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Breath acetone concentration decreases with blood glucose concentration in type I diabetes mellitus patients during hypoglycaemic clamps

Running head: Breath acetone in type 1 diabetes

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Abstract

Conventional wisdom is that breath acetone may be markedly elevated in type 1 diabetes, but that this only occurs during poor blood glucose control and/or intercurrent illness. In contrast, little is known about breath acetone at more representative everyday blood glucose levels in diabetes. We used selected ion flow tube mass spectrometry (SIFT-MS) to monitor the breath of 8 patients with type 1 diabetes mellitus during “insulin clamp” studies in which insulin and glucose were infused into patients to lower blood glucose levels in steps from normal values into the low glucose (hypoglycaemic) range. The concentration of acetone in breath and the blood sugar concentration of the patients were monitored at each blood glucose concentration. The blood glucose level at the start of the study was typically about 6mM/L, whereas the breath acetone concentration at this blood glucose level was unexpectedly variable, ranging from one part-per-million (ppm) to 21 ppm, in contrast to what was previously believed, i.e. that type 1 diabetes mellitus (T1DM) is characterised by high acetone levels. In all 8 patients, the breath acetone declined linearly with blood glucose concentration.

1. Introduction

Diabetes mellitus comprises a group of complex metabolic disorders in which elevated blood glucose levels can result in serious medical complications such as blindness, kidney failure, heart disease, gangrene, limb amputation and premature death. Type 1 diabetes, making up 15% of the total, arises from the lack of insulin caused by the auto-immune destruction of the insulin producing β cells in the pancreatic islets of Langerhans and requires lifelong insulin therapy. For many patients, insulin regimens can be complex to juggle, even with multiple daily expensive and painful capillary finger-prick tests to monitor glucose levels. Too much insulin relative to blood glucose levels can result in acute hypoglycaemic episodes with confusion, lethargy or even unconsciousness, a complication greatly feared by patients with diabetes. In contrast, insufficient insulin (either because of under-dosing and/or during intercurrent illness with the bodies stress response exerting an anti-insulin effect) may result in hyperglycaemia and ketosis may develop. Ketosis is a consequence both of increased lipolysis in adipose tissue and increased ketogenic flux in the liver resulting in a rise in circulating ketone bodies: acetoacetate, beta-hydroxybutyrate and acetone. Acetoacetate may then be decarboxylated to volatile acetone (with a small contribution to breath acetone also from dehydrogenation of 2-propanol (Kalapos, 2003)) so that during ketosis, the breath of patients with type 1 diabetes has been described as smelling like “decaying apples”. Ketosis in uncontrolled diabetes has been assumed to be a consequence of low or absent circulating insulin levels releasing a tonic inhibition on

lipolysis in adipose tissue and ketogenesis in liver. In the healthy (non-diabetic) state, ketone bodies are thought to be a protective response to fasting, increasing as both glucose and thus insulin levels fall and circulating stress hormones such as catecholamines and glucagon rise. (Kalapos, 2003; Laffel, 1999; Turner et al., 2008). This fasting scenario in non-diabetes is different from that seen in type 1 diabetes during hypoglycaemia in a number of key aspects. During diabetic hypoglycaemia, high rather than low insulin levels are experienced. In addition, most patients with type 1 diabetes fail to increase glucagon secretion during hypoglycaemia and many will also display diminished catecholamine responses to a low blood glucose (Evans and Amiel 2002). To our knowledge, the effects of changes in blood glucose *per se* independent of alterations in insulin have not been previously reported.

Although breath acetone is generally regarded as an indicator of a serious loss of metabolic control in diabetes, we hypothesised that less extreme changes in breath acetone might also occur in diabetes as metabolic state- insulin and glucose levels- change. A non-invasive method of monitoring internal changes in metabolism in diabetes- particularly blood glucose levels- would be enormously useful as a clinical alternative to finger-prick testing (Evans et al.,1999). We have previously examined breath acetone in healthy subjects showing that breath acetone levels fell following oral glucose intake (Turner et al., 2008). However, in healthy subjects, oral glucose intake results in reciprocal changes in both blood glucose and insulin. We wanted to examine the relationship between breath acetone and blood glucose levels in type 1 diabetes where insulin levels can be controlled by exogenous delivery. In particular, we wanted to examine whether acetone levels changed as blood glucose levels were lowered into the hypoglycaemic range, something that has not previously been examined.

