Novel Solid-Phase Assay of Ketone Bodies in Urine

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This simple dip-and-read color-matching assay measures ketone bodies in urine. The result is matched with any of the seven colors provided in a color chart. The intensity and hue of the color formed depends on the concentration of acetoacetic acid and follows a gradation starting from light purple (0.2 mol/L acetoacetate) to dark purple and finally to dark violet (9 mol/L). The color developed is independent of pH in the range pH 4–9.5, and is stable for at least 1 h. 3-Hydroxybutyric acid produced no color at the concentrations tested (≤100 mmol/L), and acetone was detectable only when ≥15 mmol/L. The test was compared with the Ames Ketostix assay in subjects who were on restricted-calorie weight-loss programs. The two methods showed good agreement, except that 20% (21/103) of samples that were negative by Ketostix were read as positive by the new method, which may be more sensitive (detection limit = 0.2 mmol/L). Measurement of urine ketones was unreliable (in comparison with measurement of blood or breath acetone) for monitoring the rate of fat loss during a negative calorie balance.

Additional Keyphrases: intermethod comparison • effect of weight loss on ketones • dipsticks

Ketone bodies (acetoacetic acid, acetone, and 3-hydroxybutyric acid) are metabolites of fat catabolism. They are produced in excessive amounts in dieting subjects or those who metabolize more calories than they consume (1), and in insulin-dependent (type 1) diabetic patients, whose hepatic metabolic pathways are activated (2–9). Fatty acid metabolism under such conditions is increased, resulting in increased concentrations of ketone bodies in plasma (2, 3, 10–12), which are then excreted in urine. Although urine ketones are not reliable markers in some individuals because they are not excreted in proportion to increased blood ketone concentrations (10), many diabetics in both home and clinical settings still monitor ketone bodies in urine because of the simplicity and low cost of this assay.

Numerous methods are known in the literature for measuring ketone bodies, particularly acetoacetic acid and acetone in urine (13–17). Many assays take advantage of the "Legal" method, in which a carbonyl compound reacts with a nitroprusside (nitroferricyanide) salt in the presence of an amine to form a colored complex (13, 14). The color reaction is believed to involve a coupling reaction of the nitroso group of nitroprusside with the carbonyl group of the ketone to form an intermediate that complexes with the amine to produce a color characteristic of the specific amine. In forming the complex, the trivalent ion of the nitroprusside is reduced to its divalent state. The color complex, however, is unstable in alkaline solutions. Further, nitroprusside salts are subject to decomposition in the presence of moisture and high pH. Frequently during storage a brown decomposition product is formed that interferes with sensitive color detection in the assays. Thus, numerous attempts have been made to stabilize the color complex by using mixtures of nitroprusside and amine or amino acids in combination with various buffers, metal salts, organic stabilizers, and polymeric materials (13–16). Although many devices known in the literature involve dry tablets or powders, other assay devices use adsorbent carrier upon which some or all of the reagents have been dried. The adsorbent carriers are used in the form of dipstick strips that are immersed in a urine sample for analysis of a visible color reaction. Because the indicator reagents are merely adsorbed onto the carriers, the strips exhibit some bleeding of the color complex into the aqueous environment.

Here we describe a novel, solid-phase assay for measuring ketone bodies in urine that involves preparation of a color indicator comprising a nitroprusside ionically bonded and an amine covalently bonded to a solid phase. The solid phase in aqueous solution (urine) is maintained between pH 10.0 and 10.5 to provide an optimum color with ketones. The color is independent of the pH of the urine specimen, and the intensity and hue of the color depend on the concentration of ketones in the assay sample. The color formed does not bleed from the solid phase and is stable for >2 h, compared with the most widely used Ames Ketostix assay (Ames Co., Elkhart, IN) in which the color is stable for <1 min.

