Breath Acetone as a Measure of Systemic Ketosis Assessed in a Rat Model of the Ketogenic Diet

Sergei S. Likhodii,1,2* Kathy Musa,1 and Stephen C. Cunnane1

**Background:** The mechanism of a high-fat, low-carbohydrate ketogenic diet (KD) in alleviating drug-resistant epilepsy is unknown but may be related to systemic ketosis induced under this treatment. The need for frequent measurement of systemic ketosis, which is essential for improving maintenance of the KD in patients and for studying mechanism of the KD action, has prompted us to validate the breath acetone test as a fast, reliable, and noninvasive tool for ketosis assessment.

**Methods:** A rat model of the KD that allowed frequent blood sampling was used to investigate how well breath acetone reflects plasma β-hydroxybutyrate (β-HBA), the most commonly measured ketone body. Rat pups (20 days of age) were introduced to and maintained on a KD or control diet for 33 days. During this period, breath acetone, plasma β-HBA, blood glucose, and body weight were measured approximately every 4th day. A correlational analysis of breath acetone and plasma β-HBA was conducted.

**Results:** Breath acetone was found to be a significant predictor of plasma β-HBA over a clinically relevant range of β-HBA concentrations ($r^2 = 0.75$; $P < 0.001$). We have proposed a general formula that allows the value of plasma β-HBA to be estimated based on breath acetone measurement.

**Conclusions:** Breath acetone is an accurate measure of mild to moderate systemic ketosis. The noninvasive nature of this test will be useful for day-to-day implementation of the KD, searching for better forms of this diet, and understanding the role of ketosis in the mechanism of the KD action.

© 2002 American Association for Clinical Chemistry
makes this assessment noninvasive. Breath acetone values have occasionally been reported in individuals with chronic ketosis or undergoing short test periods using high-fat meals \((7, 11–13)\). It is believed that breath acetone could be a better index of overall ketosis than other ketones because acetone in the portal venous blood and alveolar air readily equilibrates, whereas differences in muscle and renal metabolism may affect acetoacetate and \(\beta\)-hydroxybutyrate (\(\beta\)-HBA) concentrations in plasma and urine \((7)\). However, experimental data on how well breath acetone reflects blood ketones, e.g., \(\beta\)-HBA, which is the most commonly measured ketone in clinical practice, are lacking.

The objective of our study, therefore, was to investigate breath acetone and plasma \(\beta\)-HBA concentrations in a rat model of the KD that allows frequent blood sampling. The rat model provided the additional benefits of clinical studies of the KD. The noninvasive tool for ketosis assessment suitable for clinical conditions. The overall goal of our project was to validate the breath acetone test as a fast, reliable, and noninvasive tool for ketosis assessment suitable for clinical studies of the KD.

### Materials and Methods

#### ANIMALS

The University of Toronto Animal Care Committee approved all procedures involving animals. Twelve 17-day-old male albino Wistar pups (Charles River Canada) arrived at the facility with their dam. The rats were housed in plastic cages with wood-chip bedding and were placed on a 12 h light/12 h dark cycle (lights were turned on at 0700). The rats were given 3 days to acclimatize to their new environment. During this period they had unrestricted access to regular rat chow and drinking water. At 20 days of age, the rat pups were weaned and randomly assigned to either a control or a KD group so that each group consisted of six animals. Thereafter, the rats were housed individually in plastic cages with wood-chip bedding. The day the control diet and the KD replaced the rat chow was regarded as day 0 of the "days on diet" variable.

#### DIETS

The formulations for the control diet and the KD used in these experiments are shown in Table 1. The American Institute of Nutrition 93G diet (AIN-93G; Dyets Inc.) served as a control diet and was provided freely to control rats.