To do this we needed to measure accurately exhaled breath acetone, a highly volatile and reactive compound. The accurate measurement of acetone concentrations in breath (or indeed, the concentration of any breath metabolite) is very difficult (Deng et al., 2004). Nevertheless, the new science of breath analysis is starting to receive more attention in several laboratories worldwide because of its growing potential as a non-invasive clinical diagnostic tool (Amann and Smith, 2005). A recently developed technique, selected ion flow tube mass spectrometry, SIFT-MS, enables the accurate measurement of endogenous volatile compounds. Using SIFT-MS, on line real time analyses of even single breath exhalations can be achieved down to the parts-per-billion, ppb, level, with a wide dynamic range up to % levels for compounds such as water and several tens of parts-per-million, ppm, for acetone (Smith & Spanel, 2005). Another technique which has shown promise for monitoring breath acetone is use of a light emitting diode based photometric method (Teshima et al., 2005), however the upper concentration limit is little more than 1 ppm, which is insufficient for even the range of acetone in healthy volunteers (Turner et al., 2006d).

Because of its wide dynamic range, sensitivity and accuracy, SIFT-MS was used to determine the concentration distributions of the most abundant breath metabolites ammonia, acetone, methanol, ethanol, propanol, acetaldehyde and isoprene in 30 healthy volunteers over 6 months (Turner et al., 2006 a, b, c &d). In this study of 30 healthy volunteers, we found that the average non-fasting level of acetone in breath has a geometric mean of 477 ppb with a multiplicative standard deviation of 1.58, with the values ranging from 148 to 2744 ppb (Turner et al., 2006d). Deng et al (2004) found similar results for healthy volunteers with a median value of 15 volunteers as 510ppb. They also examined breath of 15 patients with type 2 diabetes, reporting an elevated median value of 2350 ppb acetone. No further data were presented on the treatments received, blood glucose levels or even the time of day and conditions of sampling so it is difficult to interpret these results. Other differences in breath composition may be present in diabetes which may be monitored with non-specific analytical devices such as gas sensors. For example, Yu et al. (2005) found a significant difference between the breath of diabetics and healthy volunteers using a conducting polymer sensor array, although the identity of the components causing this difference was not elucidated.

2. Experimental

2.1 Glucose clamp details; glucose and insulin infusion.

We recruited 8 subjects with type 1 diabetes with a relatively long duration of diabetes (mean 28 ± 3 years), BMI 26.4 ± 1.0 and, on average, sub-optimal although not disastrous glycaemic control (mean HbA1c level

of $8.8 \pm 0.4\%$). Studies were approved in advance by an independent research ethics committee and all subjects provided *a priori* written informed consent. Volunteers were admitted to the clinical research facility the evening prior to studying for overnight stabilization of glucose using intravenous insulin infusion (using an intravenous catheter in the antecubital fossa of the non-dominant arm placed using local anaesthesia with lidocaine) to avoid nocturnal hypoglycaemia during the night immediately preceding studies.

The following morning, we used an “insulin clamp” technique to achieve a controlled stepwise reduction in plasma glucose values through the course of the morning. Insulin clamps have been widely used as a metabolic tool for manipulating blood glucose, including use as a method for creating controlled hypoglycaemic challenges as described below (e.g. Evans et al. 2004). Starting at 8 am, a second intravenous catheter was placed distally in a retrograde fashion in the non-dominant hand for blood sampling and the hand warmed using a specially designed heating box to 55°C in order to “arterialize” the venous sample. After at least 45 minutes for stabilization/ acclimatization, a primed continuous infusion of regular insulin (Humulin S, 60 mU/kg/min) was then initiated, together with a variable infusion of 20% dextrose. The latter was adjusted on the information from regular bedside measures of arterialized venous plasma glucose. Using this insulin clamp technique, the blood glucose concentration was lowered in 40 minute steps aiming for 5, 3.8, 3.3, 2.8 and 2.4 mM respectively (the latter step was only 20 minutes). This stepped lowering in 40 minute steps has been used extensively in hypoglycaemia research (e.g. Evans et al. 2004). Typically, metabolic responses to hypoglycaemia occur within 20 minutes after a glucose threshold is reached (Evans et al. 2000) so the use of 40 minute steps allows sufficient time for equilibration of body glucose levels and for measurements to be made. In one subject (1B), the baseline level was much higher (13.9 mM), hence the steps were at different blood glucose concentrations. Subjects were kept unaware of their actual plasma glucose values throughout studies.

