Megazyme

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D-3-HYDROXYBUTYRIC ACID (D-3-HYDROXYBUTYRATE)

ASSAY PROCEDURE

K-HDBA 04/18

(*60 Manual Assays per Kit) or
(740 Auto-Analyser Assays per Kit) or
(600 Microplate Assays per Kit)

** The number of tests per kit can be doubled if all volumes are halved*

INTRODUCTION:

In fertilised or incubated eggs, the level of D-3-hydroxybutyric acid rises from trace levels to > 5 mg/kg (dry weight) and can reach approximately 800 mg/kg in badly spoiled eggs. As D-3-hydroxybutyric acid producing processes only occur after approximately 6 days, the level of this acid is used to indicate how fresh eggs are, or how fresh eggs were, when they were employed in the manufacture of processed egg products. In clinical chemistry, the level of D-3-hydroxybutyric acid is determined in the diagnosis of diabetic ketoacidosis.

PRINCIPLE:

The determination of D-3-hydroxybutyric acid requires two enzyme reactions. In the first reaction catalysed by 3-hydroxybutyrate dehydrogenase (3-HBDH), D-3-hydroxybutyric acid is oxidised to acetoacetate and reduced nicotinamide-adenine dinucleotide (NADH) by NAD^+ (1).

$$\text{(1)}\quad \text{D-3-Hydroxybutyrate} + NAD^+ \xleftrightarrow{\text{(3-HBDH)}} \text{acetoacetate} + NADH + H^+$$

However, since this is an equilibrium reaction, a further reaction catalysed by diaphorase is required, in which NADH reduces iodonitrotetrazolium chloride (INT) to an INT-formazan product, leading to a rapid and quantitative conversion of D-3-hydroxybutyric acid (2).

$$\text{(2)}\quad \text{INT} + NADH + H^+ \xrightarrow{\text{(diaphorase)}} NAD^+ + \text{INT-formazan}$$

The amount of INT-formazan formed in this reaction is stoichiometric with the amount of D-3-hydroxybutyric acid. It is the INT-formazan which is measured by the increase in absorbance at 492 nm.

SPECIFICITY, SENSITIVITY, LINEARITY AND PRECISION:

The assay is specific for D-3-hydroxybutyric acid. L-Lactic acid, L-succinic acid and fumaric acid do not react.

The smallest differentiating absorbance for the assay is 0.005 absorbance units, this corresponds to 0.037 mg/L of sample solution at the maximum sample volume of 2.00 mL (or to 0.74 mg/L with a sample volume of 0.1 mL). The detection limit is 0.074 mg/L, which is derived from an absorbance difference of 0.010 and the maximum sample volume of 2.00 mL.

The assay is linear over the range of 0.4 to 12 µg of D-3-hydroxybutyric acid per assay. In duplicate determinations using one sample solution, an absorbance difference of 0.005 to 0.010 may occur. With a sample volume of 2.00 mL, this corresponds to a D-3-hydroxybutyric acid concentration of approx. 0.037 to 0.074 mg/L of sample solution. If the sample is diluted during sample preparation, the result is multiplied by the dilution factor, F. If, in sample preparation, the sample is weighed, e.g. 10 g/L, a difference of 0.02 to 0.05 g/100 g can be expected.

INTERFERENCE:

If the conversion of D-3-hydroxybutyric acid has been completed within the time specified in the assay (approx. 6 min), it can be generally concluded that no interference has occurred. However, this can be further checked by adding D-3-hydroxybutyric acid (approx. 6 µg in 0.1 mL) to the cuvette on completion of the reaction. A significant increase in the absorbance should be observed.

Interfering substances in the sample being analysed can be identified by including an internal standard. Quantitative recovery of this standard would be expected. Losses in sample handling and extraction are identified by performing recovery experiments, i.e. by adding D-3-hydroxybutyric acid to the sample in the initial extraction steps.