Materials and Methods

Test Matrix for Urine Ketones

The test matrix for measuring ketones in urine is a mixture of nitroprusside—Mg–diethylaminoethyl (DEAE)—silica and diethanolamine (DEA)-coated amipropyl silica. The preparative details are outlined below.

Solid-phase selection: Because of its unique properties—high surface area, exceptional dimensional stability, availability of particles with different sizes and pore diameters, easy chemical modification of the silanol surface, and low cost—we selected porous silica gel to optimize the measurement of acetoacetic acid in biological fluids. Although the formation of a purple to violet color for urine ketones can be adopted with porous silica derivatives of different sizes and porosities, the preferred choice was particle sizes between 40 and 60 μm and average pore diameters of 2000 nm, with a surface

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area of 180 m²/g, and a pore volume of 0.63 m³/g. The DEAE-coated silica gel (purchased from Separation Industries, Metuchen, NJ) was prepared as previously described (18, 19). The materials (three different manufacturing lots) ranged in particle size from ~40 to 60 μm, with mean pore diameters of 2000 nm, surface area of 180 m²/g, mean settle volume of 1.8 cm³/g, and elemental analyses ranges of 10.30–10.80% C, 0.70–0.90% N, and 1.8–2.4% H.

Nitroprusside–DEAE–silica: The DEAE–silica was chosen as an ion-exchange medium to prepare nitroprusside salt. The materials were in hydrochloride form (18). In preparing the nitroprusside–silica, we added magnesium salt to promote chelate formation; this helps to stabilize the color formed and enhances the kinetics of the reaction between the carbonyl function of the ketones, the amine, and the nitroprusside. The total binding capacity of nitroprusside to DEAE–silica was ~50 mg/g of dry solid. The preparative procedure for nitroprusside–DEAE–silica is as described previously (20). The matrix was stored in a dark glass bottle. A portion of the solid matrix was analyzed for percent moisture by use of a Karl Fisher Autotitrator (Brinkmann Instruments, Inc., Westbury, NY) and another portion was analyzed for carbon, hydrogen, and nitrogen content. The percent moisture must be ≤0.35% and the nitrogen content between 2.30% and 2.80%. The DEAE–nitroprusside salt is stable for more than one year at room temperature when stored dry and protected from light. The color of the matrix is faint rose.

DEA-coated aminopropyl silica: To generate a pH of ~9 to 9.5, we selected aminopropyl silica in its fully basic form because of its high ionization. The aminopropyl groups on porous silica were incorporated according to the procedures previously described (21). These materials (three different manufacturing lots purchased from Separation Industries) had the same characteristics as the above-mentioned lots except for elemental analyses ranges of 5.4–6.4% C, 1.80–2.4% N, and 1.30–1.80% H. The pH of 1 g of aminopropyl silica in 10 mL of water ranged from 9.3 to 9.8. The preparative procedure for DEA-coated aminopropyl silica is as described previously (20). The dried material was stored in a dark glass bottle with the cap tightly sealed. As above, a portion of the material was analyzed for percent moisture and another portion was analyzed for the percent of C, N, and H. The moisture must be ≤0.35% and nitrogen between 1.80% and 2.40%. The pH of the DEA-coated matrix was between 10.0 and 10.5. The matrix is stable for more than one year when stored dry and protected from light. The matrix color is white.

Preparation of test matrix for urine ketones: We evaluated various mixtures of DEAE–Mg–nitroprusside silica and DEA-coated aminopropyl silica for sensitivity, intensity, and hues of the purple and background colors, and read the discrimination between different concentrations of acetoacetic acids. The most preferred composition was found to be a mixture of DEAE–Mg–nitroprusside silica containing 5 mg of nitroprusside per gram of DEAE–silica with an equal weight of DEA-coated aminopropyl silica treated with DEA (10 g/kg). The two matrices were mixed for 30 min in a V-blender for homogeneous blending. The mixed matrix was stored in a dark glass bottle with a tightly sealed cap; under these conditions, it is stable for more than one year at room temperature.

