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Short Communication

Method for the collection and analysis of volatile compounds in the breath

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ABSTRACT

A new method is described for the collection and assay of volatile compounds in the breath. Subjects expired into a pump-assisted collecting apparatus in which the breath was drawn through a water trap and then through an adsorptive trap where the volatile compounds were captured on graphitized carbon and molecular sieve. The sample was subsequently eluted from the trap by thermal desorption, concentrated by two-stage cryofocusing, then assayed by gas chromatography with flame ionization and flame photometric detection. Several compounds were regularly observed in the breath of normal human volunteers, including peaks eluting with the same retention times as isoprene, ethanol, acetone, acetaldehyde and carbon disulfide. As a quantitative assay for endogenous isoprene in the breath, the method was sensitive, linear, accurate and reproducible. This method provided a number of advantages: the collection technique was acceptable to volunteers and could be used at sites remote from the laboratory. The automated assay allowed isoprene and several other volatile compounds in the breath to be observed consistently and with improved sensitivity.

INTRODUCTION

Analysis of volatile compounds in the breath makes it possible to observe several chemical processes in the body through a non-invasive window. In practice, breath assays are of two main types: those which require pre-concentration of the sample and those which do not. Breath assays which require no pre-concentration of the sample are generally easier to perform and have therefore attracted the most attention and clinical use; these include breath assays of ethanol for medical and legal purposes [1,2] and the measurement of breath hydrogen and $^{14}CO_2$ in intestinal disorders such as malabsorption syndromes and bacterial overgrowth [3,4].

Breath analyses which require pre-concentration of the sample necessitate a greater investment of effort and equipment; however, they offer a unique tool for the investigation of abnormal metabolism in a number of disease states, including

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lung cancer [5,6], hepatic cirrhosis [7,8], alcohol abuse [9,10] and poisoning with environmental toxins [11,12]. The major advantage of breath analysis by concentration methods is that there is no theoretical lower limit to the sensitivity of the assay, since the volume of the collected sample is limited only by the patience of the donor and the capacity of the concentrating system. The use of concentration techniques makes it possible to detect several volatile compounds in the breath when their concentrations are too low to be detected in the blood.

The study of volatile compounds in concentrated breath has been impeded by the lack of standardised accepted methods for both the collection and the analysis of samples. Several *ad hoc* collection devices have been described utilizing cold trapping [13], adsorptive binding [14] or chemical interaction [15] to capture the volatile compounds while allowing free passage of the nitrogen and oxygen in the breath. Common problems with these devices have been discomfort for human subjects donating a breath sample (*e.g.* expiring against resistance), complicated structure of the apparatus, proneness to contamination (especially of collecting bags), poor trapping efficiency and the need for specialized technical supervision. In addition, both adsorptive traps and cold traps may be saturated by the large quantities of water vapor and CO_2 present in normal human breath, possibly resulting in obstruction to the free flow of breath, and difficulties in the subsequent desorption of the sample. However, the second technical obstacle, analysis of the collected specimen, has been considerably simplified in recent years by commercially available microprocessor-controlled devices which automate thermal desorption, cryogenic concentration and injection of the sample into a gas chromatograph.

We describe here a new and highly sensitive method for collecting and assaying volatile compounds in the breath which was well accepted by human subjects. Breath was collected in a portable pump-assisted device in which the volatile compounds were captured on an adsorptive trap, then subsequently desorbed and cryogenically concentrated in a microprocessor-controlled apparatus, and assayed by gas chromatography (GC). Several compounds were regularly observed in normal human breath by this method; endogenous isoprene was quantitatively assayed, as one of the most abundant [16–18]. A particular advantage of this method was that it facilitated the collection of a comparatively large quantity of breath (20 l) without discomfort, and concentrated the entire sample for assay by GC.