High concentrations of L-ascorbic acid, cysteine or sulphite interfere with the assay as they react with INT causing a non-enzymic "creep" rate. These compounds should be removed by treating the sample with H_2O_2 and alkali as follows:

Weigh or pipette sample, diluted if necessary, into a 50 mL volumetric flask. Add water to a volume of approx. 40 mL, then add 1 mL of 2 M KOH and 0.01 mL of H_2O_2 (30% v/v). Incubate the solution for 10 min at approx. 70°C. Cool to 20-25°C and adjust to pH 8.0 with 1 M H_2SO_4. Fill to the mark with distilled water, mix, filter and use the solution for the assay.

SAFETY:

The general safety measures that apply to all chemical substances should be adhered to.

For more information regarding the safe usage and handling of this product please refer to the associated SDS that is available from the Megazyme website.

KITS:

Kits suitable for performing 60 assays in manual format (or 740 assays in auto-analyser format or 600 assays in microplate format) are available from Megazyme. The kits contain the full assay method plus:

Bottle 1: Buffer (35 mL, pH 8.6) plus sodium azide (0.02% w/v).
Stable for > 2 years at 4°C.

Bottle 2: (x2) NAD^+ plus INT.
Stable for > 2 years at 4°C.

Bottle 3: Diaphorase suspension (1.25 mL).
Stable for > 2 years at 4°C.

Bottle 4: 3-Hydroxybutyrate dehydrogenase suspension (1.25 mL).
Stable for > 2 years at 4°C.

Bottle 5: D-3-Hydroxybutyric acid standard solution (5 mL, 0.06 mg/mL).
Stable for > 2 years at 4°C.

PREPARATION OF REAGENT SOLUTIONS/ SUSPENSIONS:

1. Use the contents of bottle 1 as supplied.
Stable for > 2 years at 4°C.

2. Dissolve the contents of one of bottle 2 in 6.5 mL of distilled water. Divide into appropriately sized aliquots and store in a dark container below -10°C between use and on ice during use.
Stable for > 12 months below -10°C.

3 & 4. Use the contents of bottles 3 and 4 as supplied. Before opening for the first time, shake the bottles to remove any protein that may have settled on the rubber stopper. Subsequently, store the bottles in an upright position.
Stable for > 2 years at 4°C.

5. Use the contents of bottle 5 as supplied.
Stable for > 2 years at 4°C.

NOTE: The D-3-hydroxybutyric acid standard solution is only assayed where there is some doubt about the accuracy of the spectrophotometer being used or where it is suspected that inhibition is being caused by substances in the sample. The concentration of D-3-hydroxybutyric acid is determined directly from the extinction coefficient of INT-formazan (page 6).

EQUIPMENT (RECOMMENDED):

1. Glass test tubes (round bottomed; 16 x 100 mm).
2. Disposable plastic cuvettes (1 cm light path, 3.0 mL).
3. Micro-pipettors, e.g. Gilson Pipetman® (20 µL, 100 µL and 200 µL).
4. Positive displacement pipettor, e.g. Eppendorf Multipette®
   - with 5.0 mL Combitip® (to dispense 0.2 mL aliquots of NAD^+/INT solution).
   - with 12.5 mL Combitip® [to dispense 0.5 mL aliquots of buffer (bottle 1)].
   - with 25 mL Combitip® (to dispense 2.0 mL aliquots of distilled water).
5. Analytical balance.
6. Spectrophotometer set at 492 nm.
7. Vortex mixer (e.g. IKA® Yellowline Test Tube Shaker TTS2).
8. Stop clock.
9. Whatman No. 1 (9 cm) filter papers.

A. MANUAL ASSAY PROCEDURE:

Wavelength: 492 nm
Cuvette: 1 cm light path (glass or plastic)
Temperature: ~ 25°C
Final volume: 2.84 mL
Sample solution: 0.4-12.0 µg of D-3-hydroxybutyric acid per cuvette (in 0.1-2.0 mL sample volume)

Read against air (without a cuvette in the light path) or against water

Pipette into cuvettes	Blank	Sample
distilled water (at ~ 25°C)	2.10 mL	2.00 mL
sample	-	0.10 mL
solution 1 (buffer mix)	0.50 mL	0.50 mL
solution 2 (NAD^+/INT)	0.20 mL	0.20 mL
suspension 3 (Diaphorase)	0.02 mL	0.02 mL
Mix* and read the absorbances of the solutions (A_1) after approx. 2 min. Repeat the measurement after another 2 min. If a change in absorbance greater than 0.010 is observed, the sample must be treated to remove reducing substances (see "interference" section on page 2). Start the reaction immediately by addition of:		
suspension 4 (3-HBDH)	0.02 mL	0.02 mL
Mix* and read the absorbances of the solutions (A_2) at the end of the reaction (approx. 6 min). If the reaction has not stopped after 6 min, continue to read the absorbances at 2 min intervals until the absorbances either plateau or increase constantly over 2 min**.		

