Advantage. Simplicity: the patient simply inflates the collection system, e.g., a bag or balloon.

Disadvantage. Inaccuracy: the sample is diluted by dead space air in which no interchange has occurred. This degree of inaccuracy is not a constant which can be ignored, because the dilution factor varies with the tidal volume, i.e., whether the donor is breathing deeply or shallowly.

Alveolar Breath Collection

A breath capture system can be designed with special geometry which ensures that the collected sample is virtually 100% alveolar breath (6). Breath collection bags are available with a side port which diverts the first few hundred milliliters of expired breath into an ancillary side bag, so that only alveolar breath enters the main bag (Quintron Instruments Company, Menomonee Falls, WI). The author has developed a breath collection apparatus (BCA) in which breath enters a long tube, the breath reservoir. The geometry of the breath reservoir ensures that dead space air and alveolar breath are separated, so that only alveolar breath is pumped out to the collection system.

Advantage. Accuracy: the assayed sample is not diluted by dead-space air.

Disadvantage. Complexity and expense.

Breath Container Artifact

Probably the simplest and most straightforward way to capture a sample of breath is to ask the donor to blow up a balloon or a bag. This method was employed for breath ethanol testing before digital breathalyzers were available. Typically, samples are collected into a comparatively inert plastic bag (e.g., Tedlar or Teflon), which is then taken to the laboratory for analysis by any desired method (7,8). A more elaborate though conceptually identical approach is to collect breath into a partially evacuated metal cylinder or sphere (9,10). However, use of a container entails a risk of artefactual loss of sample, due to adsorption of VOCs to the walls of the container. There are two approaches to this problem, collecting breath into a container, and collecting breath directly into a trapping system.

Collecting Breath into a Container

Advantages

1. The method is simple and straightforward to use.
2. Containers can be reused after cleaning.

Disadvantages

1. Sample can be lost by adsorption to the walls of the container.
2. Containers are often difficult to render chemically clean for reuse.
3. Containers are expensive, particularly if constructed of metal.
4. Inflated containers are bulky and may be difficult to transport.
5. The sample is mixed dead space and alveolar breath, unless a specialized collecting system employed.

Collecting Breath Directly into a Trapping System

Advantages. Avoidance of the disadvantages of containers.
Disadvantages. Additional complexity of design, and expense.

B. How to Concentrate Breath VOCs

After the breath sample has been captured, it is generally necessary to concentrate the VOCs for assay with laboratory instrumentation. Four methods are in current use: cold trapping, condensate trapping, chemical trapping, and sorbent trapping.

Cold Trapping

Cold trapping was the method employed by Pauling et al. to demonstrate VOCs in concentrated breath. Typically, a donor blows through a U-tube which is immersed in a cryogenic fluid (e.g., liquid nitrogen or acetone chilled with dry ice) (11,12). The U-tube may be packed with glass beads which provide a large surface area on which the breath VOCs can condense. The sample is then heated, and the volatilized concentrated VOCs are then analyzed by conventional laboratory methods, e.g., gas chromatography (GC) possibly combined with mass spectroscopy (MS).

Advantages. Highly efficient VOC trapping; very effective when it works well.

Disadvantages

1. Difficult to employ outside a laboratory.
2. Icing of water and CO_2 rapidly blocks the U-tube and limits the volume of breath that can be expired through the system.
3. High risk of leakage when sample is heated, because volatilized CO_2 raises pressure in the system.
4. Large quantities of water in the sample may interfere with assay and damage the GC column and detector.

Condensate Trapping

Condensate Trapping is a variant of cold trapping in which the sample is cooled only moderately. A similar system to cold trapping is employed, but the cooling fluid is usually ice water at 0°C. VOCs partition into the water which condenses from the breath, and this sample of condensed water is then analyzed by conventional laboratory methods, e.g., high-performance liquid chromatography (HPLC), or gas chromatography (GC) possibly combined with mass spectroscopy (MS).

Advantages. The method is simple and inexpensive.

Disadvantages

1. VOC trapping is inefficient; only a small number of VOCs are collected in low concentrations.
2. Risk of contamination with nonvolatile components of breath, e.g., proteins, in aerosol.