In summary, the insulin clamp technique offers 2 major scientific advantages for this study. Firstly we were able to control accurately and safely, blood glucose values in our volunteers and to create a gradual change. Secondly, the technique results in fixed an unvarying insulin levels so that any changes in breath acetone should be independent of variations in circulating insulin.

2.2 Taking breath samples

For sampling, volunteers were asked to exhale into a sample bag constructed from Nalophan tubing (Kalle UK Ltd) with a ¼ inch Swagelok fitting appended to one end for direct attachment to the SIFT-MS inlet. The volunteers were asked to breathe as normally as possible, until 2 litres of breath had been taken. They were asked to start exhaling and then breathe into the bag through the fitting. This sampled whole breath, and from earlier experiments (data not shown), acetone concentrations from the whole breath measured are close to those of alveolar breath. Each volunteer provided a breath sample just prior to the start of the clamp study, when the glucose was analysed for the “baseline” data point. This was typically around 6 mmol/L blood glucose concentration. Breath samples were then taken at each step in the glucose clamp at 30 minutes into each of the 40 minute stages of the target blood glucose concentration described above. The last stage (nominally 2.2 mM blood glucose concentration) only lasted for 20 minutes so that the breath samples were taken at the end of the stage. A background air sample in the research ward was also taken using a constant flow pump. The bagged breath samples were stored in a black plastic bag and then returned to the laboratory for analysis by SIFT-MS the same day.

2.3 Blood glucose analysis

Plasma glucose concentration was measured every five minutes at the bedside using a Yellow Springs Instruments (YSI) analyzer throughout the study, so the exact concentration could be correlated with the time the breath sample was taken. These measurements were also taken to ensure that the blood glucose concentration was at steady state when the breath samples were taken. Repeat measurements (data not shown) indicate that the precision of the blood glucose monitoring is within 5%.

2.4 SIFT-MS analysis

SIFT-MS has been described in detail previously (Smith and Španěl, 1996; Španěl and Smith, 1996, Smith and Španěl, 2005, Amann and Smith, 2005) so only a brief summary is given here. Analysis requires the generation of precursor ions which are produced in a microwave discharge and are selected by the first of two quadrupole mass filters before being injected into a fast flowing helium carrier gas (Figure 1). These ions are thermalised to the helium carrier gas temperature (300K) by multiple collisions with helium atoms before reacting with the trace gases in the breath sample, including acetone introduced from the breath bag (or via a disposable mouthpiece if direct breath is analysed) at a known flow rate via a heated calibrated capillary. The available precursor ion species are H_3O^+ , NO^+ and O_2^+ . All three precursors react with acetone, producing characteristic product ions. The precursor and product ions in the carrier gas are sampled by a downstream orifice and pass into a differentially pumped second quadrupole mass spectrometer and ion counting system for analysis.

