Technical method

Rapid acetoacetate analysis on the LKB 8600 Reaction Rate Analyser

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Automation of acetoacetate analysis has previously been by continuous flow methods using colorimetric techniques (Klein and Oklander, 1966; Dickie and Gibson, 1976). The most specific methods appear to be enzymatic using the β-hydroxybutyrate dehydrogenase (β HBDH, E.C.1.1.1.30)/NADH system (Williamson *et al.*, 1962). These end-point methods, however, do not readily permit the analysis of large numbers of samples, and the incubation time of an enzymatic end-point assay can take as long as 50 minutes (Gibbard and Watkins, 1968). A kinetic technique would reduce this time considerably. The LKB 8600 Reaction Rate Analyser has many advantages including precise temperature control, ease of use, reliability (Smith *et al.*, 1970), and accuracy (Davidson, 1976). The following method uses trichloroacetic acid as protein precipitant (100 g/l), a 50 μl sample volume, a reaction rate mode of measurement, and a one-minute measuring time.

D(-)-β-Hydroxybutyrate dehydrogenase catalyses the reaction: acetoacetate + NADH + $H^+ \rightleftharpoons$ D(-)-β-hydroxybutyrate + NAD^+. Acetoacetate is quantitated by the initial rate of decrease in absorbance of NADH at 340 nm.

Material and methods

1 Trichloroacetic acid (TCA), 100 g/l and 50 g/l.
2 0·1 M phosphate buffer, pH 7·4, is prepared by dissolving 13·6 g potassium dihydrogen orthophosphate and 3·5 g sodium hydroxide in distilled water and making up to 1 litre. The pH is checked at 7·40.
3 β-Hydroxybutyrate dehydrogenase (E.C.1.1.1.30), prepared from *Rhodopseudomonas spheroides*, was supplied from the Boehringer Corporation Ltd, Grade II, Cat. No. 15055.
4 NADH, from Sigma Chemical Co Ltd, Grade III, Cat. No. N 8129, 2 g/l in 0·1 M phosphate buffer, pH 7·4.

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5 Acetoacetate standards: stock standard must be prepared fresh each day. Weigh out 57 mg lithium acetoacetate (Sigma Chemical Co Ltd, Grade II, 90-95% pure, Cat. No. A 8509) and dissolve in 100 ml TCA (50 g/l). Dilute this stock solution in TCA (50 g/l) to produce standards of the following concentrations: 0·25, 0·50, 0·75, and 1·00 mmol/l.
6 1·0 M Triethanolamine buffer (TEA), pH 10·0, is prepared by dissolving 185 g triethanolamine hydrochloride (Sigma Chemical Co Ltd, Cat. No. T 1502) in almost 1 litre of distilled water and adjusting to pH 10·0 with 10 M sodium hydroxide.

β-Glycerophosphate, 3-hydroxybutyrate, and 2,3 diphosphoglycerate were all obtained from the Sigma Chemical Co Ltd. All other reagents were obtained from BDH Chemicals Ltd, Poole, Dorset.

The LKB 8600 was set up in the following way:

Wavelength	340 nm
Reaction course	Decrease
Back off setting	0·4 A
Chart speed	60 mm/min
Reaction time	1 min
Measuring range	0-0·05 A
Starting reagent volume	100 μl
Delay/normal	Delay
Temperature	37°

Manual acetoacetate measurements were performed on a Pye Unicam SP 1800 spectrophotometer fitted with an AR 25 linear recorder.