<table>
<thead>
<tr>
<th>Macronutrient</th>
<th>Micronutrient</th>
<th>Control diet</th>
<th>KD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>Casein</td>
<td>200</td>
<td>154</td>
</tr>
<tr>
<td></td>
<td>L-Cystine</td>
<td>3</td>
<td>2.4</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>Corn starch</td>
<td>529.5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Sucrose</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Maltose dextrin</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>Sweetener</td>
<td>Sucaryl</td>
<td>0</td>
<td>32</td>
</tr>
<tr>
<td>Fiber</td>
<td>Cellulose</td>
<td>50</td>
<td>199</td>
</tr>
<tr>
<td>Vitamins</td>
<td>AIN-93G vitamin mix</td>
<td>0.33</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td>Choline bitartrate</td>
<td>2.5</td>
<td>2.0</td>
</tr>
<tr>
<td>Minerals</td>
<td>AIN-93G mineral mix</td>
<td>27.3</td>
<td>22.0</td>
</tr>
<tr>
<td>Fat</td>
<td>Soybean oil</td>
<td>70</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>Flaxseed oil</td>
<td>0</td>
<td>525</td>
</tr>
<tr>
<td></td>
<td>Fat:(protein + carbohydrate)</td>
<td>1:12</td>
<td>3.5:1</td>
</tr>
</tbody>
</table>

\(^{a}\) Values are given as g/kg with an accuracy of \(\pm 2\%\). Both control and ketogenic diets contained 0.014 g/kg tbutylhydroquinone, an antioxidant, to improve storage.

\(^{b}\) Modified AIN-93G vitamin and mineral mixes contained no carbohydrates.

\(^{c}\) Soybean oil in the KD comes from the diet premix, where it serves as a carrier for fat-soluble vitamins.

\(^{d}\) Flaxseed oil is 55% \(\alpha\)-linolenic acid (18:3n-3).

Our preliminary experiments suggested that rat pups weaned directly to a KD with a by-weight fat:(protein plus carbohydrate) ratio >3:1 have difficulty adjusting to the new diet because they rapidly lose weight. To assist in the rats’ adaptation to the KD in the current experiment, the amount of fat in the KD was gradually increased over the first 8 days by providing intermediate fat:(protein plus carbohydrate) ratios of 1:1, 2:1, and finally, 3.5:1. Rats then stayed on the 3.5:1 ratio for the rest of the experiment (see Table 1).

The KDs were prepared in our laboratory by mixing flaxseed oil, obtained locally, with a custom-formulated powdered diet mixture containing nutrients, fiber, minerals, and vitamins. The premix for the KD was obtained from Dyets and was formulated to match the control AIN-93G diet protein, mineral, and vitamin content as closely as possible. To prevent separation of the KD into layers of oil on top and solid components on the bottom of the food dishes, the 3.5:1 KD included increased amounts of cellulose, which improved the consistency of the diet. Maltose dextrin (Dyets) and Sucaryl™ (sodium cyclamate, an artificial sweetener (Abbott Laboratories), were added to improve the palatability of the diet and its intake by the rats. A fresh, limited portion of the diets, both control and the KD, was placed in rats cages daily. All rats received slightly above the amount of food they would eat in 1 day. This amount was determined empirically as providing \(\sim 1.3\) kJ/g of body weight. Water was freely available for all groups throughout the experiment.

#### BREATH SAMPLING AND ANALYSIS

Breath air and blood were sampled between 1300 and 1700 on days 8, 13, 17, 21, 25, and 29 on the diets. The breath from each rat was sampled first; the blood was collected 30–40 min later. The rat sampling order was randomized each session.

We collected rat breath by placing each rat in a 4.2-L, air-tight glass vessel for 20 min. The 20-min period was established in our preliminary experiments as sufficient for the acetone in the vessel to reach concentrations that
could be analyzed by gas chromatography without excessive accumulation of expired CO₂. Our measurements of accumulated CO₂ showed that its concentration after 20 min did not exceed 5% by volume. We used six glass pickling jars with screw-on metal lids, obtained locally, as breath collection vessels. Standard gas chromatography septa were installed in the lids to allow collection of air samples directly from the jars. The septa were “sandwiched” between the home-made triangle-shaped aluminum plates and the jar lids. The triangles were predrilled at the corners and in the center, matching holes were made in the lids, and the whole assembly was secured with three screws placed at the corners. This allowed a needle to be inserted into the jars through the septum and a 1-mL sample of the breath to be drawn with an airtight syringe.