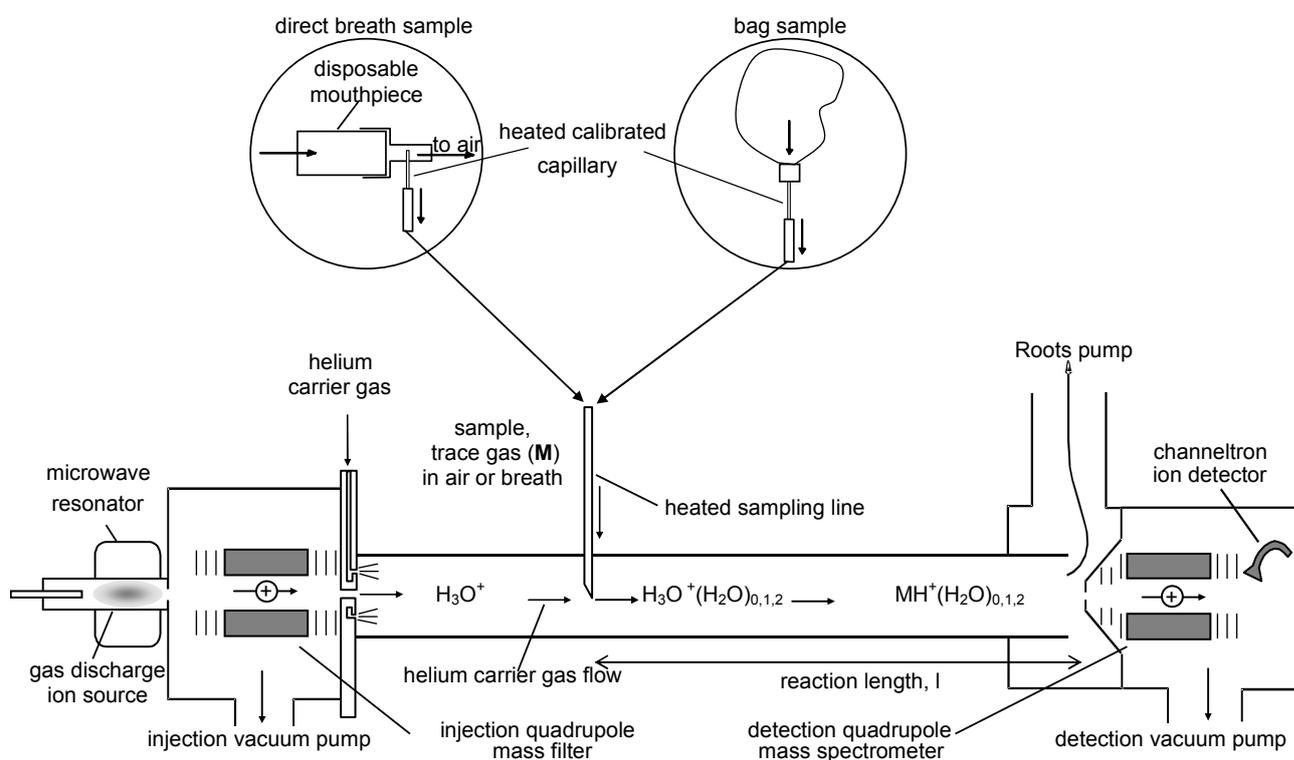


Figure 1. A schematic diagram of the SIFT-MS instrument indicating the main components. Direct breath samples, or indirect breath samples collected into a bag, may be analysed, as illustrated, although this study only used indirect bagged breath measurements.

There are two analytical modes of operation for SIFT-MS. First, the *full scan mode*, which is not used in this study, and is described elsewhere (Smith and Španěl, 2005). The second analytical mode is the *multiple ion monitoring mode*, in which the downstream analytical mass spectrometer is rapidly switched between selected m/z values for both the precursor and chosen product ions to quantify both water vapour and the targeted trace compounds in the sample. The SIFT-MS can be used to quantify the concentration of trace gas through knowledge of the sample flow rate, helium (carrier gas) flow rate, flow tube pressure and temperature, precursor and product ion intensities, which can all be measured during analysis. In addition, the reaction rate coefficient between the precursor ion and compound of interest must be known, which has been well characterised for acetone (Španěl et al., 1997). The on-line database containing reaction rate

coefficients was developed from numerous detailed selected ion flow tube (SIFT) studies of various classes of compounds (alcohols, aldehydes, ketones, hydrocarbons, etc) with the three precursor ions (Smith, and Španěl, 1996; Španěl, and Smith, 1996, Smith and Španěl, 2005). This mode was used to determine the concentrations of acetone in the breath of the diabetic volunteers in this study. The full procedure for quantifying trace gas concentration is given in Španěl et al., (2006b).

Checks are carried out at the start of analysis to ensure that the instrument is tuned and calibrated by, for example, analysing the concentration of water in direct breath: fully saturated breath at a temperature of 37°C should give approximately 6% water. Because these checks and measurements are carried out each time the instrument is used, there is no noticeable drift. Uncertainties in measurement mostly arise through inaccuracies in the determination of the rate coefficient. Acetone is also a relatively stable compound generated systemically in the body (Španěl et al., 2006a) so is easy to measure using SIFT-MS. Several studies have shown the reproducibility of analysis of acetone from subsequent breaths as to better than 10%, for example Španěl et al., 2007.

Acetone reacts with all three precursor ions, and in this study, analysis of acetone was carried out using the both H_3O^+ and NO^+ precursor ions to provide additional checks on the data obtained.

The reaction of H_3O^+ with acetone is a simple one in which a proton is transferred from the H_3O^+ ion to the acetone molecule, yielding a product ion at m/z 59:



In the presence of humid air, such as breath, the product ion clusters with water, yielding additional product ions at m/z 77 and m/z 95; these additional ions are taken into account in the kinetics database.