* for example with a plastic spatula or by gentle inversion after sealing the cuvette with a cuvette cap or Parafilm®.

** if this "creep" rate is greater for the sample than that of the blank, extrapolate the absorbances (sample and blank) back to the time of the addition of suspension 4 (3-HBDH).

NOTE: INT and the reaction system containing INT are sensitive to light. Consequently, reactions must be performed in the dark (e.g. in the spectrophotometer cuvette compartment with the photometer lid closed).

CALCULATION:

Determine the absorbance difference (A_2-A_1) for both blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample, thereby obtaining $\Delta A_{\text{D-3-hydroxybutyric acid}}$. The value of $\Delta A_{\text{D-3-hydroxybutyric acid}}$ should as a rule be at least 0.100 absorbance units to achieve sufficiently accurate results.

The concentration of D-3-hydroxybutyric acid can be calculated as follows:

$$c = \frac{V \times MW}{\varepsilon \times d \times v} \times \Delta A_{\text{D-3-hydroxybutyric acid}} \quad [g/L]$$

where:

V = final volume [mL]
MW = molecular weight of D-3-hydroxybutyric acid [g/mol]
ε = extinction coefficient of INT-formazan at 492 nm
= 19900 [l x mol^{-1} x cm^{-1}]
d = light path [cm]
v = sample volume [mL]

It follows for D-3-hydroxybutyric acid:

$$c = \frac{2.84 \times 104.1}{19900 \times 1.0 \times 0.1} \times \Delta A_{\text{D-3-hydroxybutyric acid}} \quad [g/L]$$

$$= 0.1486 \times \Delta A_{\text{D-3-hydroxybutyric acid}} \quad [g/L]$$

If the sample has been diluted during preparation, the result must be multiplied by the dilution factor, F.

When analysing solid and semi-solid samples which are weighed out for sample preparation, the content (g/100 g) is calculated from the amount weighed as follows:

Content of D-3-hydroxybutyric acid

$$= \frac{c_{\text{D-3-hydroxybutyric acid}}\ [g/L\ \text{sample solution}]}{\text{weight}_{\text{sample}}\ [g/L\ \text{sample solution}]} \times 100 \quad [g/100\ g]$$

NOTE: These calculations can be simplified by using the Megazyme ***Mega-Calc***™, downloadable from where the product appears in the Megazyme web site (www.megazyme.com).

B. AUTO-ANALYSER ASSAY PROCEDURE:

NOTES:

1. The Auto-Analyser Assay Procedure for D-3-hydroxybutyric acid can be performed using either a single point standard or a full calibration curve.
2. For each batch of samples that is applied to the determination of D-3-hydroxybutyric acid **either a single point standard or a calibration curve must be performed concurrently using the same batch of reagents.**

Reagent preparation is performed as follows:

Preparation of R1:

Component	Volume
distilled water solution 1 (buffer) solution 2 (NAD^+/INT) suspension 3 (Diaphorase)	52.6 mL 15.5 mL 6.2 mL (after adding 6.5 mL of H_2O to bottle 2) 0.62 mL
Total volume	74.92 mL

Preparation of R2:

Component	Volume
distilled water solution 4 (3-HBDH)	8.75 mL 0.62 mL
Total volume	9.37 mL

EXAMPLE METHOD:

R1:	0.200 mL
Sample:	~ 0.01 mL
R2:	0.025 mL
Reaction time:	~ 6 min at 37°C
Wavelength:	492 nm
Prepared reagent stability:	> 2 days when refrigerated
Calculation:	endpoint
Reaction direction:	increase
Linearity:	up to 99 mg/L of D-3-hydroxybutyric acid using 0.01 mL sample volume

