Kinetic Measurement of the Combined Concentrations of Acetoacetate and β-Hydroxybutyrate in Serum

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This is an automated method for the kinetic measurement of the combined concentrations of acetoacetate and β-hydroxybutyrate in a single channel of the "Multistat III" centrifugal analyzer. Acetoacetate is first reduced with high concentrations of NADH by catalysis with 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30). This reaction mixture is diluted with excess NAD⁺. The endogenous β-hydroxybutyrate and that resulting from acetoacetate are then measured kinetically. Comparing the combined concentration of acetoacetate and β-hydroxybutyrate (y) with the sum of acetoacetate and β-hydroxybutyrate measured as described by Hansen and Freier (Clin Chem 1978;24:475) (x) yielded the relationship: 

\[ y = 0.99x - 0.57 \quad (r = 0.93, \quad n = 25) \]

The run-to-run CVs for low (5 mmol/L) and high (15 mmol/L) acetoacetate controls were 12% and 6%, respectively. The method is useful for determining the concentration of ketone bodies in 2-µL samples of serum of patients with diabetic ketoacidosis. The sensitivity can be increased to determine ketone body concentration in nonketotic individuals by increasing sample volume to 10 µL.

Additional Keyphrases: centrifugal analyzer  •  ketosis  •  monitoring diabetes  •  ketone bodies

Ketosis, a metabolic derangement of carbohydrate metabolism, is frequently encountered in patients with diabetic ketoacidosis. In initial diagnosis of this condition the "Ketostix" reagent strip or the "Acetest" tablets are used, both of which are based on the nitroprusside test. A drawback of this approach is that it detects only acetoacetate (AcAc) and acetone—and then only semiquantitatively—but is insensitive to β-hydroxybutyrate (BOH).† Thus, a negative nitroprusside test might be misleading and does not necessarily preclude ketoacidosis or poor diabetic control (1, 2). The limited value of the nitroprusside test has prompted the development of the quantitative measurements of AcAc and BOH, the major ketone-body components. Automated as well as nonautomated methods based on spectrophotometric or fluorometric measurements have been described (3–12); however, these methods are based on the separate measurement of AcAc and BOH and have not been widely applied as routine tests.

Here we describe an automated method for kinetically measuring the combined concentrations of AcAc and BOH in patients with diabetic ketoacidosis and an elevated anion gap, by using a single channel in a centrifugal analyzer. Hereinafter, we refer to the combined concentration of AcAc and BOH as "total ketone bodies" (TKB).

Materials and Methods

Materials

Reagents: Lyophilized 3-hydroxybutyrate dehydrogenase (HBDH) from Rhodopseudomonas spheroides; β-NADH (grade III); β-NAD⁺ (grade III); oxamic acid, sodium salt; lyophilized salt-free l-lactate dehydrogenase (LDH) from rabbit muscle; l-(−)-lactic acid (grade L-1, crystalline); pyruvic acid, sodium salt; acetoacetic acid, lithium salt; and n-β-hydroxybutyric acid, sodium salt, were all from Sigma Chemical Co., St. Louis, MO 63178.

Specimens: We used sera from patients with diabetic ketoacidosis. Specimens submitted to our laboratory for the emergency determination of ketone bodies by use of Acetest tablets and for determination of electrolytes were aliquoted and stored at −80°C until assay. Also, sera from blood donors, submitted to our laboratory for alanine aminotransferase determination, were aliquoted and stored at −80°C until use. We did the TKB assay of all specimens within three weeks.

Reagent Preparation

Standards: Stock 200 mmol/L solutions of AcAc (2.16 g/100 mL) and n-BOH (2.52 g/100 mL) were prepared in de-ionized water. n-BOH concentration is assumed to be one-half of n-BOH. l-BOH is neither a substrate nor an inhibitor of HBDH (13, 14). Standards (0, 2.5, 5, 10, and 20 mmol of l-BOH per liter for 2-µL samples and 0, 0.5, 1, and 2 mmol of l-BOH per liter for 10-µL samples) were prepared by making the appropriate dilutions of stock solutions in de-ionized water. All stock solutions and standards were aliquoted, stored at −20°C, and used within six months. Stored frozen, aqueous solutions of AcAc and BOH are stable for at least six months (6).

