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A simple colorimetric method for the determination of serum alpha-hydroxybutyric dehydrogenase activity

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SYNOPSIS An accurate, simple colorimetric method for the determination of serum α-hydroxybutyric dehydrogenase activity is described. The method is suitable for routine laboratory use.

Determination of serum α-hydroxybutyric dehydrogenase (2-hydroxybutyrate : NAD or ireductase) activity is of considerable value in the diagnosis of myocardial infarction (Elliott and Wilkinson, 1961; Rosalki, unpublished observations; Konttinen, 1961; Pagliaro and Notarbartolo, 1961). The 'enzyme' reduces α-ketobutyric acid (2-oxobutyrate) in the presence of reduced nicotinamide-adenine dinucleotide ($NADH_2$), (formerly known as reduced diphosphopyridine nucleotide, DPNH) according to the equation:

α-ketobutyric acid + $NADH_2$ ⟶ α-hydroxybutyric acid + NAD

A spectrophotometric method is available for determining hydroxybutyric dehydrogenase (Rosalki and Wilkinson, 1960) but is unsuitable for some routine laboratories since it requires an ultra-violet spectrophotometer. An alternative simple, yet accurate, colorimetric procedure is described. This method is similar in principle to the colorimetric procedures available for serum lactic dehydrogenase determination (Berger and Broida, 1957; Cabaud and Wróblewski, 1958).

PRINCIPLE

α-Ketobutyric acid is reduced by incubation with serum in the presence of reduced nicotinamide-adenine dinucleotide. The reaction is stopped by the addition of 2-4 dinitrophenylhydrazine which reacts with the remaining unreduced α-ketobutyric acid to form α-ketobutyric acid-hydrazone. This hydrazone yields a brown colour on the addition of alkali, the intensity of which is a measure of the unreduced α-ketobutyric acid, and so inversely reflects the level of serum hydroxybutyric dehydrogenase activity.

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REAGENTS

SØRENSEN'S PHOSPHATE BUFFER, M/15, *p*H 7·4 M/15 anhydrous disodium hydrogen orthophosphate (Na_2HPO_4, 9·47 g. per litre), 80·2 ml., is added to 19·8 ml. M/15 potassium dihydrogen orthophosphate (KH_2PO_4, 9·08 g. per litre). The buffer is stored in the refrigerator and renewed if signs of contamination appear.

α-KETOBUTYRIC ACID-STOCK 0·1 M SOLUTION α-Ketobutyric acid[1], 1·02 g., is weighed in a beaker, and the contents washed into a 100 ml. volumetric flask with about 30 ml. of phosphate buffer. The *p*H of the solution is adjusted to 7·4 using British Drug Houses narrow-range indicator paper by the dropwise addition of a saturated solution of potassium hydroxide, and the volume made up to 100 ml. with further buffer. This solution is distributed in 4 ml. amounts in glass screw-capped bottles, and remains stable for several months if stored at − 18°C.

α-KETOBUTYRIC ACID SUBSTRATE The stock 0·1 M α-ketobutyric acid is diluted with phosphate buffer so that the optical density values given in column 5 of the Table are obtained when the calibration curve is prepared. A 1 in 100 dilution is usually satisfactory, but different batches of α-ketobutyric acid may require slightly different degrees of dilution. This substrate is distributed in 20 ml. amounts in glass screw-capped bottles and remains stable for up to one month if stored at −18°C.

REDUCED NICOTINAMIDE-ADENINE DINUCLEOTIDE SOLUTION A solution of reduced nicotinamide-adenine dinucleotide[2], 10 mg. per ml. phosphate buffer, is freshly prepared on the day of the test. This material is expensive and the amount prepared should depend on the number of determinations to be made. For each determination 0·1 ml. is required and a further 0·2 ml. for the substrate $NADH_2$ blanks.

[1]Supplied by L. Light and Co. Ltd., Colnbrook, England.

[2]DPNH, sodium salt, supplied by C. F. Boehringer and Soehne, Mannheim, Germany.

