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The Storage Stability of 3-Hydroxybutyrate in Serum, Plasma, and Whole Blood

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The storage stability of 3-hydroxybutyrate in whole blood, serum, and plasma was evaluated. The levels of 3-hydroxybutyrate were measured using an enzymatic rate reaction of the specific 3-hydroxybutyrate dehydrogenase with the NAD-NADH coupled reaction. 3-Hydroxybutyrate was found to be a stable analyte in whole blood, plasma, and serum. The long-term stability of β-hydroxybutyrate allows ample time for separation of blood components and offers storage of samples to meet quality control needs as well as the possibility of mailing specimens. Further studies indicate that NaF plasma, heparin plasma, and serum are the preferred specimens, because both EDTA and oxalate showed the most significant interference with the determination of 3-hydroxybutyrate. (Key words: Storage stability; 3-hydroxybutyrate) Am J Clin Pathol 1983; 80: 375–380

3-HYDROXYBUTYRATE is the major ketoacid produced by the incomplete oxidation of fatty acids by the liver in insulin-deprived conditions, such as diabetic ketoacidosis, alcoholic ketoacidosis, and starvation ketosis.

Acetoacetate, the companion metabolite of 3-hydroxybutyrate, is an unstable analyte because it undergoes nonenzymatic decarboxylation to acetone. Because of its volatile nature, acetone is lost readily from an aqueous medium. The instability of acetoacetate and acetone has necessitated refrigeration or freezing, if there is a delay in the assay, in order to preserve the specimens.

Recently, 3-hydroxybutyrate dehydrogenase[9] became available commercially, making the assay of 3-hydroxybutyrate technically practical for a clinical laboratory service. Until now, the only available methods were qualitative or semiquantitative estimates of acetoacetate and acetone. The traditional Rothera methods measured acetoacetate and acetone, while the commonly used test tablets or Ketostix® (Ames Co., Elkhart, IN) measured acetoacetate alone.[3] Neither of these two methods measure 3-hydroxybutyrate. Thus, acetoacetate and acetone were the analytes measured in most clinical laboratories.

Apparently, in view of the experience with acetoacetate, investigators utilizing 3-hydroxybutyrate assays have utilized stringent specimen-handling requirements such as collection in NaF-containing polyethylene tubes and separation of the plasma within 20 minutes after collection.[4] Because it seemed the current technology should enable one to make common use of 3-hydroxybutyrate, especially since 3-hydroxybutyrate frequently is three to five times[7] the concentration of acetoacetate and likely could replace that analyte, the storage stability of 3-hydroxybutyrate in whole blood, serum, and plasma was evaluated utilizing a previously recommended protocol.[6]

Materials and Methods

Specimens

Blood was drawn from healthy laboratory personnel into 7-mL Vacutainer® (Becton, Dickinson & Co.) tubes with fluoride/oxalate anticoagulant and 7-mL Vacutainer tubes with no anticoagulant. Both samples were spiked with 10 μL 1.4 M 3-hydroxybutyrate. After mixing by inversion five to ten times, the blood was transferred into Becton-Dickinson Microtainer tubes with serum separator and stored as a whole blood sample at either 4°C (3°C–5°C) or 24°C (22°C–26°C) room temperature. Immediately before assay, the tubes were centrifuged for 90 seconds in a Beckman Microfuge B®. Analyses were done in duplicate at 30 minutes; two, six, 24, 48 hours, and at seven days. Twenty-five patient samples collected in SST tubes (Becton-Dickinson serum separator) received for acetoacetate determination by Ketostix, were assayed for 3-hydroxybutyrate, then stored in the stoppered SST tube at 4°C (3°C–5°C) for five days or more and then reassayed. Anticoagulant interference studies were performed with quality-control serum, spiked with 3-hydroxybutyrate to levels of approximately 0.8 mmol/L and 2.4 mmol/L in volumes in 1.0 mL, 2.0 mL, and 5.0 mL to a 7-mL tube with anticoagulant present in constant amounts. The 3.5-mL volume would correspond to a 7-