Controls: We prepared 5 and 15 mmol/L AcAc controls by adding, respectively, 0.5 and 1.5 mL from the AcAc stock solution to 18.5 mL of pooled serum and adjusting the total volume to 20 mL for the 5 mmol/L control with de-ionized water. The controls, stored in aliquots at −80°C, were used within a month. We saw no significant decrease in serum AcAc stored at −80°C for up to 21 days (7).

Tris HCl buffer, pH 9.5: Dissolve 60.5 g of tri(hydroxymethyl)methylamine in 800 mL of de-ionized water, adjust the pH to 9.5 with 0.1 mol/L HCl, and dilute to 1 L with de-ionized water.

Phosphate buffer, pH 6.98: Mix 60 mL of 0.2 mol/L (28.4 g/L) Na₂HPO₄ with 40 mL of 0.2 mol/L (27.2 g/L) KH₂PO₄.

HBDH: Dissolve 25 U of lyophilized HBDH in 1250 µL of de-ionized water. Aliquot and store at −20°C until use. This is stable for at least two weeks.

NADH: Dissolve the contents of a 50-mg (97% pure) vial in 1710 µL of de-ionized water. This gives a final concentration of 40 mmol/L. Aliquotted and stored at −20°C, this is stable for at least 10 days.

NAD⁺: Dissolve 20 mg, purchased in a pre-weighed vial, in 1850 µL of de-ionized water. The final concentration of this solution is 16.2 mmol/L. Aliquot and store at −20°C.

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Nonstandard abbreviations: AcAc, acetoacetate; BOH, β-hydroxybutyrate; HBDH, 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30); LDH, lactate dehydrogenase (EC 1.1.1.27); TKB, total ketone bodies.


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Reagent 1: Mix 22.5 \( \mu L \) of pH 6.98 phosphate buffer (200 mmol/L), 22.5 \( \mu L \) of NADH (40 mmol/L), and 155 \( \mu L \) of HBDH (20 kU/L) just before TKB determination. Two-hundred microliters of this reagent suffices for nine determinations. The reagent is stable for 48 h.

Reagent 2: Mix 420 \( \mu L \) of pH 9.50 Tris HCl buffer (500 mmol/L), 620 \( \mu L \) of NAD\(^+\) (16.2 mmol/L), and 560 \( \mu L \) of de-ionized water just before TKB determination. This suffices for nine determinations, and the reagent is stable for at least 6 h.

Method

Measurement of total concentration AcAc and BOH: The principle of this method is to reduce AcAc completely to BOH by using excess NADH and HBDH at optimal pH. Endogenous BOH and that produced from the reduction of AcAc are then measured kinetically during the oxidation of all BOH back to AcAc. The measured BOH thus represents the combined concentration of AcAc and BOH contained in the sample. We used the Multistat III centrifugal analyzer and its loader (MCA; Instrumentation Laboratory, Lexington, MA 02173), as follows:

Load sample (2 \( \mu L \)), water diluent (8 \( \mu L \)), and reagent 2 (20 \( \mu L \)) into the sample (inner) well of the MCA rotor. Load Reagent 1 (160 \( \mu L \)) and water diluent (10 \( \mu L \)) into the reagent-well cuvette of the MCA rotor. Transfer the rotor to the analyzer and pre-incubate (without centrifugation) for 10 min at 30 \(^\circ\)C, then start centrifugation to mix the sample-well components with those in the reagent well. Measure BOH by recording for 2 min the increase in absorbance at 340 nm after a delay of 10 s, using water as the reference blank. If 10-\( \mu L \) samples are used, add oxamic acid to both reagents to give a final concentration of 1.5 g/L without changing the total volume of each reagent.

We used the following setting for IL MCA analyzer: program, absorbance/time; delay time, 10 s; interval time, 30 s; number of intervals, 8; filter code, 1 (340 nm); start mode, 1 (pre-incubation); temp code, 1 (30 \(^{\circ}\)C).

Comparison Method

For comparison, we also measured the sum of AcAc and BOH separately as described by Hansen and Freier (6). We then compared the sum of these components with TKB concentration measured as described in sera from 25 patients with diabetic ketoacidosis.