EXPERIMENTAL

Breath collection device

The device is shown schematically in Fig. 1. In this portable pump-assisted apparatus, breath was drawn through two routes: a high-impedance pathway (comprising a water trap and an adsorptive trap) and a low-impedance bypass. The water trap was found to be necessary, since without it, water was captured in

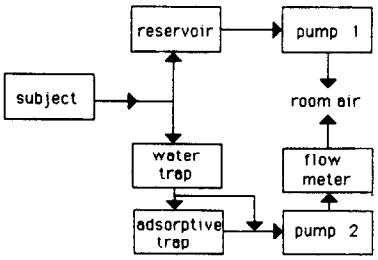

Fig. 1. Breath collecting apparatus (schematic). The breath sample was pumped through two routes: a high-impedance pathway (water trap and adsorptive trap) and a low-impedance bypass through the reservoir. A three-way tap between the water trap and the adsorbent trap shunted breath to a second bypass route, so that the subject's breath purged the apparatus of other gases prior to collection of the sample. The structural components of the apparatus are detailed in the text.

the adsorptive trap, resulting in icing and obstruction in the cryogenic trap during subsequent desorption of the sample. The water trap comprised a transparent gas purifier (Alltech Assoc., Deerfield, IL, U.S.A.) containing hygroscopic crystals of calcium sulfate impregnated with a color indicator of water content (Dry-Rite®, Fisher Scientific, Springfield, NJ, U.S.A.). The adsorptive trap comprised an air-tight metal container (air sampling adapter 14-1486-000, Tekmar, Cincinnati, OH, U.S.A.) holding a stainless-steel tube (15.9 mm diameter) containing 4.4 g of Carbotrap C, 3.2 g of Carbotrap and 2.0 g of Carbosieve S III (No. 2-0371, Supelco, Bellefonte, PA, U.S.A.). All connecting tubing taps and fittings upstream from the adsorptive trap were constructed from stainless-steel, brass, or Teflon®, in order to minimize the possibility of introducing contaminants into the collecting system. The flow-rate through the traps was controlled by a needle-valve leading to an air pump and a flow meter. The low-impedance bypass ensured that human subjects could breath into the device without the discomfort or potential hazards of blowing against resistance. The mouthpiece comprised a length of plastic tubing containing a small amount of Dry Rite to prevent condensation in the system. Two air pumps were used: a Pulmo-Aide (DeVilbiss, Somerset, PA, US.A.) in the low-impedance pathway and a 1/25 HP air pump (Magne Tek Universal Electric, Owosso, MI, U.S.A.) in the high-impedance pathway. The flow meter was a Wright respirometer (Herman Berman, Van Nuys, CA, U.S.A.). The reservoir comprised an empty metal gas purifier (approximate volume 300 ml) (Alltech Assoc.).

Breath collection method

Subjects breathing room air were instructed to inhale through the nose and exhale through the mouth, while keeping the lips firmly applied around the disposable plastic mouthpiece. Subjects breathed into the breath-collecting apparatus for 1 min with the flow bypassing the trap, in order to purge the system, with

the flow-rate adjusted to approximately 3.0 l/min. The flow was then directed through the adsorptive trap, and a sample of 20 l breath was collected. At the completion of the collection, the adsorptive trap was removed and sealed in an air-tight container (P/N 14-1463-000, Tekmar) for transport to the laboratory. A control sample of 20 l air was also collected, in order to determine background levels of the assayed compounds.

Sample desorption and concentration

The sample was desorbed from the trap and cryogenically concentrated using a microprocessor-controlled thermal desorber (5010 GT automatic on-line desorber, Tekmar). The trap was flushed with helium (10 ml/min), first at room temperature for 5 min to remove any water, and then at 300°C for 8 min; the desorbed volatile compounds were captured in an internal cryogenic trap cooled to −150°C with liquid nitrogen. The internal cryogenic trap was then heated to 225°C and the sample was flushed with helium through a fused-silica transfer line (heated to 250°C) to the external cryogenic trap (cooled to −150°C) mounted on the inlet port of a gas chromatograph. After 1.2 min, the external cryogenic trap was heated to 250°C and the concentrated sample was injected into the gas chromatograph. Prior to re-use, the trap was baked at 325°C for 60 min with helium flowing at 10 ml/min.

