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Short communication

Mass spectrometric investigations to obtain the first direct comparisons of endogenous breath and blood volatile organic compound concentrations in healthy volunteers

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ABSTRACT

Volatile organic compounds (VOCs) in breath could be clinically useful for the early detection and diagnosis of diseases, physiological disorders and therapeutic monitoring. However, it is crucial to compare the reliability and precision of breath measurements with those from blood if endogenous VOCs on breath are to be used as biomarkers. Few studies have been undertaken to investigate this, none of which relate to endogenous VOCs in freely breathing subjects. Here we establish the reliability and precision of breath measurements to determine endogenous VOC concentrations in comparison to blood measurements in order to assess the viability of using breath measurements for potential diagnostic and screening purposes. Acetone and isoprene concentration levels in the breath, radial arterial blood and peripheral venous blood and *in vivo* arterial blood/breath ratios for freely breathing subjects have been determined using mass spectrometric techniques. Mean (range) breath concentrations in parts per billion by volume are 1090 (515–2335) for acetone and 465 (308–702) for isoprene. The mean (range) blood concentrations are: for acetone in radial arterial blood 26 (10–73) μmol/l and in peripheral venous blood 18 (9–39) μmol/l; for isoprene in radial arterial blood 6.8 (3.7–11) μmol/l and in peripheral venous blood 14 (5.5–30) μmol/l. Arterial blood/breath ratios mean (range) are 580 (320–860) for acetone and 0.38 (0.19–0.58) for isoprene. An important finding is that the coefficients of repeatability as a percentage of mean are less than 30% in breath but greater than 70% in blood. This study suggests that breath VOC measurements could provide a more consistent measure for investigating underlying physiological function or pathology than single blood measurements.

1. Introduction

Volatile organic compounds (VOCs) are produced in the body as a result of metabolic processes. They exchange across the alveolar-blood capillary membrane into exhaled air and so provide an insight to biochemical processes occurring in the human body. Breath analysis is consequently attracting clinical attention as a potential means for delivering non-invasive, real-time, rapid screening and diagnosis of complex diseases such as cancers and acute infections. An overview of the field has recently been published [1]. Notably, Phillips pioneering work provides compelling evidence for the detection of molecular biomarkers in the breath related to lung and breast cancers [2–4]. However, it is crucial to compare the reliability and precision of breath measurements with those from blood if endogenous VOCs on breath are to be used as biomarkers. Few studies have been undertaken to investigate this, none of which relate to endogenous compounds in freely breathing subjects [5–11].

In this article we compare the precision of measurements of the concentrations of two endogenous VOCs, acetone and isoprene, in breath and blood (arterial and venous). Also reported are the first values of *in vivo* arterial blood/breath acetone and isoprene concentration ratios for freely breathing subjects.

There are a large number of VOCs which could have been selected for study, but we selected acetone and isoprene because: they are normally present in trace concentrations in breath and blood; they have very different solubilities (and solubility is known to alter the dynamics of gas exchange [12]); and they are common biomarkers associated with a number of common disorders [13].

2. Experimental details

2.1. Volatile organic compound details

Isoprene is considered to be a by-product of cholesterol biosynthesis [14]. It has been shown that there is a fairly constant level

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of breath isoprene excretion over long periods of time [15]. Thus blood isoprene concentrations should not have changed significantly over the period of 1–2 h in which the samples were taken for this study. Acetone in breath predominantly derives from oxidation of free fatty acids. It is influenced by glucose metabolism, having been found to be elevated in fasting subjects [16]. Therefore changes in the level of blood acetone over the time-scale of the sampling may be expected. To minimise this, volunteers were asked to eat a good breakfast using food they normally ate.

2.2. Volunteer details

The healthy volunteers were six males (M1–6), mean (range) age 34 (22–50) years and four females (F7–10), mean (range) age 26 (20–42) years. Approval from the regional ethics committee of Walsgrave Hospital, Coventry was obtained and the volunteers gave their informed consent. Volunteers came to the clinic on separate days with measurements taken at approximately the same time on each of these days (10:00 a.m. to 1:00 p.m.). None of the subjects were smokers or suffered from any lung problems.

Prior to the start of the clinical measurements all subjects performed spirometry tests to check for normal lung function. An attending anaesthetist inserted one cannula into the radial artery of the non-dominant wrist and another into the antecubital vein of the other arm.