2-4 DINITROPHENYLHYDRAZINE Concentrated hydrochloric acid, 85 ml., is added to 400 mg. 2-4 dinitrophenylhydrazine (British Drug Houses, AnalaR grade) and the solution made up to 1 litre with distilled water. The solution is stable for several weeks in the refrigerator.

0·4 N SODIUM HYDROXIDE 16g. NaOH per litre.

PROCEDURE

To ensure satisfactory results it is essential that all glassware is chemically clean. Sera must be free from any trace of haemolysis and should be separated from the red cells within two hours of collecting the blood. The serum enzyme is stable for at least seven days at 4°C.

For each serum tested, a serum test and serum blank are required. For each batch of tests a substrate blank and a substrate-$NADH_2$ blank should be set up in duplicate.

The following reagents are accurately pipetted into stoppered 6 in. × ⅝ in. test-tubes:

	Serum Test (ml.)	*Serum Blank (ml.)*	*Substrate Blank (ml.)*	*Substrate $NADH_2$-blank (ml.)*
α-Ketobutyric acid substrate	1·0	1·0	1·0	1·0
Serum	0·1	0·1	—	—
Phosphate buffer	—	0·1	0·2	0·1
$NADH_2$	—	—	—	0·1

All tubes are allowed to reach 37°C. by immersion in a water-bath for five minutes.

The reaction in the serum test tube is started by the addition of 0·1 ml. of $NADH_2$ solution, and the time when it was added is accurately recorded. If a number of sera are being examined, the $NADH_2$ is added to the serum test tubes at convenient, *e.g.*, 15 seconds, intervals. Subsequent steps are then carried out making allowance for this interval.

The serum test is incubated for exactly 60 minutes after the addition of the $NADH_2$; 1 ml. of 2-4 dinitrophenyl hydrazine reagent is then added to all tubes and the contents mixed by gentle shaking. The tubes are then removed from the water-bath and allowed to stand at room temperature.

Twenty minutes after the addition of the 2-4 dinitrophenylhydrazine 10 ml. 0·4 N NaOH is added to all tubes and the contents mixed by inversion.

After standing at room temperature for a further 10, but not more than 30 minutes, the optical densities of all tubes are recorded at 490 mμ, using cuvettes of 1 cm. light path and setting zero optical density with distilled water. The EEL portable photoelectric colorimeter with an Ilford 623 filter is suitable.

CALCULATION

The serum test optical density is subtracted from that of the serum blank. To the resultant figure is added the value obtained by subtracting the substrate blank optical density from the substrate $NADH_2$ nicotinamide-adenine dinucleotide blank optical density. The value thus obtained is converted into milliunits (defined below) of hydroxybutyric dehydrogenase activity per millilitre of serum by reference to the calibration curve. If the activity is in excess of 310 milliunits per millilitre, the test is repeated using serum diluted 1 in 5 with phosphate buffer (1 volume of serum + 4 volumes of buffer) and the result from the calibration curve multiplied by five.

CALIBRATION CURVE

With each batch of substrate a calibration curve is prepared. Dilutions of the α-ketobutyric acid substrate with phosphate buffer are made as shown in the Table.

These dilutions are treated at room temperature with 1 ml. of 2-4 dinitrophenylhydrazine reagent, and after standing for 20 minutes, 10 ml. 0·4 N NaOH is added. Optical densities are recorded 10 minutes after adding the NaOH at 490 mμ as before. Zero optical density is set with distilled water. A calibration curve is obtained by plotting the net decrease in optical density compared with tube 1 (see Table) against the values for hydroxybutyric dehydrogenase activity shown in column 6 of the Table. The optical density of tube 1 should approximate to 0·550, and for the values set out in column 6 to apply, the values for the net decrease in optical density of tubes 2 to 5 should be identical to those given in column 5 of the Table. This is ensured by suitably diluting the stock 0·1 M α-ketobutyric acid solution when the substrate is prepared. The calibration curve should be repeated at weekly intervals to check the stability of the substrate.