Results and Discussion

Conversion of AcAc to BOH

The described method is based on the enzyme-catalyzed (HBDH) reversible reaction:

\[
\text{pH 7.0} \\
\text{AcAc} + \text{NADH} + \text{H}^+ \rightleftharpoons \text{BOH} + \text{NAD}^+ \\
\text{pH 9.5}
\]

The forward reaction is favored by excess NADH and low pH (pH 7.0) (12); the reverse reaction is favored by excess NAD\(^+\) and high pH (pH 9.5). Our approach is to convert AcAc into BOH and then measure the concentration of BOH obtained. Thus, the measured concentration of BOH represents the total concentration of BOH and AcAc (TKB) originally present in the sample. Reduction of AcAc rather than oxidation of BOH was chosen as the first step, because it is thermodynamically more favorable and is easily driven to completion (12). Moreover, a large excess of NAD\(^+\) over NADH can be used in the oxidation of BOH in the second stage of the assay, because NAD\(^+\) has little background absorbance at 340 nm.

General conditions for the reduction of AcAc and oxidation of BOH were based on those described by Hansen and Freier (6). For the reduction step that takes place in the inner well of the MCA rotor, we used high concentrations of NADH and HBDH, a small reaction volume (30 \( \mu L \)), and pre-incubation of the reaction for 10 min—the shortest pre-incubation needed to achieve complete reduction. Under these conditions, an average reduction of 101% (range, 87–106%) is obtained with 2.5–20.0 mmol of AcAc per liter. Theoretically, complete reduction could have been attained within a shorter period if a higher concentration of HBDH was used. However, we chose not to increase the enzyme concentration because preparation of a stock enzyme solution with higher concentration was not possible, owing to solubility problems with some enzyme batches.

The extent of AcAc reduction is assessed from the number of millimoles of NADH consumed. The relatively low reduction value (87%) is only observed at 2.5 mmol of AcAc per liter, and it could have been due to low signal/noise ratio, which is to be expected at an absorbance reading exceeding 1.0 A. At 2.5 mmol of AcAc per liter the NADH/BOH mole ratio is 15, and only 6.5% of NADH is consumed for its complete reduction. The concentration of NADH used in the reduction mixture was adequate for the complete reduction of 20.0 mmol of AcAc per liter. The molar ratio of NADH to AcAc when the concentration of AcAc is 20 mmol/L is 1.9.

In the reagent-well cuvette, where oxidation takes place, the concentration of NADH is diluted 10-fold (optical light path is 0.5 cm) and corresponds to a background absorbance reading of 1.2 A for a water blank. Conditions under which oxidation of BOH takes place are similar to those of Hansen and Freier (6) with the exception of a smaller total reaction volume and the 10-fold diluted components of the reduction reaction.

Analytical Variables

Assay linearity: When 2-\( \mu L \) samples containing different concentrations of AcAc were assayed by the method described, the rate of the reaction (mA/2 min) was linear up to 25 mmol/L (Figure 1). A similar rate response and range of linearity were obtained when equivalent concentrations of
BOH were used. The relationship between reaction rate \( y \) and concentration of AcAc and BOH (\( z \)) was: 
\[
y = 14.4x + 4.5 \\
(\text{r} = 0.999) \\
y = 14.2x + 6.6 \\
(\text{r} = 0.998),
\]
respectively. The similar slopes of the two lines support our previous conclusion that reduction of AcAc is complete during the pre-incubation period. It also indicates that either AcAc or BOH can be used as a standard curve, because the reaction rates are superimposable. Similarly, the rate of the reaction was linear up to at least 2.0 mmol/L when a 10-\( \mu \)L sample volume was used instead of 2 \( \mu \)L. The regression line was: 
\[
y = 52x - 4.2 \\
(\text{r} = 0.995).
\]

**Analytical recovery of AcAc and BOH:** When different concentrations of aqueous AcAc and BOH were mixed and assayed for TKB concentration, we could account for an average of 101% (range, 98–104%) (Table 1). This result further supports our conclusion that reduction of AcAc and oxidation of BOH are independent, with no interaction. That is, the presence of different concentrations of BOH does not interfere with the reduction step, nor do various concentrations of AcAc affect the oxidation step. Therefore, the various concentrations of NADH remaining after the reduction step do not affect the oxidation step.