GC assay

A Shimadzu GC9A (Shimadzu Scientific Instruments, Columbia, MD, U.S.A.) was used, equipped with a CR2AX/INP-R2A microprocessor and printer, fused-silica capillary column ("007" series, methylsilicone bonded phase, 25 m × 0.53 mm I.D., 8.0 μm film thickness) (Quadrex, New Haven, CT, U.S.A.). The column effluent outflow was split between a flame ionization detector (FID) and a flame photometric detector (FPD). The following temperatures were maintained: inlet port, 200°C; oven, 35°C for 10 min, then rising to 250°C at 7°C/min; FID, 200°C; FPD, 200°C. The carrier gas was helium flowing at 5–7 ml/min.

Isoprene assay

Standard isoprene vapor was prepared by volatilizing 50 μl of pure isoprene (Fisher Scientific, Springfield, NJ, U.S.A.) in a 1000-ml glass flask sealed with a rubber septum and heated in a water bath to 45°C. A standard curve was obtained by injecting known quantities of the vapor into the mouthpiece of the breath-collecting apparatus, and measuring the amount collected on the adsorbent trap. Using a gas-tight syringe, duplicate samples of vapor (ranging from 0 to 2.0 ml) were aspirated from the flask, injected into the mouthpiece of the breath collecting apparatus and collected as described above. A Tedlar bag containing 24 l zero-grade helium was affixed to the mouthpiece in order to provide a source of carrier gas to flush the isoprene standard on to the adsorptive trap. The low-impedance bypass was closed off during injection of the standards. All iso-

prene samples captured on the adsorptive trap were desorbed and assayed as described above. The area under curve (AUC) of each FID peak arising from isoprene was determined automatically by the GC microprocessor, and the line of best fit relating the mean AUC to the quantity of isoprene in the sample was determined by linear regression. The efficiency of the assay and the concentration factor were determined by comparing FID response to similar quantities of isoprene which were either directly injected into the inlet port of the GC system or loaded onto the resin trap and desorbed and assayed as described above. An isoprene vapor standard (140 nmol) was assayed six times during a single day, in order to determine precision and accuracy of the assay. The cycle time, from commencement of an assay to readiness for the next assay, was approximately 90 min.

Clinical study

A group of five normal male volunteers was studied, drawn from employees at this institution. Subjects were not asked to fast or otherwise modify their diet. This research was approved by the Institutional Review Board of St. Vincent's Medical Center of Richmond.

RESULTS

Isoprene assay

The mean efficiency of isoprene recovery from the adsorbent trap was 24.4%. The concentration factor achieved for isoprene by sequential adsorption and cryofocusing of breath was estimated as follows:

$$\text{concentration factor} = \frac{\text{initial sample volume}}{\text{final sample volume}} \times \text{assay efficiency}$$

The initial sample volume was 20 l, the final sample volume in the second cryogenic trap was 17.6 μl; hence the isoprene was concentrated approximately 280 000 times in this procedure.

There was a linear relationship between the AUC of the FID response and the amount of isoprene injected into the breath collecting apparatus ($y = 1.8 \cdot 10^4 x + 3.1 \cdot 10^5$, where y = AUC and x = nmol isoprene in the sample; $r^2 = 0.97$). The mean intra-assay observation of a 140 nmol standard was 124.60 nmol (89% of expected, coefficient of variation = 7.0%).

Clinical study

No subject experienced any discomfort or adverse effects while donating a specimen of breath. A chromatogram obtained from a typical volunteer is shown in Fig. 2. Breath isoprene levels ranged from 0.49 to 1.99 nmol/l (mean ± S.D. = 0.99 ± 0.58 nmol/l). Peaks arising from isoprene as well as several other com-