Assay Test Device for Urine Ketones

Microcolumn assay device: Figure 1A depicts a microcolumn device suitable for measuring the concentrations of ketone in urine samples. The column is a 2 mm (i.d.) × 60 mm clear polystyrene or polypropylene tube. We inserted a hydrophilic polyethylene frit (Porex Technologies Corp., Fairburn, GA; cat. no. 4897) in one end of the column. Next, we fed 15 mg of the test matrix through a funneling device to the other end of the column. The column was vibrated to pack the column uniformly. Another Porex frit was then introduced and pushed into place with a stainless steel rod. After packing, the columns were stored in a dark bottle at room temperature over a silica gel pouch desiccant (Minipax Multiform Desiccants, Buffalo, NY). The columns are stable for one year at room temperature.

Dipstick assay device: Polystyrene sheets, 0.025 mm thick (Vinyl Plastics, Inc., Milwaukee, WI; cat. no. 1045), were sprayed uniformly with Scotch Spray Mount Adhesive (3M, St. Paul, MN; cat. no. 6065) and then sprayed with dry test matrix. Excess test matrix that did not bind to the glue surface was removed by tapping the sheets. The coated strips were cut in small pieces (4 × 2 mm) and attached to double-faced adhesive tape mounted on 60-mm-long dipsticks (Figure 1B). The dipsticks, when stored in a dark bottle at room temperature over a silica gel pouch desiccant (as above), are stable for one year at room temperature.

Clinical Study

We evaluated the assay device for monitoring the effectiveness of a weight-loss program and compared it with the Ames Ketostix assay. The study was limited to normal, healthy men and women between ages 24 and 54, with no chronic medical disorder except obesity. Participating in the diet study were 58 volunteers, 20 men and 38 women, divided into three groups. Each study period covered 30 consecutive days. We also in-

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**Fig. 1. Measurement of acetoacetic acid in urine:** A, microcolumn assay; B, dipstick assay
cluded 72 volunteers, 29 men and 43 women, in a separate nondieting control group to obtain baseline values. The selection of volunteers the diets used, and the experimental design are described in detail elsewhere (20).

Blood acetone concentrations of all dieting and nondieting groups were measured once every week with gas-chromatographic head-space analysis (2, 20, 22). Breath acetone concentrations at the time of blood acetone analysis were also determined simultaneously for comparison. Urine ketone body concentrations of some of the dieting subjects (seven men and seven women) were measured by the volunteers at home and by a laboratory technician at the clinical site. Early-morning urine specimens were assayed within 2 h after collection. The microcylinder assay devices we developed and the Ketostix were used simultaneously in all assays. For the nondieting control group, early-morning urine specimens were analyzed at the clinical site by a laboratory technician.

Results

Color formation with ketone bodies: To test the devices, we added various amounts of acetoacetic acid, lithium salt (Sigma Chemical Co., St. Louis, MO; cat. no. A-8509), to buffer solutions at pH 4.0, 7.3, 8.0, and 9.5, and also to fresh urine samples at pH between 5.0 and 7.0 from normal, healthy, nondieting subjects (see Table 1). The devices were dipped in the test samples and the colors obtained were read after 1–5 min. The colors appeared on the microcylinder devices within 1 min, and no visible color changes were observed after 2 min for at least 1 h. With our dipstick devices, the optimum color development took 3 min and remained the same for at least 1 h. The colors are stable for >2 h at room temperature, although the reading at that time may not be accurate because the test matrix dries up. For the most accurate results, we read the colors within 5 min. Buffer solutions not supplemented with acetoacetic acid gave a slightly yellow color, whereas urine samples gave a slightly yellowish-tan color, possibly because of trace amounts of ketones inherent even in normal subjects. The results showed virtually no differences between buffers and urine samples with added acetoacetic acid.