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Table 1. Settings for the Centrifichem 400 Pipettor and Analyzer

Sample size	20 μL
Sample and diluent	70 μL
Reagent volume	350
Analyzer run temperature	37°C
Wavelength	340 nm
Standard concentration	2 mmol/L
T_0, time delay	10 sec
ΔT, time interval	30 sec
Abnormal absorbance	0.25 A
Blank	Auto
Test mode	Term
Print out	Conc.
Number of prints calibrate	1
Test code	00

mL fill volume with a hematocrit of 50%. The tubes were mixed on a rocker for two hours before analysis in duplicate. Forty specimens selected for the normal range verification study had normal glucose values (normal range = 70–115 mg/dL) and negative acetoacetate levels as measured by Ketostix.

Procedure

3-Hydroxybutyrate was measured by enzymatic assay[1,2,4,5,9] using the Centrifichem 400 Pipettor® and Analyzer (Baker Instruments Corporation). The enzyme catalyzed the reaction, 3-D-Hydroxybutyrate + β-NAD ⇌ acetoacetate + β-NADH. The production of β-NADH was followed at 340 nm. Table 1 shows the settings used for the Centrifichem System.

Reagents

3-Hydroxybutyrate dehydrogenase (EC 1.1.1.30) and D, L-3-hydroxybutyrate were purchased from Boehringer Mannheim Corp., Indianapolis, Indiana, 46250 (cat. no. 127814 and 106569). Tris (hydroxymethyl) methylamine and β-NAD were purchased from Sigma Chemical Co. St. Louis, Missouri 63178.

A working reagent was prepared by mixing 11.76 mL 0.1 M tris, (*p*H 9.5), 0.238 mL of 117 mM NAD, and 0.238 mL of enzyme solution (3 kU/g, 25 mg/5 mL). A blank reagent was prepared by substituting water for the enzyme solution.

The working standard was 2.0 mmol/L-3-hydroxybutyrate. Ortho Normal (Ortho Diagnostics, Raritan, NJ 08869) was used as a run control.

Lactic acid interference was monitored by subtracting concentrations of 3-hydroxybutyrate determined without enzyme using the blank reagent.

Ketostix were used as described in the Ames enclosure.

Table 2. Storage Stability for 3-Hydroxybutyrate at Room Temperature (22°C–26°C) for Whole Blood and Serum Collected with no Anticoagulant (Unit = mmol/L)

Storage Time Before Analysis	<1 hr	2 hr	6 hr	24 hr	48 hr	7 days
Clotted whole blood						
1	2.96	3.00	3.03	2.98	2.76	2.50
2	2.58	2.57	2.64	2.59	2.50	2.29
3	2.47	2.44	2.52	2.46	2.28	2.19
4	2.38	2.35	2.48	2.39	2.26	2.11
5	2.60	2.54	2.61	2.62	2.65	*
Range of change from initial value		−0.06 to 0.04	0.01 to 0.10	−0.02 to 0.02	−0.20 to 0.05	−0.46 to −0.27
	$\bar{x}$ = 2.60					
Mean change from initial value		−0.02	0.06	0.01	−0.11	−0.32
Serum						
1	3.06	3.04	3.17	3.18	3.14	3.16
2	2.63	2.63	2.74	2.75	2.65	2.71
3	2.48	2.51	2.60	2.62	2.55	2.68
4	2.36	2.38	2.45	2.48	2.40	2.53
5	2.66	2.68	2.81	2.93	2.96	*
Range of change from initial value		−0.02 to 0.03	0.09 to 0.15	0.12 to 0.27	0.02 to 0.30	0.08 to 0.17
	$\bar{x}$ = 2.64					
Mean change from initial value		0.01	0.12	0.15	0.10	0.14

* Assay not performed

Table 3. Storage Stability for 3-Hydroxybutyrate at Room Temperature (22°C–26°C) for Whole Blood and Serum Collected with NaF/Oxalate. Unit = mmol/L