C. MICROPLATE ASSAY PROCEDURE:

NOTES:

1. The Microplate Assay Procedure for D-3-hydroxybutyric acid can be performed using either a single point standard or a full calibration curve.
2. For each batch of samples that is applied to the determination of D-3-hydroxybutyric acid **either a single point standard or a calibration curve must be performed concurrently using the same batch of reagents.**

Wavelength: 492 nm
Microplate: 96-well (e.g. clear flat-bottomed, glass or plastic)
Temperature: ~ 25°C
Final volume: 0.284 mL
Linearity: 0.1-1.2 µg of D-3-hydroxybutyric acid per well (in 0.01-0.20 mL sample volume)

Pipette into wells	Blank	Sample	Standard
distilled water	0.210 mL	0.200 mL	0.200 mL
sample solution	-	0.010 mL	-
standard solution	-	-	0.010 mL
solution 1 (buffer)	0.050 mL	0.050 mL	0.050 mL
solution 2 (NAD^+/INT)	0.020 mL	0.020 mL	0.020 mL
suspension 3 (Diaphorase)	0.002 mL	0.002 mL	0.002 mL
Mix*, read the absorbances of the solutions (A_1) after approx. 2 min and start the reactions by addition of:			
suspension 4 (3-HBDH)	0.002 mL	0.002 mL	0.002 mL
Mix* and read the absorbances of the solutions (A_2) at the end of the reaction (approx. 6 min). If the reaction has not stopped after 6 min, continue to read the absorbances at 2 min intervals until the absorbances increase constantly over 2 min**.			

* for example using microplate shaker, shake function on a microplate reader,or repeated aspiration (e.g. using a pipettor set at 50-100 µL volume).

** if this "creep" rate is greater for the sample than for the blank, extrapolate the sample absorbances back to the time of addition of suspension 4.

CALCULATION (Microplate Assay Procedure):

$$g/L = \frac{\Delta A_{sample}}{\Delta A_{standard}} \times g/L \text{ standard} \times F$$

If the sample is diluted during preparation, the result must be multiplied by the dilution factor, F.

SAMPLE PREPARATION:

1. Sample dilution.

The amount of D-3-hydroxybutyric acid present in the cuvette (i.e. in the 0.1 mL of sample being analysed) should range between 0.4 and 12 µg. The sample solution must therefore be diluted sufficiently to yield a D-3-hydroxybutyric acid concentration between 0.004 and 0.12 g/L.

Dilution Table

Estimated concentration of D-3-hydroxybutyric acid (g/L)	Dilution with water	Dilution factor (F)
< 0.12	No dilution required	1
0.12-1.2	1 + 9	10
1.2-12	1 + 99	100
> 12	1 + 999	1000

If the value of $\Delta A_{\text{D-3-hydroxybutyric acid}}$ is too low (e.g. < 0.100), weigh out more sample or dilute less strongly. Alternatively, the sample volume to be pipetted into the cuvette can be increased up to 2.00 mL, making sure that the sum of the sample and distilled water components in the reaction is 2.10 mL and using the new sample volume in the equation.

2. Sample clarification.

a. Solutions:

Carrez I solution. Dissolve 3.60 g of potassium hexacyanoferrate (II) $\{K_4[Fe(CN)_6].3H_2O\}$ (Sigma cat. no. P9387) in 100 mL of distilled water. Store at room temperature.

Carrez II solution. Dissolve 7.20 g of zinc sulphate ($ZnSO_4.7H_2O$) (Sigma cat. no. Z4750) in 100 mL of distilled water. Store at room temperature.

Sodium hydroxide (NaOH, 100 mM). Dissolve 4 g of NaOH in 1 L of distilled water. Store at room temperature.

b. Procedure:

Pipette the liquid sample into a 100 mL volumetric flask which contains approx. 60 mL of distilled water, or weigh sufficient quantity of the sample into a 100 mL volumetric flask and add 60 mL of distilled water. Carefully add 5 mL of Carrez I solution, 5 mL of Carrez II solution and 10 mL of NaOH solution (100 mM). Mix after each addition. Fill the volumetric flask to the mark, mix and filter.