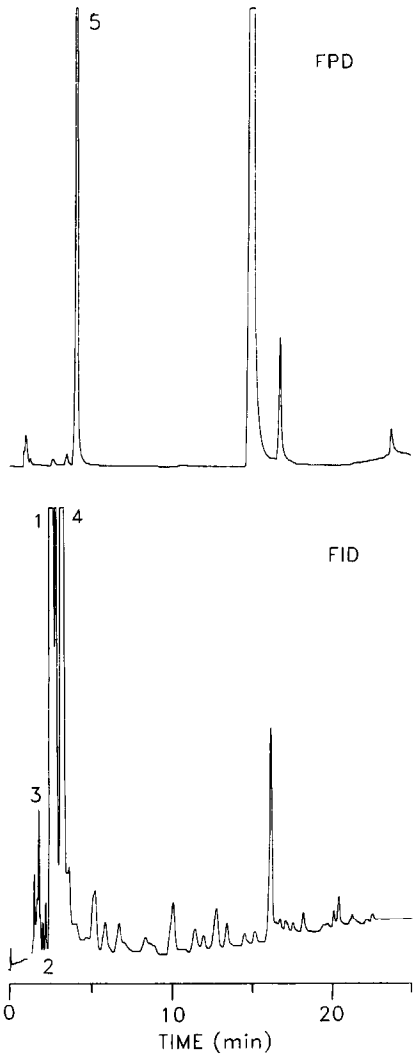

Fig. 2. Gas chromatogram of volatile compounds in the breath of a normal subject. The sample was split and diverted to two detectors: flame photometric (FPD) (upper panel) and flame ionization (FID) (lower panel). The following compounds were identified on the basis of elution times: (1) isoprene, (2) ethanol, (3) acetaldehyde, (4) acetone and (5) carbon disulfide.

pounds were observed in all subjects. Peaks were observed with the same retention times as acetaldehyde, acetone, ethanol and methanol (on FID) and carbon disulfide (on FPD).

DISCUSSION

The collecting procedure was found to be well accepted by human volunteers and the breath-collecting apparatus was readily transported to sites outside the laboratory for the collection of samples. The collecting apparatus channeled the breath sample through two routes: a high-impedance pathway (comprising the water trap and the adsorbent trap) and a low-impedance bypass and reservoir. The advantage of this design was that the breath sample could be drawn through the high-impedance adsorbent trap at a constant rate, while sparing the volunteer the discomfort and potential hazards (*e.g.* a Valsalva maneuver) of blowing against resistance.

Isoprene has been previously identified as a major component of human breath by gas chromatography–mass spectroscopy of enriched samples [17]. The metabolic sources of isoprene have also been investigated. Gelmont *et al.* [18] demonstrated *in vitro* production of isoprene in rat kidney and liver slices. Isoprene may be formed from acetate or squalene; it is of metabolic significance as a precursor of cholesterol [19]. DeMaster and Nagasawa [16] observed a diurnal variation in human breath isoprene concentrations, rising during the night to an early morning peak, then falling to a trough in the late afternoon. In addition to isoprene, several other peaks were consistently observed in the breath of normal volunteers. Some have been tentatively identified on the basis of their elution times; these include acetone, ethanol, acetaldehyde and carbon disulfide. With the exception of carbon disulfide, all of these compounds have been previously reported as constituents of normal human breath. Further studies are required, in order to confirm the presence of carbon disulfide by mass spectroscopy.

Earlier researchers, particularly Pauling *et al.* [20], pioneered the field of breath analysis by GC more than two decades ago, and it is necessary to clarify where advances have been made in the methodology that has been previously described.

The advantages of the method described in this report lie in three main areas: the improved technique of breath collection, the improved trapping material and the automated analysis of the samples. The breath collecting apparatus was convenient to use in a clinical setting, due to its portability, ease of use and acceptability to human subjects. Unlike most previously described methods, it was possible to take the breath-collecting apparatus to a patient's bedside in a hospital, rather than requiring the patient to come to the laboratory. Second, the advantage of the trapping material used in this study (Carbotrap/Carbosieve, a combination of activated carbon and molecular sieve) was that it could be baked completely free of contaminants, unlike trapping materials such as Tenax®. In practice, this resulted in a chromatogram with fewer artefactual peaks and an enhanced signal-to-noise ratio. The third advantage of this method was the automated analysis of samples. The microprocessor-controlled automatic desorber provided a convenient elution technique which concentrated the volatile compounds in the breath by a factor of 280 000 in a reproducible and standardized fashion.

Several compounds were observed in addition to isoprene, so that this collecting method might serve as a flexible general technique for the study of volatile organic compounds in human breath. We have previously described other methods of collecting and analyzing breath for volatile compounds in the breath, including endogenous ethanol [21] and acetone [22]; the method described in this report provides an improvement in ease of use and sensitivity, as well as detecting a greater number of compounds in the collected sample.

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