Storage Time Before Analysis	<1 hr	2 hr	6 hr	24 hr	48 hr	7 days
Anticoagulated whole blood						
1	2.44	2.46	2.45	2.38	2.23	2.08
2	2.27	2.30	2.33	2.26	2.13	2.01
3	2.28	2.25	2.30	2.25	2.13	2.00
4	2.34	2.33	2.37	2.34	2.23	2.12
5	2.25	2.15	2.25	2.21	2.20	*
Range of change		−0.10 to +0.03	0.03 to 0.06	−0.06 to 0.0	−0.21 to 0.05	−0.36 to −0.22
	$\bar{x}$ = 2.32					
Mean change from initial value		−0.02	+0.02	−0.03	−0.13	−0.28
Plasma						
1	2.45	2.43	2.54	2.51	2.46	2.45
2	2.35	2.30	2.42	2.44	2.32	2.38
3	2.27	2.28	2.35	2.36	2.26	2.37
4	2.32	2.39	2.43	2.43	2.36	2.48
5	2.25	2.24	2.32	2.43	2.40	*
Range of change		−0.05 to +0.07	+0.07 to 0.11	0.06 to 0.18	−0.03 to +0.15	0. to +0.16
	$\bar{x}$ = 2.33					
Mean change from initial value		0.00	+0.08	+0.11	+0.03	+0.07

* Assay not performed.

Results

The normal serum 3-hydroxybutyrate level of <0.3 mmol/L[8] was verified using 40 hospital-derived specimens that were negative for acetoacetic acid by Ketostix. The glucose values for these specimens ranged from 66–108 mg/dL, the 3-hydroxybutyrate levels from 0.04–0.48 mmol/L with a mean of 0.14 ± 0.14 mmol/L (1 SD). The technical performance of the 3-hydroxybutyrate assay revealed linearity up to 2.5 mmol/L and showed a sensitivity of 0.04 mmol/L. The sensitivity was determined as the mean + 2 SD as determined from 20 de-ionized water specimens. The day-to-day precision was ±0.04 mmol/L (2 SD) at a concentration of 0.13 mmol/L 3-hydroxybutyrate and was 0.10 mmol/L (2 SD) at 2.58 mmol/L. The number of assays used to calculate the precision was 20 over a one-month period.

The storage stability of 3-hydroxybutyrate at room temperature is shown in Table 2 for clotted whole blood and serum (collected with no anticoagulant) and in Table 3 for anticoagulated whole blood and plasma (collected with NaF/oxalate). At room temperature, the clotted whole blood average change after 48 hours from the initial value was −0.11 mmol/L (−4.1%) with a range of change −0.20 mmol/L to +0.05 mmol/L. The anticoagulated whole blood changed after 48 hours to an average of −0.13 mmol/L (−5.6%) with a range of change from −0.21 mmol/L to −0.05 mmol/L. Again, at room temperature, the serum change after seven days averaged 0.14 mmol/L (+5.3%), with a range of change from 0.08 mmol/L to 0.17 mmol/L. The plasma change at room temperature after seven days averaged 0.07 mmol/L (3.0%) with a range of change from 0.00 mmol/L to 0.16 mmol/L.

The storage stability for 3-hydroxybutyrate at 4°C (3°C–5°C) is shown in Table 4 for clotted whole blood and serum and in Table 5 for anticoagulated whole blood and plasma. At 4°C (3°C–5°C), the clotted whole blood change after seven days averaged 0.03 mmol/L (2.3%), with a range of change from −0.06 mmol/L to 0.08 mmol/L. The NaF/oxalate anticoagulated whole blood change after seven days averaged −0.07 mmol/L (−3.0%) with a range of change from −0.16 mmol/L to 0.03 mmol/L. At 4°C (3°C–5°C), the serum change after seven days averaged 0.14 mmol/L (5.3%), with a range of change from −0.06 mmol/L to 0.08 mmol/L. The plasma change averaged 0.05 mmol/L, (2.1%) with a range of change from −0.03 mmol/L to 0.12 mmol/L.