2.3. Sampling

4.9 ml blood samples were taken from each cannula into EDTA monovettes (Startstedt) at the start of each trial. Five breath samples and a further five arterial and venous blood samples were then sequentially taken over a time period of approximately 2 h for each volunteer.

2.4. Protocol for breath sampling and analysis

Breath samples were obtained using a simple cyclic rebreathing method developed by the authors [17]. In brief, a volunteer took a deep breath and expired this into a specially designed Teflon® bag (max. volume of 5 l) maintained at a temperature of 40 °C. The volunteers used this as a reservoir for 20 rebreaths (corresponding to 4 cycles of 5 rebreaths with short breaks in between the cycles). Whilst the first breath sample was being analysed, blood samples were taken from the subject followed immediately by the next breath sample using a second bag. During this time the first measurement bag was flushed three times with dry nitrogen, checked for residual VOCs, and sent back to the clinical room ready for use for the third breath sample. This cycle continued, using two bags per volunteer, until the completion of the breath measurements resulting in the analysis of five breath samples per volunteer.

To determine the concentrations of acetone and isoprene in breath a standard proton transfer reaction mass spectrometer (PTR-MS) was used. The standard PTR-MS (Ionicon, www.ptrms.com/products/sptrms.html) is an analytical instrument designed to detect extremely low concentrations (parts per billion by volume) of VOCs in air samples and has been described in detail elsewhere in the literature [18]. In brief, the PTR-MS exploits some unique features of the reactions of protonated water, H_3O^+ ($m/z = 19$ amu), with neutral molecules (M): $H_3O^+ + M \rightarrow MH^+ + H_2O$. This reaction does not occur for any of the gases present in clean air for energetic reasons. The operating conditions used for these experiments are similar to those described in reference 16. The pressure of the drift tube was accurately maintained at 2.060 ± 0.005 mbar and a temperature of 40 °C. The operating voltage was kept at 600 V.

The acetone and isoprene present in the breath were detected by monitoring the protonated parent molecules. Count rates (counts per second) are converted to parts per billion by volume (ppbv) by use of measured calibration factors. These calibration factors were obtained by using known low concentrations of acetone and isoprene mixed into high purity nitrogen (Linde Gas UK). The calibration factors obtained are 27 ± 2 cps/ppbv for isoprene and 54 ± 3 cps/ppbv for acetone. The isoprene calibration factor is smaller than that for acetone. This reflects differences in the transmission properties and detection efficiency of the two product ions (i.e., the protonated species) and also the fragmentation pathway leading to a loss of C_2H_4 from protonated isoprene (branching ratio ~50% at the electric field strengths and pressures used in the drift tube). The effect of humidity on the calibration factors is difficult to quantify. The uncertainty in this has been estimated and incorporated into the conservative experimental errors on the measured concentrations. Linearity of the PTR-MS has been investigated by a number of groups [19,20], and these demonstrate that the measurement capabilities of the instrument are linear over the range of concentrations measured in this study.

2.5. Protocol for blood analysis

An independent commercial analytical laboratory (Trace Laboratories, Birmingham) determined the VOC concentrations in the blood. Whereas the breath samples were analysed immediately using a PTR-MS (see below), the blood samples were carefully labelled and stored in refrigerator in the clinic at 4 °C. At the end of each trial all blood samples were transferred to a cool box and taken on ice to Trace Laboratories (15 min drive), where they were transferred to a refrigerator maintained at 4 °C. They were stored overnight and analysed the following day, i.e., all blood samples were analysed within 24 h of collection.

The approach adopted by this laboratory for the blood measurements was similar to that described in detail by Miekisch et al., who analysed arterial and venous blood samples taken from mechanically ventilated patients [21]. The blood samples were analysed by means of solid-phase microextraction and gas chromatography-mass spectrometry. Repeatability of measurement was checked by taking five samples from a mixed pool of blood and measuring the acetone and isoprene intensities, giving a standard deviation of 7% for acetone and 8% for isoprene.