Using NO^+ , the process involved is an ion-molecule collisional association resulting in an ion of type NO^+ .M at m/z 88 as follows (Španěl et al 1997):



Data from the analysis of acetone with both these precursors were recorded for all eight type 1 diabetes volunteers in this study.

3. Results & discussion

Baseline breath acetone concentrations

Of note, during clamp studies, circulating insulin levels rose to 600 pM when first assayed 40 minutes into the clamp but then remained constant at that value throughout the duration of the 180 minute study.

Table 1 gives the baseline breath acetone concentration and blood glucose concentrations for each of the diabetic volunteers. The results reported here are from data obtained using the H_3O^+ precursor ion. However, results obtained using the NO^+ precursor showed a similar pattern.

Table 1. Baseline blood glucose and breath acetone concentrations for type 1 diabetes volunteers

Volunteer	Baseline blood glucose (mmol/L)	Baseline breath acetone (ppm)
4A	8.0	1.2
5A	5.8	3.4
1B	13.9	8.5
8A	5.7	1.7
7B	7.0	4.3
6B	4.8	3.5
10A	5.8	21.0
11A	5.0	1.0

These results show a very wide range of baseline breath acetone concentrations (range 1.0 to 21 ppm) for all the volunteers, and there appears to be no correlation ($r = 0.134$, $n = 8$) between baseline glucose and breath acetone among volunteers. Of note, three volunteers had baseline breath acetone levels within the range reported for normal healthy volunteers (Turner et al., 2006d), data which contradict the widely held view that breath, blood or skin acetone levels are almost invariably higher in patients with diabetes (Deng et al., 2004; Yamane et al., 2006; Jones et al., 1993, Chakraborty et al., 2008). However, three out of 8 patients may not be significant, and it is likely that the heterogeneity is greater in diabetics (Owen et al., 1982) than that observed in healthy volunteers (Turner et al, 2006d).

We then examined how breath acetone changed as blood glucose levels were lowered. Given the inter-individual variability at baseline, we have presented how changes in plasma glucose and breath acetone are correlated for each individual rather than a group average in figure 2. We omitted one value - the baseline sample for volunteer 4A was markedly lower than other values (shown in table 1), most likely because in this subject, the plasma glucose value was changing rapidly just prior to the start of the clamp. The data portrayed in figure 2 show that, under these experimental conditions, there is a linear correlation between plasma glucose and breath acetone, albeit with the correlation in some volunteers being stronger than for others. Parenthetically, the relationship appears to be the opposite of that seen in healthy volunteers, where low blood glucose following fasting yields higher breath acetone concentration (Turner et al., 2008; Smith et al., 1999). We feel, however, that this is likely due to changing insulin levels in the latter. As set out in the introduction, hypoglycaemia in type 1 diabetes presents the body with a number of conflicting metabolic signals. For example, blood glucose levels are low- analogous with fasting- but circulating insulin levels are high- a signal more generally associated with food intake. We found that under these hyperinsulinaemic hypoglycaemic conditions, breath acetone levels rose suggesting increased generation of circulating ketones. Our study design did not allow us to distinguish between an effect predominantly on lipolysis or on ketogenesis or a mixed pattern. Clearly more elaborate studies would be needed to identify the physiological explanation for our findings.

A further limitation of our approach in these studies is that we had no control arm in which we maintained plasma glucose at baseline levels to see if the study conditions themselves resulted in a change in breath acetone and/or the time line involved. Additionally, given the inter-individual variation in baseline acetone, we plan further work to characterise further this relationship. Nevertheless, our results do indicate that we can reliably measure breath acetone and that there appears to be a relationship between breath acetone and metabolic state in type 1 diabetes when examined at “everyday” levels.

One of the main difficulties in the past in investigating the relationship between acetone and blood glucose is the difficulty in measuring acetone accurately. Techniques such as GC-MS may be sensitive, however they are difficult to make fully quantitative and certainly do not give rapid results. SIFT-MS is a direct technique (results almost in real time) and is fully quantitative. This makes it ideally suited to accurately monitoring breath acetone concentrations during glucose clamps and with the development of smaller instruments, placement of a SIFT-MS in the clinical laboratory is planned for direct breath measurements.