We determined breath acetone concentrations immediately after sampling, using a gas chromatograph equipped with a flame ionization detector (Model 5890; Hewlett-Packard). A 180 cm × 4 mm glass column packed with 80/100-µm mesh Carbopack was purchased from Supelco. The 3-min chromatographic run was performed at 70 °C with helium as the carrier gas at a flow rate of 20 mL/min. The injector temperature was 150 °C, and the detector temperature was 200 °C. The acetone peaks that resulted were calibrated against a gaseous acetone calibrator (37.41 nmol/L). This gaseous acetone calibrator was prepared on each day of sampling by injection of 500 µL of pure acetone into a 4.264-L airtight vessel and then, after allowing 1 h for acetone evaporation and equilibration, by injection of 500 µL of gaseous sample from the first jar into another, 4.253-L airtight vessel. The final calibrator concentration of 37.41 nmol/L was obtained by drawing 0.2 mL from the last vessel into an airtight syringe and diluting it by a factor of 5, by drawing 0.8 mL of clean air into the same syringe. The resulting 1.0-mL calibrator was immediately injected into the gas chromatograph. Analogous procedures were used to prepare calibrators in a wide range of acetone concentrations (0.015, 0.03, 0.06, 0.12, 0.24, 0.48, 0.72, 0.96, 1.44, 1.92, 2.88, and 4.80 µmol/L) to test the linearity of the analysis. These calibrators covered a broad range of acetone concentrations expected in experiments with animals and in humans.

The procedures for preparing calibrators and breath acetone analysis required special attention to the quality of the laboratory air. During pilot tests, we recognized that volatile solvents in the laboratory or even perfume worn by laboratory staff might interfere with the analysis. For that reason, we used hydrogen peroxide, not alcohol, as the antiseptic for blood collection in the rats (see below). We routinely controlled the quality of the laboratory air during the experiment by injecting a sample of the air into the gas chromatograph; the background concentration of acetone was negligible.

**Results**

**BLOOD SAMPLING AND ANALYSIS**

Thirty to 40 min after breath samples were collected, ~200–300 µL of blood was collected from the tail vein of each rat. The tip of a 23-gauge needle (Becton-Dickinson) was cut to a length of ~2 cm and inserted with its sharp end into the tail vein where it stayed for the duration of blood collection. The blood dripping from the open end of the needle tip was collected into three to four 75-µL heparinized capillary tubes (BD Microtromatrit tubes; Becton-Dickinson). Blood glucose was analyzed immediately by the Glucometer Elite (Bayer Inc.), using a small drop of blood. The rest of the collected blood was transferred into 2.5-mL heparin-containing microcentrifuge tubes where it was kept on ice for 20–30 min until centrifugation. The blood samples were centrifuged at 1000g and 0 °C for 9 min to separate the plasma. Plasma samples were transferred into 500-µL centrifuge tubes and stored at −20 °C until analyzed for β-HBA by an enzymatic assay (Sigma).

**STATISTICAL ANALYSIS**

To compare group means, we performed a repeated measures two-way ANOVA using SAS statistical software (SAS Institute Inc.). The “diet type” variable was the repeated factor in this analysis and was assigned values of 0 and 1 for the control and the KD diet, respectively. The “days on diet” variable was a second but nonrepeated factor. Tukey’s honestly significant difference was used as the post-F-test. Significance was set at P <0.05. To determine whether “breath acetone” was a significant predictor of “plasma β-HBA”, we performed a multiple linear regression analysis using Stata Statistical Software (Stata Corporation). Plasma β-HBA was set as the dependent variable, whereas breath acetone and body weight were set as the independent variables. Significance in this test was set at P <0.05.

**Analytical performance of acetone GC method**

The acetone peak appeared in chromatograms, with a retention time of 1.03–1.06 min. It was well resolved from the injection front and was the only major peak in chromatograms of the rat breath. The linearity/calibration curves were obtained by plotting the peak areas against the gaseous acetone calibrators at 12 concentrations (0.015, 0.03, 0.06, 0.12, 0.24, 0.48, 0.72, 0.96, 1.44, 1.92, 2.88, and 4.80 µmol/L). The resulting linear regression equation was: \[ y = (182.773.75 \pm 2013.24)x + (2271.89 \pm 3622.04); r^2 = 0.9988. \] The within- and between-day imprecision (CV) of the instrument was estimated by analysis of two calibrator samples with acetone concentrations of 37.41 and 960.0 nmol/L, respectively. The CVs were 5.7% (n = 6) and 4.8% (n = 6) for within-day and 16% (n = 6) and 18% (n = 6) for between-day measurements, respectively. We attributed the relatively large between-day imprecision mainly to degradation of the gaseous calibrator, perhaps because of diffusion of acetone from...
the vessel containing the calibrator. Because of the difficulty in preparing and storing multiple gaseous calibrators, in these experiments we used a single acetone calibrator with a concentration of 37.41 nmol/L for the breath samples. This calibrator was prepared fresh each sampling day. The CV across triplicate calibrator measurements typically was ~3%.