2.6. Determination of blood/breath ratios

In vivo blood/breath ratios for acetone and isoprene were calculated as follows: breath measurements were first converted into the same unit as the blood measurement (μmol for acetone and nmol for isoprene). Five individual blood/breath ratios (CAB_i/CBr_i) were calculated for each subject using paired blood and breath values (the paired blood concentration was considered to be the mean of the blood samples before and after each individual breath sample. Data are not shown). Each subject's mean blood/breath ratio $(CAB/CBr)_m$, was calculated from $\sum(CAB_i/CBr_i)/n$, where $n = 5$ (except in the case of M4, where $n = 4$). The sample mean $(CAB/CBr)_{SM}$ was then calculated from $\sum((CAB/CBr)_m)_i/n$, where $n = 10$. The uncertainty on each $(CAB/CBr)_m$ was taken to be the twice the standard deviation of the mean (SDM) of the five individual CAB_i/CBr_i values used to calculate the mean.

3. Results

The means of the breath concentration (CBr), arterial blood concentration (CAB), venous blood concentration (CVB) and arterial blood/breath ratio (CAB/CBr) for each volunteer are presented in Table 1. In comparison to other studies measuring blood and breath

Table 1
Individual mean concentrations of isoprene and acetone in breath (CBr), arterial blood (CAB) and venous blood (CVB) determined from the five breath samples and six blood samples (unless indicated) from each volunteer. Values for the standard deviations in the measurements are provided in the parentheses.

Subject	Acetone				Isoprene			
	CBr (nmol/l), $n=5$	CAB (μmol/l), $n=6$	CVB (μmol/l), $n=6$	CAB/CBr	CBr (nmol/l), $n=5$	CAB (nmol/l), $n=6$	CVB (nmol/l), $n=6$	CAB/CBr
M1	22 (1.2)	10 (3)	9 (2)	430 (95)	16 (1.9)	9.9 (1.6)	13.9 (6.0)	0.58 (0.05)
M2	37 (2.1)	30 (5)	28 (3)	820 (100)	21 (2.8)	8.0 (1.8)	9.3 (1.5)	0.39 (0.06)
M3	37 (4.9)	35 (19)	39 (8)	860 (240)	23 (1.4)	8.9 (3.2)	15.8 (6.0)	0.38 (0.10)
M4[a]	45 (1.0)	19 (7)	12 (4)	420 (121)	24 (1.4)	4.7 (1.0)	7.1 (0.6)	0.19 (0.02)
M5	60 (3.0)	38 (8)	20 (4)	620 (110)	27 (2.5)	11 (4.0)	30 (4.2)	0.38 (0.06)
M6	20 (0.3)	13 (3)	13 (3)	660 (85)	13 (0.9)	5.4 (0.6)	5.5 (0.9)	0.42 (0.05)
F7	27 (0.4)	13 (2)	11 (2)	470 (77)	12 (1.2)	5.9 (1.0)	15.9 (4.8)	0.48 (0.07)
F8	40 (4.1)	13 (2)	9 (2)	320 (29)	14 (1.5)	4.9 (0.8)	14.1 (5.3)	0.35 (0.06)
F9	91 (17)	73 (27)	34 (16)	830 (170)	18 (0.9)	5.1 (0.8)	13.9 (3.2)	0.29 (0.04)
F10	46 (1.2)	16 (4)	11 (4)	360 (63)	13 (1.8)	3.7 (1.1)	14.4 (3.9)	0.31 (0.09)

[a] The first breath sample was inadequate for M4 because the volunteer did not initially follow the prescribed protocol, so the results for breath and blood are obtained from four breath samples and five arterial and venous samples only.

concentrations our results are in reasonable agreement [21–28], especially for those studies involving healthy patients. For some of the studies there are some differences which need some explanation. Schubert et al. [8] values for measured acetone in the blood and breath are not in good agreement with ours. However, their study involved mechanically ventilated patients, which means direct comparison may not be possible. In particular, the exhaled breath acetone concentrations measured by Schubert et al. (means of 145 μmol/l for septic patients and 183 μmol/l for non-septic patients) are significantly higher than the values we have obtained. In contrast recent selected ion flow tube studies (SIFT) [23,27,28] all present breath acetone and isoprene concentrations lower than our values. Wilson et al. [28] provide values for breath acetone and isoprene to be 100–200 ppb and 200–500 ppb, respectively. Turner et al. [23,27] present mean values of 477 ppb (range 148–2744 ppb) for acetone and 118 ppb (range 0–474 ppb) for isoprene. We have shown [17] that end exhaled concentrations of isoprene are particularly sensitive to the duration of exhalation. The uncontrolled end-exhaled sampling employed in all the SIFT studies could account for the lower concentration values from the SIFT measurements. Direct comparison of acetone is more problematic. Acetone in breath has been shown to be elevated in fasting subjects. Therefore acetone concentrations are very much dependent on the time since the last meal [16,29,30].

