Head-Space Gas-Chromatographic Determination of 3-Hydroxybutyrate in Plasma after Enzymic Reactions, and the Relationship among the Three Ketone Bodies

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In this sensitive, reproducible method for determination of 3-hydroxybutyrate (3-OHB) in plasma, it is converted to acetone by use of 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30)/lactate dehydrogenase (EC 1.1.1.27) coupled with acetoacetate decarboxylase (EC 4.1.1.4). The resulting acetone is detected by head-space gas chromatography. The lowest concentration of 3-OHB detectable in plasma was 2 μmol/L. The calibration curve showed a linear relationship for 3-OHB concentration from 0 to 5 mmol/L (r = 0.989). Analytical recovery of 3-OHB (50 μmol/L) was 97.9 (SD 3.8)%.

Additional Keyphrases: acetoacetate · acetone · diabetes

Investigators have reported a wide range of values for the concentrations of acetoacetate and 3-hydroxybutyrate (3-OHB)1 in normal human plasma or blood (1–8), reflecting the difficulties of measuring the low concentrations of ketone bodies in plasma. On the other hand, acetone in plasma has seldom been determined (9, 10), having been thought to be negligible in amount and ambiguous in its role in ketosis. Sullivan and Malins (10), however, reported a case in which the concentration of acetone exceeded that of acetoacetate. Accurate determination of all three ketone bodies in various ranges of concentration would be useful for investigating the metabolism of carbohydrates and lipids.

Although sensitive detection of acetone is possible by head-space gas chromatography, the use of strong acid and heat during chemical conversion of acetoacetate and 3-OHB to acetone by the method of Eriksson (11) yields undesirable interfering byproducts (12). Here we describe an enzymic method for determining 3-OHB by head-space gas chromatography. We adapted our previously reported (12) method for determining acetoacetate and acetoacetate to the determination of all three ketone bodies. Further, we studied the relationships among the three ketone bodies as measured in normal and diabetic subjects.

Materials and Methods

Apparatus

Gas chromatography: We used the Hitachi gas chromatograph Model 073 (Hitachi Ltd., Tokyo, Japan), with a 2 m × 3 mm (i.d.) glass column packed with 10% polyethylene glycol 600 Chromosorb W AW 80/100 mesh (Gasukuro Kogyo Inc., Tokyo, Japan) and equipped with a flame ionization detector. A MS-GAN 100 syringe (Termo Co., Tokyo, Japan) was used for gas sampling.

"High-performance" liquid chromatography: A Jasco Tri Rotar pump was combined with a Uvidec 100-II detector (both from Japan Spectroscopic Co., Ltd., Tokyo, Japan) and a 30 cm × 6 mm (i.d.) Shodex Ionpak KC-811 column (Showa Denko Co., Ltd., Tokyo, Japan).

Reagents

Lithium acetoacetate and L-(+)-lactic acid (grade L-1, crystalline) were from Sigma Chemical Co., St. Louis, MO 63178. dl-3-OHB, pyruvate, acetone, methyl ethyl ketone, phosphoric acid, and other chemicals were analytical reagent-grade products of Wako Pure Chemical Industries Ltd., Osaka, Japan.

3-Hydroxybutyrate dehydrogenase (3-OHBDH; EC 1.1.1.30; grade II, from Rhodopseudomonas spheroides; 16.5 kU/L) was from Boehringer Mannheim GmbH, Penzberg, F.R.G. Lactate dehydrogenase (LDH; EC 1.1.1.27) from rabbit muscle and β-NAD+ (grade I) were from Oriental Yeast Co., Ltd., Osaka, Japan.

Acetoacetate decarboxylase (AADC, EC 4.1.1.4) was prepared from a cell-free extract of Bacillus polymyxa (A-57 strain),5 which had been selected by screening for AADC activity and cultured in maltose medium, as reported previously (12).

Phosphate solutions were kept at room temperature. Acetoacetate and lactate were stored at −20 °C. All other reagents were refrigerated. Enzyme reagents were prepared just before use.

First enzyme reagent: 3-OHB is oxidized to acetoacetate by adding 0.1 mL of the first enzyme reagent—which consists of 4.2 mL of 3-OHBDH (16.5 kU/L), 0.3 mL of LDH (1 × 105 U/L), 0.5 mL of β-NAD+ (20 mmol/L, pH 7.0), 1.0 mL of pyruvate (80 mmol/L, pH 7.0), and 4.0 mL of Na2HPO4 (0.4 mol/L, pH 13.7)—to 0.5 mL of deproteinized (see below) plasma sample. The pH of reagent plus sample is 9.