To evaluate the reactivity of the assay devices with acetone, we added various amounts of acetone (see Table 1) to buffer solutions at pH 5, 7.0, and 9.0, and also to fresh urine samples (between pH 5 and 7) from normal subjects. The urine samples chosen in the study gave readings of 0 with Ketostix. The microcylinder devices were dipped in the test samples and the colors obtained were read after 1–5 min. The colors started to fade after 30 min. The results showed virtually no differences between buffers and urine samples supplemented with acetone.

The assay was also tested with 3-hydroxybutyrate at various concentrations in buffer solutions and added to urine samples similarly to the acetone experiments described above. No color was observed at hydroxybutyrate concentrations ≤100 mmol/L.

Table 1 shows the reactivity of the ketone bodies with the assay. The assay is completely nonreactive with 3-hydroxybutyrate and measures only acetoacetic acid when acetone is ≤15 mol/L. At acetone concentrations >15 mmol/L, the color produced is due mainly to acetoacetic acid, and only to a lesser extent to acetone. Therefore, if the acetone concentration in urine is >15 mmol/L, the resulting color should be interpreted as total ketone concentration (except 3-hydroxybutyrate). Figure 2 shows a color chart for measuring ketone concentrations. Seven discriminating colors are provided for matching and reading the results. At ketone concentrations ≤4.6 mmol/L, the color intensity and hue follow a stepwise gradation, but at 9.2 mmol/L, the color is fairly dark and violet.

Assay sensitivity: The sensitivity (detection limit) of the assays was evaluated by analyzing solutions of acetoacetate in buffers at pH 5, 7, and 9 at 0, 0.05, 0.1, 0.2, 0.25, 0.3, and 0.4 mmol/L in replicates of five. Colors were read at 1–5 min; changes in color at 5 min were considered positive. Acetoacetate concentrations ≤0.1 mmol/L had readings of 0. Positive readings were observed for all samples with acetoacetate concentrations.

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\begin{align*}
&\text{ACETOACETIC ACID (mM)} \\
&9.2 \quad 4.5 \quad 2.3 \quad 0.92 \quad 0.46 \quad 0.23 \quad 0 \\
&\text{ACETONE (mM)} \\
&90 \quad 50 \quad 30 \quad 20 \quad 15 \quad 0
\end{align*}
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**Fig. 2.** Color comparison chart for measurements of ketones in urine

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≥0.2 mmol/L. Because 0.2 mmol/L acetoacetate can be distinguished from 0, the observed sensitivity is ≤0.2 mmol/L. Similar experiments with acetone solutions indicate that the detection threshold for acetone is 15 mmol/L. That is, the method is 75-fold more sensitive to acetoacetic acid than to acetone on a molar basis.

**Correlation with Ketostix:** We compared Ketostix and the present method for measuring the urine ketone concentrations of normal and dieting subjects (Table 2). The results indicate a good agreement between the two methods except on the low side. We found that 20% (21 of 103 samples) of samples that gave 0 readings in the Ketostix method provided readings of 0.23 mmol/L in the present method, possibly indicating enhanced sensitivity of the present method over the Ketostix method.

**Correlation with blood acetone:** Figure 3 shows the comparison of average blood acetone concentrations with average urine ketone concentrations for 13 dieting subjects. The blood acetone and urine ketone concentrations were determined by head-space analysis (22) and with the microcylinder assay device (y), respectively. The correlation curve gave a linear regression of $y = 0.144x - 0.479$ ($r = 0.57$).

**Correlation with rate of fat catabolism:** The correlation between urine ketone concentrations (y) and the rate of fat loss (x) of dieting subjects (n = 14) is shown in Figure 4. The control subjects (n = 20) who showed 0–0.23 mmol/L readings in the urine ketone assay did not show any fat loss and are not included in Figure 4. The correlation curve gave a linear regression of $y = 0.158x - 0.706$ ($r = 0.54$). The urine ketone concentrations were determined with the microcylinder assay devices, and daily fat losses (g/day) were determined with a Bioelectrical Impedance Analyzer (20).