In vivo arterial blood/breath ratios were calculated for each individual and used to determine the sample mean and ranges. These are found to be 580 (320–860) for acetone and 0.38 (0.19–0.58) for isoprene. To our knowledge, this is the first report of the determination of *in vivo* blood/breath ratios for endogenous VOCs in freely breathing subjects. By way of comparison, *in vitro* blood/air partition coefficients have been determined to be 341 [31] and 301 [32] for acetone and 0.75 for isoprene [15] which are of a comparable order of magnitude to the values we have obtained.

To illustrate the type and quality of measurements obtained, Fig. 1 shows CBr, CAB and CVB for volunteers M6 (acetone) and F7 (isoprene) presented as a function of the times at which the various samples were taken from the start of the trials. Error bars show a conservative estimate for the experimental uncertainties of 10%.

Fig. 1 demonstrates that the trends in both blood and breath concentrations are reasonably flat over the sampling period. Notably, this figure demonstrates the variability in blood concentration measurements from one sample to the next for a given volunteer. This variability must reflect both biological fluctuations in blood acetone/isoprene levels and potentially procedural and measurement variations. However, given that the blood measurement repeatability (one standard deviation) is approximately 8%, we suggest that the major part of the blood VOC variability is biological. In comparison, it can be seen in Fig. 1 that breath concentrations are less variable. These statements can be quantified by determining repeatability coefficients [33]. Taking all samples obtained from the ten volunteers we find that, taken as a percentage of the mean, the repeatability coefficient for acetone is found to be 19% for breath, 103% for radial arterial blood and 79% for peripheral venous blood. The corresponding values for isoprene are 27% for breath, 79% for radial arterial blood and 81% for peripheral venous blood.

The larger repeatability coefficients for the blood concentration value, compared to those obtained from the breath samples, indicate that single blood samples do not necessarily provide reliable and reproducible values of the systemic blood VOC concentrations.

The lower variability observed in the VOC concentrations determined from the breath samples must reflect averaging processes occurring in the lungs where a large volume of blood will have been involved in the gas transfer. In contrast, blood is taken from a vein

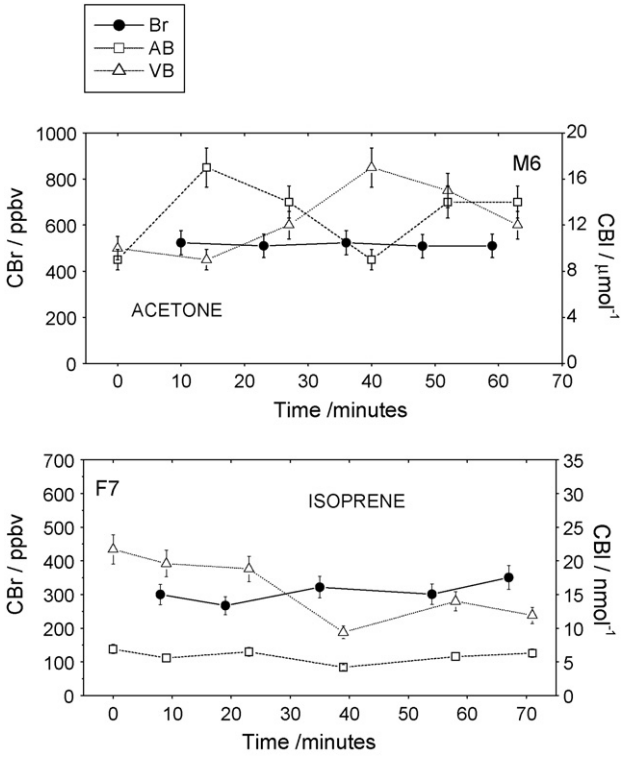

Fig. 1. Concentrations of isoprene and acetone in breath (CBr) and blood (CBl) for acetone (volunteer M6) and isoprene (volunteer F7) plotted as a function of time at which the samples were taken. AB refers to arterial blood and VB to venous blood. The lines drawn connecting the data points are visual guides only. Error bars represent a conservative experimental uncertainty of 10%. Note the variability in the measured concentrations of acetone and isoprene in blood from one sample to the next.