DISCUSSION

The Commission on Enzymes of the International Union of Biochemistry (1961) has recommended

TABLE

1	*2*	*3*	*4*	*5*	*6*	*7*
Tube No.	*Volume of Substrate (ml.)*	*Volume of Buffer (ml.)*	*Optical Density Readings*	*Net Decrease in Optical Density Compared with Tube 1*	*Milliunits of H.B.D. Activity per ml. Serum after One Hour*	*Milliunits H.B.D. Activity per ml. Serum after 30 Minutes*
1	1·0	0·2	—	0	0	0
2	0·8	0·4	—	0·090	53	103
3	0·6	0·6	—	0·180	114	206
4	0·4	0·8	—	0·270	183	309
5	0·2	1·0	—	0·395	310	—

that where possible enzyme units should be expressed so that one unit represents the amount of enzyme that will catalyse the transformation of one micromole of substrate per minute at 25°C., and has suggested that where the value for the unit is inconveniently large results may be expressed in milliunits. In accordance with this recommendation all results are expressed in milliunits per millilitre serum, the value of the milliunit having been calculated on the basis of $NADH_2$ oxidation in the spectrophotometric method (Rosalki and Wilkinson, 1960; Elliott and Wilkinson, 1961, 1 milliunit = 2·1 spectrophotometric units).

The colorimetric method above has been calibrated using the spectrophotometric method. The values for hydroxybutyric dehydrogenase activity per millilitre serum incubated at 37°C. are consequently interchangeable with the values at 25°C. using the spectrophotometric method, and no adjustment for temperature is required.

Using the colorimetric method duplicate determinations on 27 sera with activities for hydroxybutyric dehydrogenase evenly distributed over the range 71 to 310 milliunits per millilitre were found to have an average percentage difference from the mean of 3·0%, S.D. ± 2·0%. This degree of reproducibility is satisfactory for clinical use and compares with an average percentage difference from the mean of 1·4% S.D. ± 0·4% obtained by replicate determinations on 23 of these sera using the spectrophotometric method.

The observed range of hydroxybutyric dehydrogenase activity of 43 normal sera using the colorimetric method was found to be 53 to 131 milliunits per millilitre (mean 91 milliunits per ml., S.D. ± 15 milliunits per ml.). Values in excess of 140 milliunits per ml. may be regarded as definitely increased. Using the spectrophotometric method the range of activity of these sera was 56 to 125 milliunits per ml. (mean 89 milliunits per ml., S.D. ± 15 milliunits per ml.). Enzyme activities of 80 sera were determined by both procedures. The average percentage difference from the mean of determinations on individual sera by the two methods was 4·9%, S.D. ± 4·2%, and enzyme activities determined by the two procedures showed a highly significant degree of correlation ($r = 0{\cdot}98$, $P<0{\cdot}001$).

If for the sake of speed it is desired to shorten the incubation time to 30 minutes, the values shown in column 7 of the Table should be substituted for those of column 6 when the calibration curve is prepared. With 30 minutes' incubation the difference in optical density between serum test and serum blank is decreased, but reproducibility is but little impaired. The values in column 7 do not represent twice those in column 6, for at the low concentration of substrate keto-acid used, first-order kinetics are obeyed; the progress curve of the reaction is therefore logarithmic rather than linear.

The low substrate concentration is required so that serum-test and serum-blank optical densities may be within a suitable range of galvanometer deflection on the colorimeter. Optical densities in the region 0·40 to 0·60 are yielded by serum blanks and by serum test sera within the normal range.

The $NADH_2$ solution contributes to the final optical density of the serum test (up to 0·05). A 'true' serum blank could be prepared by adding 0·1 ml. of $NADH_2$ solution to the serum blank immediately before the addition of the 2-4 dinitrophenylhydrazine, the 0·1 ml. of buffer being omitted. The use of substrate blanks and substrate $NADH_2$ blanks as described above, however, reduces the expense of the method when a number of sera are being examined. Using the values given in the calibration table it is not necessary to adjust for change in final optical density consequent upon $NADH_2$ oxidation in the reaction.

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