Chemical Trapping

For chemical trapping, breath is bubbled through a solution which interacts chemically with the analyte of interest. The solution may change color, and/or the sample may be analyzed in the laboratory. This was the basis of Lavoisier's method for detecting CO_2 in the breath and of the colorimetric methods for ethanol and acetaldehyde described above. These methods are of great antiquity, dating from the eighteenth and nineteenth centuries, and have been largely displaced by more modern techniques. However, modern variants have been described for the detection of carbon disulfide and mercury in breath, as well as for the capture of radiolabeled analytes in load tests for GI diseases such as *H. pylori* infection.

Advantages

1. Simple and inexpensive.
2. Capture and concentration steps are combined.
3. Convenient for sample collection outside a laboratory.
4. May give color change which can be read by eye without instruments.
5. Convenient for analytes present in high concentrations.

Disadvantages

1. Repertoire limited to a single analyte.
2. Poor sensitivity; cannot detect most endogenous VOCs.
3. High resistance to expiration; may limit use in elderly and in respiratory disease.

Sorbent Trapping

In sorbent trapping, breath is passed through a bed of a material such as activated carbon or a specialized resin (e.g., Tenax) which captures the VOCs. The process is reversible, so that the captured VOCs may be subsequently eluted by heating (13) or by chemical stripping with a solvent (14,15). This method of breath VOC analysis has been in use in one form or another for many years, but has recently become the method of choice for many breath researchers. This was an indirect result of federal clean air regulations in the United States; the U.S. Environmental Protection Agency now mandates the monitoring of volatile pollutants in the air. These regulations stimulated manufacturers to develop a new generation of instruments for the capture and analysis of air VOCs. Manufacturers and researchers were pleasantly surprised to discover that this technology also provided a sensitive and convenient new method for the analysis of VOCs in breath. A breath VOC sample is captured on to a sorbent trap, typically a stainless steel tube packed with activated carbon or resin. In the laboratory, the VOCs are

eluted with an automated thermal desorber, then analyzed by gas chromatography.

Advantages

1. Convenient for sample collection outside a laboratory.
2. Captures a wide variety of VOCs in breath.
3. Highly sensitive; can detect VOCs in picomolar concentration.
4. Traps can be reused several times.

Disadvantages

1. Traps are too resistant to blow through, so that a specialized pump-assisted breath collecting apparatus is required.
2. Technology is expensive and complicated.
3. Trapping material may be selective, capturing some VOCs efficiently and others inefficiently.
4. Water and CO_2 in breath may interfere with VOC trapping.
5. Cleaning traps for reuse requires scrupulous and time-consuming quality assurance.

C. How to Analyze Breath VOCs

Portable Hand-Held Instruments

The most familiar hand-held instruments are the "breathalyzers" for ethanol which have been employed by police forces for several years. These devices generally employ a fuel cell which oxidizes ethanol in breath to acetaldehyde, and the resulting electrical current (which varies with the concentration of ethanol in breath and blood) is displayed digitally. In recent years, newer devices have become available for analysis of other volatiles in breath such as nitric oxide, sulfur derivatives, and carbon monoxide.

Advantages

1. These instruments are very convenient to use and deliver results in seconds.
2. Small sample volume ensures that alveolar breath sample is collected, uncontaminated by dead-space breath.

Disadvantages

1. Analytical repertoire is usually restricted to a single VOC.
2. Accuracy, precision, and sensitivity are generally poorer than laboratory instruments.
3. Calibration in the field may be difficult.

Conventional Laboratory Instruments

The instrument of choice in most breath research laboratories is currently gas chromatography. A wide variety of detectors may be employed (e.g., flame ionization detection, flame photometric detection), depending on the analyte of interest. In recent years, mass spectroscopy has become increasingly popular as a universal detector. However, other instruments may potentially be employed for breath VOC analysis. Absorption spectrometry offers the potential of direct analysis of breath VOCs without prior concentration or separation of the sample.

Advantages. High sensitivity, accuracy, and precision.

Disadvantages

1. Large, immobile instruments generally mandate sample analysis in a laboratory.
2. Expensive and complicated.
3. Labor-intensive; require dedicated laboratory staff and frequent extensive maintenance.