or artery in small quantities making the blood VOC concentration measurements more sensitive to short term metabolic fluctuations. This is an important finding, especially when considering the practical advantages of using breath rather than blood for screening purposes: painless, non-invasive, requires no medical personnel and results can be obtained immediately. We can add another benefit in that breath analysis provides an improved method for determining blood VOC concentrations with better repeatability than that obtained from a single blood sample.

4. Discussion

To our knowledge, this is the first study to investigate the precision of measurements to determine endogenous breath and blood VOC concentrations for freely breathing volunteers. Furthermore, we have determined the first *in vivo* blood/breath ratios, which have shown considerable variation from one volunteer to the next. As a result of the superior repeatability of breath measurements using a rebreathing sampling technique, breath concentrations can provide an easier way of estimating systemic VOC blood concentrations than can be obtained directly from single blood samples, despite inter-individual variations in blood/breath ratio. However, the conversion of breath to blood concentrations is most probably not crucial for diagnostic or screening purposes. It is the changes in the VOC profiles which could be used to discriminate healthy from ill patients. To monitor these requires that reliable and consistent VOC concentrations can be obtained, and this study has clearly demonstrated that breath analysis using our sampling protocol provides such measurements.

There are a number of limitations that could be associated with this study. One is the small number of volunteers (10). Another is that only two VOCs were investigated. Both of these limitations were related to the costs of using a commercial company to analyse the blood samples. However, we considered it important in this clinical study to have completely independent measurements of the VOCs concentrations in the blood samples. In addition it is important to have the blood concentrations measured by techniques commonly using in medical laboratories (GC–MS). Although only ten volunteers were involved in this study, it should be recalled that each volunteer had 5–6 blood (venous and arterial) and breath samples analysed. Thus a significant amount of data was involved in the analysis. That only two VOCs, acetone and isoprene, were selected for study is also not a major limitation in terms of the results and general conclusions reached. As mentioned in the introduction, these two chemicals cover the different physico-chemical VOC properties in the body, which are mainly associated with their solubilities. For example, very little acetone is cleared from the venous blood in the lungs because of its high solubility. In contrast, isoprene's low solubility means that it has a greater affinity for the gas phase and hence more will be released from the venous blood as it passes through the lungs. Another limitation associated with this study is that only peripheral venous blood concentrations were measured, rather than mixed venous concentrations. However, in terms of using VOCs as a diagnostic tool, it is the peripheral venous blood which would be clinically sampled, and therefore it is important to compare breath and arterial blood measurements with those obtained from peripheral venous blood.

5. Conclusions

It is known that changes in metabolic processes alter the trace molecular VOC profile to be found in blood or in breath. Consequently this altered VOC profile presents a potential method for screening and so classifying a patient's status (healthy or diseased) providing that reliable VOC measurements, not susceptible to short-term fluctuations, are available. This study demonstrates that breath VOC analysis gives more consistent results than blood VOC measurements and thereby highlights the viability of using VOCs on breath for investigating underlying physiological function or pathology.

6. Future direction

Using a PTR-MS and suitable breath sampling protocols, this study demonstrates that breath analysis is a viable technique capable of providing reliable VOC concentration measurements. Real time and non-invasive disease diagnosis or therapeutic monitoring for a range of clinical applications using breath sampling is therefore a reality. To date the majority of research associated with breath analysis has been limited to small proof-of-principle trials. We do not only need to revise and validate preliminary measurements, but we also need to undertake more rigorous clinical studies. Comparisons of molecular VOC biomarkers from one disease to another are required. The main goals of any future research programme are to identify the key chemical biomarkers in the breath of patients resulting from major diseases, and to evaluate if they can be accurately and reliably used as a clinical differential diagnostic tool. Detailed modelling will be required, particularly in the use of pattern recognition software—a set of biomarkers instead of a single VOC will increase the diagnostic accuracy and viability of breath analysis. This holds enormous promise for improved healthcare.

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