Second enzyme reagent: This reagent consists of one volume of crude AADC solution (20 kU/L) plus two volumes of phosphate buffer (1 mol/L, pH 3). Adding 0.2 mL of this reagent to the above mixture after the first reaction is terminated changes the pH of the reaction mixture to 5.8.
Sample Collection

We studied 31 normal subjects (ages 21 to 45 years) and 86 diabetic patients (ages 13–80 years) who were undergoing medical treatment in the Hyogo College of Medicine Hospital.

Blood samples (2 mL) collected 1.5 to 3 h after meals, were pipetted into heparinized tubes and centrifuged immediately to separate the plasma. We deproteinized the plasma by Somogyi's method (13)—i.e., by adding 1 mL of a 10 g/L solution of Ba(OH)₂·H₂O to 0.5 mL of plasma—then added to the sample 1 mL of 10 g/L ZnSO₄·7H₂O containing 4.03 mg of methyl ethyl ketone per liter as the internal standard.

Methods

Principle of the 3-OHB determination: Conversion of 3-OHB to acetacetate by 3-OHBDDH/β-NAD⁺ is completed by coupling the reaction with the oxidation of β-NADH + H⁺ by pyruvate/LDH.

\[
\begin{align*}
&\text{(I)} \\
&\text{3-OHB} \rightarrow \text{3-OHBDDH} \rightarrow \text{acetacetate} \rightarrow \text{AADC} \rightarrow \text{acetone} \\
&\beta-\text{NAD} \rightarrow \beta-\text{NADH} + \text{H⁺} \\
&\text{lactate} \rightarrow \text{LDH} \rightarrow \text{pyruvate} \\
\end{align*}
\]

β-NADH produced in the first reaction (I) is consumed in the reduction of pyruvate to lactate in the second reaction (II). The resulting β-NAD⁺ is used again to oxidize 3-OHB. The acetacetate produced is converted to acetone by AADC, and the acetone is detected by head-space gas chromatography.

Assay of enzymic activity: We determined the activity of 3-OHBDDH/LDH in the multienzyme system, and the amount of acetacetate in the time course experiment, by liquid chromatography. The eluent, 20 mmol/L phosphoric acid solution (pH 2.3), was filtered through a 0.22-μm (av. pore-size) GS filter (Millipore Corp., Bedford, MA 01730) before use. At appropriate intervals, we deproteinized 0.5 mL of the reaction mixture with 50 μL of 6 mol/L perchloric acid, centrifuged (2800 × g, 10 min), and injected 100 μL of the supernate onto the chromatographic column. Peaks for 3-OHB, acetacetate, pyruvate, and lactate were clearly resolved. We calculated concentrations of 3-OHB, acetacetate, and lactate by comparing the peak heights with those for the standards.

To examine the inhibitory effect of pyruvate on AADC, we used head-space gas chromatography or noted the decrease of absorbance at 210 nm caused by decarboxylation of acetacetate at room temperature.

Determination of ketone bodies: Except for the incubations, carry out all procedures with the samples in an ice bath. To determine 3-OHB, seal 0.5 mL of deproteinized plasma and 0.1 mL of the first enzyme reagent in a 10-mL vial with a rubber and aluminum seal, and incubate for 30 min at 37 °C. Then cool the mixture, add to it 0.2 mL of the second enzyme reagent, reseal the vial, and incubate for 30 min at 50 °C. Inject 1 mL of head-space gas into the gas chromatograph for acetone assay. Use nitrogen carrier gas at a flow rate of 30 mL/min, with injection and detector temperatures at 140 °C and oven temperature 77 °C.

To measure acetone in plasma, seal 0.5 mL of deproteinized plasma alone in a vial without enzymic treatment and incubate for 15 min at 50 °C. Add the second enzyme reagent and proceed as above, measuring acetone plus acetacetate as acetone. [We have described this principle previously (12).]

Determine the concentration of acetone from the calibration curve of the acetone concentration and the peak height ratio of acetone to the internal standard. Concentrations of acetone, acetacetate, and 3-OHB in plasma were calculated by comparing the three acetone concentrations with and without enzyme treatments.

Results and Discussion

Determination of 3-OHB

Conversion of 3-OHB to acetacetate: In the reaction mixture containing the multienzyme system of 3-OHBDDH/LDH, 3-OHB was almost completely converted to acetacetate and the number of moles of lactate produced was equal to the number of moles of acetacetate converted from 3-OHB. Accordingly, we measured lactate rather than acetacetate, which was spontaneously decarboxylated during the incubation. The enzyme activity of the system was optimized at pH 9 (14). On the other hand, when the LDH/pyruvate was not present, conversion of 3-OHB to acetacetate depended only on 3-OHBDDH and β-NAD⁺, so that even though the molar concentration of β-NAD⁺ was 2.5-fold that of 3-OHB, production of acetacetate was about half that of 3-OHB. Studies of the time course of formation of lactate, and therefore of acetacetate, showed that incubation for 30 min sufficed to convert 5 mmol of 3-OHB per liter to acetacetate.