3. General considerations.

(a) Liquid samples: clear, slightly coloured and approximately neutral, liquid samples can be used directly in the assay.

(b) Acidic samples: if an acidic sample is to be used undiluted (such as red wine or coloured fruit juice), the pH of the solution should be increased to approx. 8.6 using 2 M NaOH, and the solution incubated at room temperature for 30 min.

(c) Carbon dioxide: samples containing carbon dioxide should be degassed by increasing the pH to approx. 8.6 with 2 M NaOH and gentle stirring, or by stirring with a glass rod.

(d) Coloured samples: an additional sample blank, i.e. sample with no 3-HBDH, should be performed in the case of coloured samples.

(e) Strongly coloured samples: if used undiluted, strongly coloured samples should be treated by the addition of 1 g/100 mL of activated carbon. Stir for 2 min and then filter.

(f) Solid samples: homogenise or crush solid samples in distilled water and filter if necessary.

(g) Samples containing fat: extract such samples with hot water at a temperature above the melting point of the fat, e.g. in a 100 mL volumetric flask. Adjust to 20°C and fill the volumetric flask to the mark with distilled water. Store on ice or in a refrigerator for 15-30 min and then filter. Discard the first few mL of filtrate and use the clear supernatant (which may be slightly opalescent) for assay. Alternatively, clarify with Carrez reagents.

(h) Samples containing protein: deproteinise samples containing protein by adding an equal volume of ice-cold 1 M perchloric acid with mixing. Centrifuge at 1,500 *g* for 10 min and neutralise the supernatant with 1 M KOH. Alternatively, use Carrez reagents.

SAMPLE PREPARATION EXAMPLES:

(a) Determination of D-3-hydroxybutyric acid in whole liquid egg.

Accurately weigh approx. 5 g of homogenised whole egg into a 25 mL volumetric flask, add 15 mL of distilled water and 1 drop of *n*-octanol. Mix and incubate for 15 min in a boiling water bath (approx. 100°C). Cool to 20-25°C and carefully add 1 mL of Carrez I solution, 1 mL of Carrez II solution and 2 mL of NaOH solution (100 mM). Mix after each addition. Fill the volumetric flask to the mark, mix, filter and use the filtrate for the assay. *Typically, no dilution is required and a sample volume of 0.1-1.0 mL is satisfactory. With fertilised eggs, the volume is 0.1 mL, while with unfertilised eggs, the volume is 1.00 mL. Take the altered sample volume into account in the calculations.*

Recovery of D-3-hydroxybutyric acid can be determined by adding 1.0 mL of a solution of D-/L-3-hydroxybutyrate, monosodium salt (0.4 g/100 mL) (equivalent to 0.16 g of D-3-hydroxybutyric acid/ 100 mL) to the 5 g of egg solution in the 25 mL volumetric flask and proceeding with sample extraction as per the standard procedure.

(b) Determination of D-3-hydroxybutyric acid in egg products.

Carefully homogenise the egg component (whole egg, white or yolk) at 20-25°C. Weigh 5 g of the sample into a 25 mL volumetric flask and treat as described under "whole liquid egg".

(c) Determination of D-3-hydroxybutyric acid in whole egg powder.

Accurately weigh approx. 1 g of whole egg powder into a 25 mL volumetric flask, add 15 mL of distilled water and 1 drop of *n*-octanol. Mix and incubate for 15 min in a boiling water bath. Cool to 20-25°C, carefully add 1 mL of Carrez I solution, 1 mL of Carrez II solution and 2 mL of NaOH solution (100 mM). Mix after each addition. Fill the volumetric flask to the mark, mix, filter, and use the filtrate for the assay. *Typically, no dilution is required and a sample volume of 0.2 mL is satisfactory. Take the altered sample volume into account in the calculations.*

REFERENCE:

Kientsch-Engel, R. I. & Siess, E. A. (1990). D-(-)-3-Hydroxybutyrate and Acetoacetate. *"Methods of Enzymatic Analysis"* (Bergmeyer, H. U., ed.), 3rd ed., **Vol. VIII**, pp. 60-69, VCH Publishers (UK) Ltd., Cambridge, UK.

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