In the course of these studies, it was noted that the specimens collected with NaF/oxalate from the same volunteers and stored under the same conditions as the specimens without anticoagulants seemed to be systematically lower in value. The initial <1 hour Naf/oxalate

Table 4. Storage Stability for 3-Hydroxybutyrate at 4°C (3°C–5°C) for Whole Blood and Serum Collected with no Anticoagulants. Unit = mmol/L

Storage Time Before Analysis	<1 hr	2 hr	6 hr	24 hr	48 hr	7 days
Clotted whole blood						
6	2.68	2.68	2.79	2.79	2.74	2.72
7	2.48	2.53	2.61	2.61	2.43	2.42
8	2.63	2.63	2.74	2.69	2.62	2.71
9	2.54	2.48	2.57	2.57	2.50	2.59
10	2.46	2.43	2.51	2.65	2.61	*
Range of change from initial value		−0.06 to 0.05	0.03 to 0.11	0.03 to 0.19	−0.05 to 0.06	−0.06 to −0.08
	$\bar{x}$ = 2.56					
Mean change from initial value		−0.01	+0.09	+0.10	+0.02	+0.03
Serum						
6	2.78	2.76	2.89	2.93	2.86	2.91
7	2.59	2.58	2.67	2.68	2.60	2.71
8	2.60	2.58	2.70	2.72	2.64	2.73
9	2.52	2.54	2.66	2.63	2.56	2.68
10	2.49	2.55	2.62	2.81	2.75	*
Range of change from initial value		−0.02 to 0.06	0.08 to 0.14	0.09 to 0.32	0.04 to 0.26	0.12 to 0.16
	$\bar{x}$ = 2.60					
Mean change from initial value		+0.01	+0.11	+0.16	+0.09	+0.14

* Assay not performed.

Table 5. Storage Stability for 3-Hydroxybutyrate at 4°C (3°C–5°C) for Whole Blood and Plasma Collected with NaF/oxalate. Unit = mmol/L

Storage Time Before Analysis	<1 hr	2 hr	6 hr	24 hr	48 hr	7 days
Anticoagulated whole blood						
6	2.36	2.36	2.51	2.67	2.22	2.21
7	2.36	2.42	2.42	2.37	2.26	2.20
8	2.39	2.38	2.43	2.41	2.29	2.42
9	2.27	2.24	2.29	2.25	2.17	2.26
10	2.24	2.22	2.10	2.22	2.21	*
Range of change from initial value		−0.03 to 0.06	−0.14 to 0.15	−0.02 to 0.31	−0.14 to 0.03	−0.16 to 0.03
	$\bar{x}$ = 2.32					
Mean change from initial value		0.00	+0.03	+0.06	−0.09	−0.07
Plasma						
6	2.43	2.45	2.53	2.53	2.45	2.47
7	2.41	2.39	2.50	2.49	2.39	2.38
8	2.42	2.41	2.45	2.47	2.40	2.49
9	2.27	2.26	2.32	2.35	2.22	2.39
10	2.23	2.24	2.36	2.37	2.31	*
Range of change from initial value		−0.02 to 0.02	0.03 to 0.13	0.05 to 0.14	0.05 to 0.08	−0.03 to 0.12
	$\bar{x}$ = 2.35					
Mean change from initial value		0.00	+0.08	+0.09	0.00	+0.05

* Assay not performed.

values were lower by approximately 0.3 mmol/L and prompted our investigation of the effects of anticoagulants on β-hydroxybutyrate determination. Table 6 presents the interference of different types of anticoagulants, including NaF/oxalate, oxalate, EDTA, heparin, and NaF. The greatest effect was seen with the EDTA anticoagulant, which showed a 37% decrease in comparison with the serum aliquot of the same specimen, going from 2.00 mmol/L to 1.26 mmol/L. The interference was approximately the same at the four concentrations of EDTA tried. Estimating 2.8 SD at the level of 2.2 mmol/L as 2.8 × 0.044 mmol/L or ± 0.12 mmol/L, the difference of 0.74 mmol/L is statistically significant. Serum, plasma collected with heparin, or plasma collected with NaF are the recommended specimens.