Conversion of acetacetate to acetone: Pyruvate has a competitive inhibitory effect on AADC (15). Using head-space gas chromatography, we determined the inhibition constant (Kᵢ) of pyruvate to AADC to be 3 mmol/L at 50 °C. Although the pyruvate concentration was necessarily higher than the 3-OHB concentration, we thought that the excess pyruvate might inhibit AADC activity. 3-OHB at 5 mmol/L in a plasma sample corresponds to 625 μmol/L in the reaction mixture. When we added pyruvate (1.00 mmol/L) to the mixture, we found an apparent Kᵢ value for AADC at 26 °C of 1 mmol/L. The time course of decarboxylation of acetacetate by AADC in the presence of pyruvate was completed in 20 min at 50 °C, as determined by liquid chromatography (data not shown). We therefore chose an incubation time of 30 min for this process. Methyl ethyl ketone (2-butanone) in a high concentration also inhibits AADC activity, by 15% at 143 mmol/L, but at a low concentration such as we use for the internal standard, 7.0 μmol/L.

Calibration curve: The concentration of 3-OHB and the peak-height ratio (3-OHB/internal standard) were linearly related from 0 to 5 mmol of 3-OHB per liter (r = 0.999). If the concentration of 3-OHB in plasma exceeds 5 mmol/L, dilute the sample.

Sensitivity, precision, specificity, and column durability: The lowest concentration of 3-OHB detectable in plasma, defined as a signal-to-noise ratio of 3, was 2 μmol/L. The analytical recovery and precision of 3-OHB assay are shown in Table 1. Day-to-day precision (CV) of measuring 100 μmol of 3-OHB per liter (n = 19) was 3.7%. The specific and complete enzymic conversion of 3-OHB to acetacetate and

| Table 1. Analytical Recovery and Precision of 3-OHB Assay |
|----------------|----------------|
| 3-OHB added, μmol/L | Mean recovery, % | CV, % |
| 10.0            | 104.1          | 6.1  |
| 50.0            | 97.9           | 3.9  |
| 200.0           | 100.9          | 2.6  |
| n = 20 each.    |                |      |

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then to acetone was verified by time-course and recovery experiments. No peaks interfered with the acetone peak on the chromatogram. The column packing was stable for at least six months.

Determination of the Three Ketone Bodies

Figure 1 shows gas chromatograms we used for calculating the three ketone bodies in the plasma of a diabetic patient. In the normal subjects \((n = 31)\), the mean (and SD) concentrations of acetone, acetoacetate, and 3-OHB were 7.0 (3.0), 21.6 (9.8), and 25.3 (13.2) \(\mu\text{mol/L}\), respectively, and total ketone body was 53.8 (22.2), range, 25 to 124 \(\mu\text{mol/L}\). In the 86 diabetic patients, the mean (and SD) concentrations of the individual ketone bodies varied widely: 17.6 (35.7) \(\mu\text{mol/L}\) for acetone, 50.1 (77.5) \(\mu\text{mol/L}\) for acetoacetate, 94.0 (242.9) \(\mu\text{mol/L}\) for 3-OHB, and 161.6 (362.9) \(\mu\text{mol}\) of total ketone body per liter, with a range of 25 to 2240.

The mean (and SD) ratios of acetone to acetoacetate were 0.36 (0.18) for 31 normal persons and 0.32 (0.15) for 86 diabetic. These values were not significantly different \((p > 0.2, \text{Student's t-test})\). The mean (and SD) 3-OHB/acetoacetate ratios were 1.20 (0.44) for the 31 normal persons and 1.21 (0.70) for the 86 diabetic. Although these mean values are so similar, the 3-OHB/acetoacetate ratios in diabetics increased concomitantly with the increase of total ketone body concentrations. The coefficient of correlation \((r)\) between the 3-OHB/acetoacetate ratio and total ketone body concentration in diabetics was 0.674. However, the correlation between the 3-OHB/acetoacetate ratio and the logarithm of total ketone body concentration was better in diabetics \((r = 0.826)\), but not in normal subjects \((r = 0.326)\) (Figure 2). Changes in ketone body concentrations and 3-OHB/acetoacetate ratios in plasma may reflect the rate of production of ketone bodies and the free \(\beta\)-NADH/\(\beta\)-NAD\(^+\) ratio in liver mitochondria, as well as the metabolism of acetoacetate in peripheral tissues (16).

Because this method is sensitive, reproducible, and specific, it might be applicable to determining the three ketone bodies in low concentration (even less than 100 \(\mu\text{mol/L}\)) in other biological fluids and tissues.

References