METHOD

Approximately 1 ml of blood is collected without preservative and immediately delivered into a preweighed glass tube containing 1·0 ml of ice-cold TCA solution (100 g/l). This is then mixed and packed in ice for immediate delivery to the laboratory. The tube is reweighed to obtain the weight of blood added and spun in a refrigerated centrifuge for 10 minutes, and the clear supernatant is removed and stored in a deep freeze at −15° until it is ready for analysis. By assuming a relative density of blood of 1·06, the volume of blood added can be calculated. Subsequent measurements of TCA centrifugate may be corrected to the original blood concentration by multiplying by the dilution factor $(1{\cdot}06 + x)/x$, where x is the weight of blood added in grams. Fifty microlitres of TCA centrifugate, standard or blank solution (TCA, 50 g/l), is added to 1·0 ml of phosphate buffer in an LKB measuring cuvette, which contains 20 μl of neat enzyme suspension (βHBDH), and the racks are then fed into

the instrument (see note below). The starting reagent pump is primed with NADH solution. The initial rate of reaction is then determined by measurement of the change in absorbance over one minute at 340 nm for blank, standards, and test samples. The results of the standard concentrations are plotted graphically, and the results of the unknowns are determined from the graph. A measuring time of one minute is selected since the progress curve is linear over this time. Longer measuring times were found to yield a non-linear response.

NOTE: There is a batch to batch variation in the reactivity of the commercial enzyme suspension. When a new bottle of HBDH is to be used then the minimum amount of enzyme which will give an initial rate of reaction of not less than 0·05 A/min at a standard concentration of 1·00 mmol/l should be determined. This would not normally exceed 20 μl.

Results

A linear response of initial rate of reaction to increasing concentration is found up to a value of at least 2·00 mmol/l.

The mean recovery for an aqueous solution of acetoacetate (5·0 mmol/l) added to heparinised blood was 103% (range 100%-106%) for six samples.

Specificity was determined by analysing, by the method described above, a series of solutions (in TCA, 50 g/l) of substances which might conceivably interfere in the reaction. The results are presented in Table 1.

A series of patient samples were assayed for acetoacetate by both the present method and an end-point technique (Mellanby and Williamson, 1965). The data are presented in the Figure.

Acetoacetate is a highly labile substance which

Table 1 *Specificity of acetoacetate method on the LKB 8600*

Potentially interfering substance	*Concentration (mmol/l)*	*Apparent acetoacetate concentration (mmol/l)*
Oxaloacetate	4·0	0·73
Dihydroxyacetone	4·0	0
α Ketobutyrate	4·0	<0·04
β Glycerophosphate	4·0	0
L-Cysteine	4·0	0
Succinate	4·0	0·04
α Ketoglutarate	4·0	<0·04
L-Serine	4·0	0
Malate	4·0	<0·04
Lactate	1·0	0
Pyruvate	0·4	0
3-Hydroxybutyrate	1·0	0
Glycerol	2·26	0
2,3-Diphosphoglycerate	1·0	0

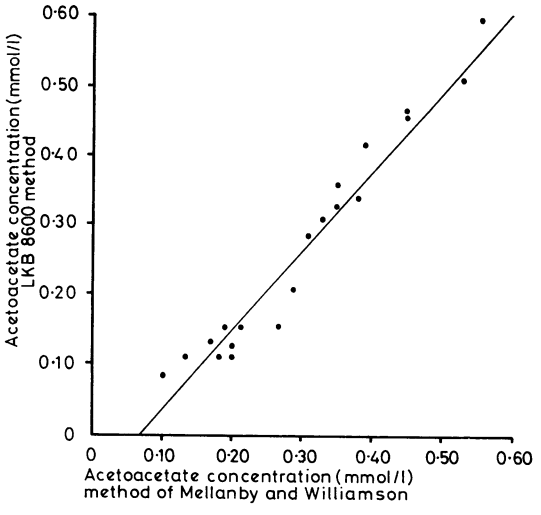

Figure *Correlation between LKB 8600 method and that of Mellanby and Williamson (1965). Number of pairs = 20, intercept = −0·071, slope = 1·10, r = 0·975.*

can undergo non-enzymatic decarboxylation (Williamson *et al.*, 1962). A solution of acetoacetate in 50 g/l TCA was found, using this method, to lose as much as 20% of its concentration after storage for 24 hours at 4°. However, storage at −15° resulted in no more than a 7% loss in concentration over a period of 48 hours. A similar study (Gibbard and Watkins, 1968) showed that storage of heparinised blood samples before acetoacetate determination was unsatisfactory, and even after preservation at −20° for 24 hours large losses could be demonstrated. These workers concluded that blood must be deproteinised immediately after it has been taken.