To eliminate the possibility of some unexpected behavior of clinical specimens, a variety of patient specimens (mostly abnormal) were checked for storage stability. Twenty-five different sera with a mean concentration of 2.09 mmol/L were stored at 4°C (3°C–5°C) for variable periods of time from five to 14 days. The overall mean change was 0.02 mmol/L, with a range of change of −0.2 mmol/L to 0.14 mmol/L. This stability for at least 14 days agrees with a literature report indicating stability at 4°C for up to 30 days.[4] Nine other sera with an initial mean of 4.33 mmol were stored at 23°C (22°C–24°C) for seven days. Aliquots of the sera were assayed at the following times during storage: zero days, one day, four days, and seven days. The respective overall mean changes were −0.07 mmol/L, 0.20 mmol/L, and −0.11 mmol/L. The respective range of changes were −0.50 mmol/L to 0.24 mmol/L, −0.20 mmol/L to 0.48 mmol/L, and −0.50 to 0.10 mmol/L.

Table 6. Interference Study Using NaF/K Oxalate, NaF, K Oxalate, EDTA and Heparin

Anticoagulant	Volume of Control* Added to tube (ml)	Level I (mmol/L)†	Level II (mmol/L)‡
NaF/K oxalate (31.5 mg)/tube	Control	0.80	2.36
	2.0	0.61	2.06
	1.0	0.57	1.94
NaF (17.5 mg)/tube	Control	0.72	2.22
	5.0	0.69	2.20
	2.0	0.66	2.16
	1.0	0.64	2.08
K oxalate (14 mg)/tube	Control	0.72	2.13
	5.0	0.63	1.82
	2.0	0.56	1.81
	1.0	0.53	1.74
EDTA (10.5 mg)/tube	Control	0.67	2.00
	5.0	0.44	1.26
	2.0	0.46	1.28
	1.0	0.44	1.27
	0.5	0.42	1.26
Heparin Li (143 IU)/tube	Control	0.72	2.13
	5.0	0.72	2.10
	2.0	0.72	2.09
	1.0	0.73	2.13

* Control represents final concentration of 3-hydroxybutyrate with no anticoagulants, after quality control serum had been spiked with 3-hydroxybutyrate to varying levels.

† Level I is measured concentration of 3-hydroxybutyrate (approximately 0.7 mmol/L) with varying concentrations of anticoagulants present.

‡ Level II is measured concentration of 3-hydroxybutyrate (approximately 2.2 mmol/L) with varying concentrations of anticoagulants present.

Discussion

This study was initiated because it was suspected that 3-hydroxybutyrate was a stable analyte in blood. The findings presented in this article verify this suspicion to a remarkable extent in whole blood, serum, and plasma. β-hydroxybutyrate is stable for a period of time compatible with all preanalytic storage circumstances, as well as prolonged storage requirements for quality control. The whole blood storage and serum storage stability at room temperature (22°C–26°C) for up to two days allows ample time for separation of serum or plasma. The serum or plasma stability at room temperature for at least up to seven days enables mailing of specimen. Stability at 4°C (3°C–5°C) in the refrigerator for at least 14 days provides storage needs for possible confirmation request investigations and meets the overall needs of an analytic quality control specimen. This striking storage stability also was associated with demonstrable analytic reliability based upon quality control experiences of recovery and day-to-day precision data.

Therefore, based on the stability findings reported in this paper, 3-hydroxybutyrate may emerge as the most important, or at least a very important, analyte for monitoring diabetic patients. Obviously the convenience of being able to do reliable 3-hydroxybutyrate assays should encourage greater use and clinical explorations to the net worth of this analyte in patient care. In addition, normal values as stated in the literature were verified to further substantiate the potential net worth and convenience of this analyte in clinical laboratories generally.

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