**Precision and analytical recovery:** Within-run precision was determined by assaying 13 samples of two different concentrations of the prepared controls. Day-to-day precision was determined by assaying 10 duplicates with the same controls on 10 different days. For AcAc concentrations of 5.0 and 15.0 mmol/L the respective within-run CVs were 4% and 6%, the run-to-run CVs 12% and 9% (Table 2). We chose AcAc rather than BOH to serve as a control material because it undergoes both reactions: reduction in the inner well followed by oxidation in the outer well. We used 5.0 and 15.0 mmol of AcAc per liter and not lower concentrations as controls, because we designed our method to measure high concentrations of TKB such as those found in patients with diabetic ketoacidosis. Respective average recoveries of 96% and 94% were obtained for 5.0 and 15.0 mmol/L AcAc controls (Table 2) when assayed vs BOH standards.

**Reference interval.** In diabetic ketoacidosis, TKB concentration was reported to be more than 5 mmol/L by several investigators (7, 16, 17). Because our assay was designed to measure high TKB concentrations in patients with diabetic ketoacidosis, a larger sample volume (10 \( \mu \)L) was necessary to increase the sensitivity of the assay enough for determination of TKB concentrations in nonketoetic individuals. Also, oxamic acid was added to reagents 1 and 2 to inhibit lactate dehydrogenase activity (8). When TKB concentration in 30 healthy blood donors (13 men and 17 women, ages 20–55) was measured by the described method with 10-\( \mu \)L samples, an average value of 184 (SD 95, range, 78–503) mmol/L was obtained. Such a wide range might be expected, because there were no sampling restrictions concerning the time of the day (15) or the fasting state for blood donors in our hospital. Moreover, our result is consistent with values of 280 ± 220 mmol/L reported by Li et al. (5) and 400 ± 200 mmol/L reported by Siegel et al. (10). In the former report, TKB concentration represents the sum of separate measurement of AcAc and BOH; in the latter it represents the total concentration of AcAc, BOH, and acetone.

Lower normal values for the fasting subject—62 ± 31 mmol/L (6) or 74 ± 7.8 mmol/L (7)—as well as higher values (896 ± 58 mmol/L) (9) have also been reported. This wide variation among reported results could result from differences in methodology, standardization, fasting state, time of the day, and (or) sensitivity of the different assays.

**Effect of oxamic acid:** Addition of 10 mmol of lactate or 5 mmol of pyruvate per liter to the pooled serum with LDH activity of 152 kU/L and use of 2-\( \mu \)L samples of the pooled serum did not affect TKB measurement, nor did it change the initial absorbance reading when compared with that in the absence of lactate or pyruvate. Furthermore, the addition of oxamic acid to reagents 1 and 2 to give a final concentration of 1.5 g/L did not affect the measured concentration of TKB in pooled serum or in sera from blood donors, nor did it affect the standard curve significantly. We did not do detailed studies of lactate interference at high LDH activity. Oxamic and oxalic acids have been shown to effectively prevent this interference (8, 11). Addition of oxamic acid had no measurable effect on the performance of our assay system.

**Validation of Method**

Further to evaluate the described method, we measured TKB concentration in patients with diabetic ketoacidosis (\( y \)) and compared it with the sum of AcAc and BOH concentrations (\( z \)) determined separately (6). The means were 5.53 ± 2.49 mmol/L (\( y \)) (range, 2.21–12.15) and 5.84 ± 2.31 mmol/L (\( z \)) (range, 2.67–11.43). The regression equation was 
\[
y = 0.99x - 0.57 \\
(\text{r} = 0.93, n = 25).
\]

Table 3 shows the decrease in TKB concentration, as measured by both methods, after treatment was begun in a patient with diabetic ketoacidosis. This decrease was accompanied by a decrease in the electrolyte balance. Assuming a mean anion gap ([\( \text{Na}^+ \)] – [\( \text{Cl}^- \]) – [\( \text{HCO}_3^- \)]) of 12 mmol/L, TKB probably accounts for most of the anion gap in the illustrated patient.