**Discussion**

The present assay device is well correlated to the Ames Ketostix method for measuring the concentrations of ketones (acetoacetic acid and acetone) in urine samples from dieting and nondieting subjects. Despite the equivalency of the two methods, the major limitation of the Ketostix assay is the time it takes to read the color. For accurate results, the Ketostix color must be read in exactly 15 s; moreover, the strips must be dipped into urine and removed immediately to avoid leaching of the reagents from the strips into the sample. In the present assay device, the reagents that produce color are chemically bonded to the solid phase and cannot leach into the sample, whereas with Ketostix and other available methods the reagents are physically adsorbed onto the solid phase, not bonded. Thus, the color on the present device may be read ≤1 h after assay.

The present assay device also offers a better discrimination range, which allows better quantification in visual reading. The Ketostix color chart provides 50 (0.46), 150 (2.3), 400 (3.6), 800 (7.2), and 1600 mg/L (14.4 mmol/L) for ketone concentrations, whereas the color chart of the present assay device is calibrated for 0.23, 0.46, 0.92, 2.3, 4.6, and 9.2 mmol/L ketone concentrations. This assay range is more than adequate to measure the ketone concentrations of subjects dieting moderately, as shown in this study, for subjects who are on highly ketogenic diets (20), and for a diabetic population (23).

The present assay device is also solely reactive toward acetoacetic acid at an acetone concentration ≤15 mmol/L. At an acetone concentration of >15 mmol/L the assay device reacts primarily with acetoacetic acid, and to a lesser extent with acetone (Table 1). In diabetic patients with ketocidosis, acetone and acetoacetate concentrations in plasma range between 1.55 and 8.91 mmol/L and 1.16 and 6.08 mmol/L, respectively (23). Thus, in the majority of diabetic patients, the readings obtained by the present assay device are due solely to

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**Table 2. Comparison of Ketostix and Present Assay Results for Urine Samples from Normal and Dieting Subjects**

<table>
<thead>
<tr>
<th>Ketostix reading</th>
<th>Present method, mmol/L</th>
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<tr>
<td>mg/L</td>
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<tr>
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<td>0</td>
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<tr>
<td>800</td>
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Fig. 3. Comparison of urine ketone concentrations with blood acetone of dieting subjects

Fig. 4. Correlation of urine ketone concentrations with the rate of fat loss
acetoacetic acid. This assay is highly sensitive and can measure an acetoacetate concentration of at least 0.2 mmol/L. In addition, 20% of the urine samples that gave readings of 0 with Ketostix had measurable ketone concentrations with the present device.

The results from the clinical study for normal, healthy subjects who were on a negative calorie balance indicate that ketone concentrations in urine correlate poorly with acetone concentrations in blood. An earlier study (10) also demonstrated that urine ketones are not reliable markers because some individuals do not excrete ketone bodies in urine despite increased blood acetone concentrations. On the other hand, acetone concentrations in blood correlated very well with breath acetone concentrations in normal, dieting, and diabetic subjects (2, 11, 12, 20).

Finally, concentrations of ketones in urine have been shown to be a poor indicator for monitoring the rate of fat catabolism in subjects who are on a negative calorie balance. In measuring the rate of fat catabolism in weight-loss programs, breath or blood acetone provides more accurate predictions (20). However, subjects who are on a ketogenic diet (high protein and very low or no carbohydrate) produce excessive amounts of blood ketones, commonly causing subjects to excrete ketones in urine. Despite a plateau in blood ketone concentrations after a few days on a diet program, ketone concentrations in urine fluctuate daily, which makes the prediction of the fat loss rate from this measurement very difficult. In type 1 (insulin-dependent) diabetic patients, urine ketone measurements do not provide as accurate results as do blood ketones, although measurement of urine ketone concentrations is used with highly ketogenic or poorly controlled diabetic patients (2). The present assay device has not yet been tested for possible interfering substances such as drugs containing free sulphydryl groups.

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