Within-batch precision was determined by replicate analysis of TCA supernatants of whole blood at two levels of concentration. For estimation of between-batch precision, a sample of TCA blood supernatant was prepared, divided into aliquots, and stored at −15°. Samples were removed, thawed, and analysed in 17 separate batches over the next 12 days. The results are presented in Table 2. The between-batch coefficient of variation was 7·9%. The between-batch coefficient of variation of the slope of the calibration line gave a similar value of 7·8%.

To investigate the effect of TCA concentration on initial reaction velocity, aqueous solutions of acetoacetate were prepared (0·5 mmol/l) in various concentrations of TCA ranging from 0 to 100 g/l. Acetoacetate was measured in each solution by the reaction rate method. Increasing the TCA concen-

Table 2 *Precision of acetoacetate method on the LKB 8600*

Mean	*SD*	*CV%*	*n*
Within batch precision			
0·34	0·008	2·4	10
0·75	0·014	1·9	10
Between batch precision			
0·92	0·073	7·9	17

tration from 0 to 30 g/l decreased the method sensitivity by 50%. The change from 50 g/l to 100 g/l TCA resulted in a decrease in sensitivity of less than 10%.

Discussion

From Table 2 it can be seen that oxaloacetate interferes significantly in the method to the extent of 18% on a mole for mole basis. This can probably be attributed to the fact that malate dehydrogenase is a contaminant of the commercial preparation. Oxaloacetate interference in enzymatic acetoacetate analysis has been documented previously (Mellanby and Williamson, 1965; Olsen, 1971). The very small concentration of oxaloacetate in blood is, however, unlikely to contribute significantly to the final result (Diem and Lentner, 1970).

Measurement of substrate concentration by an enzymatic reaction rate method is possible under certain reaction conditions (Ingle and Crouch, 1971; Tiffany *et al.*, 1972; Faust *et al.*, 1973). The first condition is a low initial substrate concentration (S_o) in relation to the Michaelis constant (K_m) of β-hydroxybutyrate dehydrogenase such that $S_o \leqslant 0{\cdot}2\ K_m$. The second condition is a particular relationship between the maximum reaction velocity (V_{max}), the measuring time (t), and the Michaelis constant: $V_{max}.t/K_m \leqslant 0{\cdot}1$. The apparent K_m (acetoacetate) for the preparation of HBDH used in the present method was determined to be $2{\cdot}2 \times 10^{-4}$ mol/l. The highest standard (1·00 mmol/l) under the conditions of the method gives an initial concentration of $4{\cdot}35 \times 10^{-5}$ mol/l. This corresponds to 0·2 K_m. Maintenance of the second condition for a one-minute measuring time, and assuming a value for molar absorbance for NADH of 6220 litres moles^{-1} cm^{-1}, requires that the enzyme concentration is 0·16 U/cuvette but this would result in a method which was too insensitive for practical use. A useful compromise is to increase the enzyme concentration to 0·26 U/cuvette, which corresponds to a volume of 20 μl of neat suspension/cuvette. This gives the relationship $V_{max}.t/K_m = 0{\cdot}16$.

The comparison of the two methods, as shown in the Figure, discloses a small negative intercept. This is explained by a reduced specificity on the part of the end-point method where other materials do interfere, such as D-fructose, pyruvate, and the effect of oxaloacetate. If enzyme is omitted from the reaction mix in the kinetic method, there is no measurable blank reaction even in the presence of acetoacetate in high concentration (10·0 mmol/l). The instability of acetoacetate requires that analysis should take place with the minimum of delay after blood collection.