The lowest limit of quantification, determined as the lowest acetone concentration with a CV <15% after repeated analysis (n = 9), was 1.5 nmol/L. For comparison, most breath samples collected from rats fed control diet showed an acetone concentration in the range of 4.1–12.8 nmol/L. The analogous samples from rats on the KD showed acetone concentrations of 11.1–95.6 nmol/L.

**KETONE AND BLOOD GLUCOSE CONCENTRATIONS**

The time courses of changes in blood glucose, plasma β-HBA, and breath acetone are shown in Fig. 1, B, C, and D, respectively. β-HBA and breath acetone were significantly higher, whereas blood glucose was significantly lower in the KD rats (P <0.05) throughout the entire experiment. Both β-HBA and breath acetone in the KD group increased steadily, peaking 16 days after introduction the KD, and exhibited a decreasing trend thereafter. The blood glucose concentrations in rats on the KD were consistently lower than the corresponding concentrations in controls throughout the experiment, although there was a trend toward normalization after 16 days on the KD (Fig. 1B).

**BODY WEIGHT**

The time courses of changes in body weight in the control and the KD groups of rats after they were introduced to their respective diets are shown in Fig. 1A. After 8 days on the diets (at age 28 days), control rats weighed more than rats fed with the KD. By the end of the experiment, i.e., after 33 days on the diets (at age 53 days), controls were 16% heavier than the rats from the KD group (P <0.05).

**BREATH ACETONE AS A PREDICTOR OF PLASMA β-HBA**

A scatter plot of the relationship between the variables breath acetone and plasma β-HBA is shown in Fig. 2; both experimental data and the β-HBA values predicted by the correlational model are shown. Breath acetone was found to be a significant predictor of plasma β-HBA (r² = 0.75; P <0.001). The general model for the estimation of plasma β-HBA values in the units of mmol/L is represented by
the following expression: plasma $\beta$-HBA = 1.527 + 0.00824(breath acetone) - 8.677(body weight), where breath acetone is expressed in nmol/L per kg of body weight and the body weight is expressed in kg. The variable “body weight” was included in the regression model to account for the differences in the volumes occupied in breath collection vessels by rats of different body weights. Table 2 summarizes the statistical outcomes (regression coefficients, SEs, and $P$ values) for each of the variables included in this model. The standard error of the estimate ($S_{\hat{y}|x}$) was 1.000. The ANOVA further showed the following statistics for the regression: regression sum of squares = 202.9; residual sum of squares = 69.0, with 2 and 69 degrees of freedom, respectively. The $F$-test statistic was 101.41, and $P$ was < 0.001.

**Discussion**

This study was undertaken to assess the feasibility of using breath acetone as an indicator of ketosis induced by the KD. At present, measuring urinary ketones with a urinary nitroprusside dipstick monitors a patient’s success on the KD at its initiation in a hospital or, after discharge, its maintenance at home. The goal is to maintain urinary ketones at 8–16 mmol/L (14). The nitroprusside ketone test is largely qualitative and has been criticized as not being truly representative of blood ketone concentrations (1, 5, 6, 8–10). If the value of our proposed acetone test is established and an inexpensive easy-to-use “breathalyzer” sensitive to acetone can be manufactured, it may improve a day-to-day implementation of the KD because the induction and maintenance of stable systemic ketosis are thought to be essential for the success of this therapy.

Huttenlocher (15), for example, suggested that for the KD to be clinically effective in reducing seizures in children, blood $\beta$-HBA had to be maintained at concentrations $\geq$ 2.0 mmol/L and blood acetoacetate $\geq$ 0.6 mmol/L. These conclusions, however, did not take into account the wide age range for patients (18 months to 18 years) in that study (15), which might have influenced the results by a mechanism independent of ketosis. Nevertheless, Gilbert et al. (10) recently also reported a positive correlation of blood $\beta$-HBA with seizure control in children. Sirven et al. (4) noted that in adults on the KD, a reduction in seizure frequency was best achieved when serum $\beta$-HBA was $\geq$ 2.4 mmol/L. In rat models of the KD, seizure threshold may also be directly related to the concentration of plasma $\beta$-HBA (16), but this is not always observed (5).