D. How to Compensate for VOCs in Background Air

As assays of concentrated breath became increasingly sensitive, researchers learned that a sample of normal room air also contains most of the VOCs which are present in the breath (16). Cailleux and Allain (17) reported that pentane could be detected in room air in concentrations not very different from those in breath. This finding initially caused some consternation: it raised the specter that the infant science of breath VOC analysis was not a science at all. Perhaps the phenomenon was nothing more than an artefact of room air contamination? Fortunately, we now know that this is not the case: VOCs are manufactured and cleared in the body, and the composition of inspired air is different from that of expired breath (3). Researchers who analyze VOCs in breath must compensate somehow for the VOCs that are present in background air. There are essentially three options:

1. Ignore the problem.
2. Supply the donor with hydrocarbon-free air to wash out the lungs.
3. Subtract the air background from the breath signal.

Ignoring the Problem

The option of ignoring the problem has not been stated frivolously. The overwhelming majority of papers on the topic of breath VOC analysis published in the peer-reviewed literature during the 30 years preceding the year 2000 make no mention at all of this problem.

Advantages. None.

Disadvantages. Breath VOC data are skewed and difficult (or possibly impossible) to interpret.

Supplying the Donor with Hydrocarbon-Free Air to Wash out the Lungs

The rationale for supplying the donor with hydrocarbon-free air is that VOCs in breath comprise "signal," while VOCs in air comprise "noise." Hence, if the subject inspires hydrocarbon-free air, the VOCs in the expired breath should comprise pure signal uncontaminated by noise, and therefore provide a clear picture of what VOCs are being manufactured in the body. This approach is more theoretical than actual, and has not been validated in a rigorously controlled study.

Advantages. In theory, could provide an unambiguous picture of breath VOCs manufactured in the body.

Disadvantages

1. Has not been validated in practice.
2. Commercial "hydrocarbon-free air" is generally not free of hydrocarbons, when an assay sensitive for VOCs in picomolar concentrations is employed.
3. Several VOCs in the body are of exogenous origin, and may require several hours or days to wash out completely (18).
4. The delivery system for delivering air to humans (cylinder valves, flow meter, tubing, and mask) may also introduce contaminant VOCs into the air.
5. Expensive, inconvenient, and time-consuming.

Subtracting the Air Background from the Breath Signal

Another method has been developed by the author (3,4,19,20). In practice, it requires that two samples be collected every time a patient is studied: one of breath and one of room air. Both samples are analyzed in the same way. For each VOC in breath, the alveolar gradient is then calculated as concentration in breath minus concentration in room air. Kinetic analysis has shown that the alveolar gradient of a VOC varies with the rate of synthesis minus the rate of clearance in the body. This approach has generated the surprising finding that approximately half the VOCs in the breath are present in lower concentrations than in room air; i.e., they are actively cleared from the body by metabolism and excretion.

Advantages

1. Provides rational compensation for VOCs in room air.
2. Provides an insight into the kinetics of VOCs in the body.

Disadvantages

1. Increases the investment of time and money required for analysis of samples.
2. Increases the statistical error of the assay.

IV. Conclusions

This review has focused exclusively on one question about breath VOC analysis: How should it be done? It should be apparent to the reader that breath VOC analysis is a perfectly feasible undertaking, but it is fraught with a large number of potential difficulties, pitfalls, and sources of error. All of these can be overcome, more or less, as the author has shown over some years of wrestling with these problems. His laboratory employs a number of portable breath collectors which are shared among academic medical centers around the United States and in Europe. Collaborators collect samples from patients enrolled in a variety of clinical studies and sorbent traps are mailed to the laboratory for analysis with automated analytical equipment. This has "democratized" breath testing and made it possible, for the first time, to perform large-scale evaluations of breath VOC analysis.

However, as discussed at the beginning of this chapter, there are two other important questions about breath testing: What do the results mean? And why should we do it? Slowly, some answers are emerging. One of the most exciting answers to the question of "What do the results mean?" is that breath VOCs are providing new markers of oxidative stress. Formerly, ethane and pentane were the only known breath markers of oxidative stress (21), but it is now emerging that breath VOCs contain many others. And why should we analyze breath? Clearly, because breath VOC analysis offers the prospect of sensitive and specific new markers of disease. A breath test for lung cancer, for example, could make it possible for physicians to detect the disease in its earliest stages and potentially save or extend many thousands of lives (22,23). The author's laboratory is currently performing clinical studies of breath testing in several disorders, including breast cancer, heart transplant rejection, and ischemic heart disease. That is the vision which sustains breath testing, and makes it one of the most exciting and innovative areas of biomedical research in the twenty-first century.

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