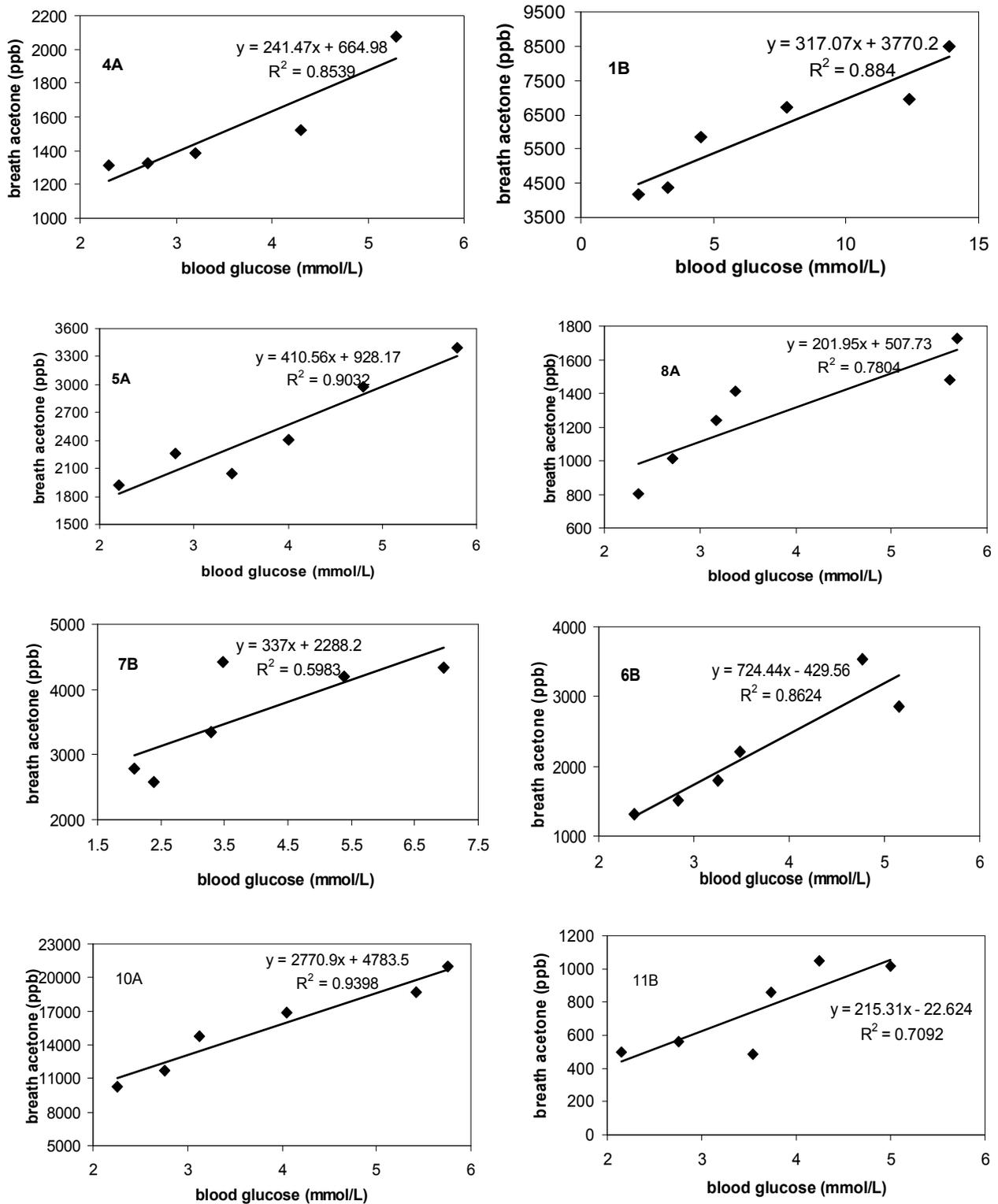


Figure 2. Relationship between blood glucose and breath acetone concentrations for all 8 volunteers. Linear regression lines have been plotted for each glucose clamp

4. Conclusions

Our data show that, contrary to some reports, absolute levels of breath acetone in people with type 1 diabetes are not invariably higher than those seen in healthy subjects (Deng et al., 2004; Yamane et al., 2006; Jones et al., 1993). Although basal levels varied widely, during carefully controlled experimental hypoglycaemia with fixed circulating insulin levels, breath acetone levels decreased in all subjects as blood glucose levels fell. Our data indicate that breath acetone does vary as glycaemia and/or metabolic status changes in type 1 diabetes. We speculate that breath acetone may be a potential future marker of metabolic state- perhaps even as an indicator of changes in blood glucose in diabetes although clearly further work in this area is warranted

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