In conclusion: We have shown that reduction of AcAc and oxidation of BOH can be carried out consecutively in the MCA. The method should be transferable to other instruments that have the capability of sequential two-reagent addition and kinetic measurement. The method was designed to measure TKB concentration in patients with diabetic ketoacidosis without diluting the sample. Often

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**Table 1. Analytical Recovery of TKB (AcAc and BOH) after Mixing Different Concentrations of AcAc with BOH**

<table>
<thead>
<tr>
<th>Concentration, mmol/L</th>
<th>AcAc</th>
<th>BOH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.5</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>10.0</td>
</tr>
</tbody>
</table>

**TKB**

<table>
<thead>
<tr>
<th>Expected</th>
<th>15.0</th>
<th>15.0</th>
<th>15.0</th>
<th>15.0</th>
<th>15.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observed</td>
<td>14.7</td>
<td>15.2</td>
<td>15.0</td>
<td>15.6</td>
<td>15.2</td>
</tr>
</tbody>
</table>

**Recovery, %**

| 98 | 101 | 100 | 104 | 101 |

**Table 2. Analytical Accuracy and Precision of TKB**

<table>
<thead>
<tr>
<th>Assay</th>
<th>Low control, 5 mmol/L AcAc</th>
<th>High control, 15 mmol/L AcAc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Within-run precision (n = 13 each)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>x, mmol/L</td>
<td>4.81</td>
<td>14.11</td>
</tr>
<tr>
<td>SD, mmol/L</td>
<td>0.27</td>
<td>0.60</td>
</tr>
<tr>
<td>CV, %</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Anal. recovery, %</td>
<td>96</td>
<td>94</td>
</tr>
<tr>
<td>range, %</td>
<td>84–112</td>
<td>87–105</td>
</tr>
</tbody>
</table>

| Day-to-day precision (n = 20 each) | | |
| x, mmol/L | 4.81 | 14.11 |
| SD, mmol/L | 0.57 | 0.98 |
| CV, % | 12 | 6 |
Table 3. Serum Ketone Bodies Measured in a Diabetic Patient Treated for Ketoadicosis

<table>
<thead>
<tr>
<th>Time, h</th>
<th>Glucose, mg/dL</th>
<th>BOH, mmol/L</th>
<th>AcAc, mmol/L</th>
<th>TKB, mmol/L</th>
<th>Anion gap</th>
<th>HCO₃⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>552</td>
<td>5.30</td>
<td>3.22</td>
<td>8.76</td>
<td>29</td>
<td>7.0</td>
</tr>
<tr>
<td>2.5</td>
<td>426</td>
<td>8.80</td>
<td>3.36</td>
<td>12.41</td>
<td>23</td>
<td>10.0</td>
</tr>
<tr>
<td>5.0</td>
<td>231</td>
<td>3.66</td>
<td>1.93</td>
<td>6.10</td>
<td>12</td>
<td>15.0</td>
</tr>
<tr>
<td>6.5</td>
<td>183</td>
<td>1.70</td>
<td>1.18</td>
<td>3.23</td>
<td>13</td>
<td>17.0</td>
</tr>
<tr>
<td>10.0</td>
<td>210</td>
<td>0.05</td>
<td>0.17</td>
<td>0.21</td>
<td>6</td>
<td>19.0</td>
</tr>
<tr>
<td>13.0</td>
<td>156</td>
<td>0.04</td>
<td>0.11</td>
<td>0.16</td>
<td>7</td>
<td>20.0</td>
</tr>
<tr>
<td>20.0</td>
<td>157</td>
<td>0.06</td>
<td>0.09</td>
<td>0.16</td>
<td>10</td>
<td>21.0</td>
</tr>
</tbody>
</table>

*Time from start of treatment with insulin. aAs determined by the method of Hansen and Freier (6). aAs determined by the described method.

Those patients have an anion gap exceeding 20 mmol/L caused by the accumulation of AcAc and BOH anions, and measurement of TKB could help determine whether the anion gap is entirely ascribable to the increased TKB or in part to the presence of other anions in the patient's serum. A feature of our TKB method is that it is not affected by changes in the BOH/AcAc ratio that occur during ketosis or during its treatment as a result of changes in NADH/NAD⁺ ratio. By increasing the sample size fivefold, we demonstrated the feasibility of measuring TKB concentrations in normal individuals with this method.

References