High concentrations of acetoacetate are found in catabolic states such as starvation or diabetes mellitus. In the response to trauma the role of ketone production and metabolism has been reviewed (Fleck, 1976). In this response, a significantly increased acetoacetate may be associated with decreased protein breakdown (Smith *et al.*, 1975). The measurement of acetoacetate in conjunction with 3-hydroxybutyrate may help to discriminate between lactic acidosis associated with diabetes and true diabetic ketoacidosis (Cohen and Woods, 1976). An increased acetoacetate has also been described in chronic renal disease that is not due to defective excretory function (Campanacci *et al.*, 1968).

The advantages which this assay system has over previous methods are that it is more specific, the analysis time is very short, the method is semi-automated using a commonly available instrument, it is a discrete system with a minimum of reagent wastage, and the analysis is performed directly on the deproteinised supernatant without the requirement of an initial step in which protein precipitant is removed.

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Book reviews

Connective Tissue Diseases. By Graham R. V. Hughes. (Pp. vi + 266; illustrated; £9·00) Oxford: Blackwell Scientific Publications. 1977.

This book is likely to be of most interest to practising physicians since its emphasis is mainly on clinical diagnosis. Some mention is made of the pathological features of the connective tissue diseases, and it is surprising that the benign localised arteritis of gallbladder and appendix, which can easily be mistaken histologically for polyarteritis nodosa, is not described in the chapters on arteritis which occupy one-fifth of the book. Current theories of aetiology are discussed, especially the role of immune complexes; the role of HLA antigens in ankylosing spondylitis (a condition not dealt with at all as such) could properly have received more attention than a brief mention in the introduction. For the clinical pathologist there is an up-to-date account of the many serological abnormalities found in the connective tissue diseases and a useful statement of the author's views of their value in diagnosis and patient management. R. B. GOUDIE

Human Hemoglobins. By H. F. Bunn, B. G. Forget, and Helen Ranney. (Pp. x + 432; illustrated; £17·00) Eastbourne: Holt-Saunders. 1977.

This is an excellent review of the abnormal haemoglobin field. Early chapters deal with the structure and function of human haemoglobin and the way in which this is modified in various disease states. Later sections deal with the various structural haemoglobin variants and their associated clinical manifestations, the thalassaemias, and the acquired disorders of haemoglobin. The book is extremely well produced, nicely illustrated, and remarkably free from errors of fact or type; it contains an extensive and up-to-date bibliography.

Although this book deals with the abnormal haemoglobin field slightly unevenly it is by far the best general account of the subject that is currently available. The sections that deal with the modification of haemoglobin function in various disease states are particularly good. *Human Hemoglobins* is recommended most strongly to all haematologists and to others interested in the abnormal haemoglobin field. Impecunious academic haematologists will be encouraged by the news that an abridged version is shortly to be published under the title 'Hemoglobinopathies' in the monograph series 'Major Problems in Internal Medicine'. D. J. WEATHERALL

Pathology of the Female Genital Tract. By Ancel Blaustein. (Pp. xx + 897; 1206 illustrations and 39 colour figures; DM 118, $52.00) New York, Heidelberg, Berlin: Springer-Verlag. 1977.

This book contains contributions from 28 different authors, many of whom are household names in the field of gynaecological histopathology. The 39 chapters give excellent descriptions of all anatomical regions of the female genital tract and include an up-to-date evaluation of cervical intraepithelial neoplasia. Six chapters are devoted to the anatomy and pathology of pregnancy and one to a concise assessment of disorders of female sex differentiation. Two other chapters deal with genital tract tumours in animals, both induced and spontaneously occurring.

The chapters vary in quality, the figures and illustrations falling below the average standard in both the endometrial and myometrial sections and also those on the placenta and the pathology of pregnancy. The section on ovarian neoplasms of childhood by Abell could be regarded as repetitious in view of the detailed coverage