The availability of an acetone breath test that is noninvasive and allows frequent sampling may permit clinical studies on the role of ketosis in seizure protection afforded by the KD. In addition to providing data on the mechanism of the KD effects, a breath acetone test may also be useful in the search for better and more efficient versions of the KD. By modifying the type of fat, for example, the KD can better conform to specific requirements of the growing brain in children for polyunsaturated fatty acids (5). At the same time, the increase in plasma cholesterol, a side effect complicating application of the classical form of the KD in adults (4), might also be prevented. In view of that, we have developed a novel form of the KD based on flaxseed oil and have shown that it has certain advantages over the classic KD formulations (5). Progress along these lines, however, will require a robust method of assessing the ability of new forms of the KD to induce and sustain ketosis.

The high-fat, low-carbohydrate KD induces the production of three ketones: $\beta$-HBA, acetoacetate, and acetone. The former two ketones are metabolites that can be readily used by the peripheral tissues and the brain as biosynthetic and energy-generating substrates. The role of acetone as an energy substrate is apparently small compared with two other ketones. A significant amount of acetone is removed from the body with the expired air, whereas the rest is metabolized by the liver via the cytochrome P450 pathway or excreted with the urine (6). The absolute concentrations of ketones in the body are dependent on many factors. In this experiment, plasma $\beta$-HBA and breath acetone both peaked in rats at approximately day 16 on the KD and decreased to a certain extent thereafter. Blood glucose showed inverted but simultaneous time-dependent changes after initiation of the KD. These patterns were consistent with our other experiments (5) and with reports from other laboratories (17, 18). We believe that the time course of absolute concentrations of ketones may reflect long-term adapta-

**Table 2. Statistical summary for the model predicting plasma $\beta$-HBA from breath acetone.**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Regression coefficient</th>
<th>SE</th>
<th>$P$</th>
<th>Variance inflation factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>1.527</td>
<td>0.454</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>Breath acetone</td>
<td>0.00824</td>
<td>0.00065</td>
<td>&lt;0.001</td>
<td>1.048</td>
</tr>
<tr>
<td>Body weight</td>
<td>-8.677</td>
<td>2.500</td>
<td>&lt;0.001</td>
<td>1.048</td>
</tr>
</tbody>
</table>
tional changes of metabolism to high fat intake. These may involve a complex interplay of lipolysis, glucogenesis, and ketogenesis and transport and utilization of glucose and ketones by the brain and peripheral tissues as discussed previously (5).

The relative concentrations of ketones were of specific interest in this study because we wanted to devise a model predicting the concentration of one of the blood ketones, β-HBA, based on the measurement of another ketone, breath acetone. The relationship between all three ketones in the body is complex and is based on the following considerations. β-HBA and acetoacetate undergo interconversion in a reaction catalyzed by β-HBA dehydrogenase, the equilibrium of which is dependent on the NAD⁺:NADH concentration ratio. It is interesting to note that, in general, the higher the total concentration of ketones, reflecting a higher rate of their synthesis from fat, the higher the blood β-HBA:acetoacetate concentration ratio (19). Acetone is formed in the body mainly by the spontaneous decarboxylation of acetoacetate and, to a lesser degree, by the enzymatic conversion of acetoacetate to acetone via the enzyme acetoacetate decarboxylase (20).

We found that breath acetone was a good predictor of plasma β-HBA. Their relationship over a clinically relevant concentration range was adequately described by a simple linear equation. Our conclusion on the correlation of breath acetone with blood ketones supports the results of a study by Tassopoulos et al. (21), who reported a significant positive relationship between breath acetone and blood β-HBA in obese patients during prolonged fasting. We are currently measuring breath acetone in adult volunteers with acute, short-term ketosis and assessing its relationship with both plasma β-HBA and acetoacetate. A study examining the relationship between breath acetone and plasma ketones in children with chronic ketosis is also planned so that the reliability of breath acetone in predicting plasma ketone concentrations can be evaluated in this pertinent clinical population.

This research was supported by a research fellowship from Epilepsy Canada/Parke-Davis (to S.S.L.), a University of Toronto Open Fellowship (to K.M.), and grants from the Stanley Thomas Johnson Foundation, the Bloorview Childrens’ Hospital Foundation, and the Dairy Farmers of Canada. We thank Mary Ann Ryan for technical assistance.

References


