JNSF13-2-235

J. Natn. Sci. Coun. Sri Lanka 1985 **13(2)** : 235 – 258

AN EVALUATION OF A COLORIMETRIC PROCEDURE FOR THE ESTIMATION OF GLYCOSYLATED HAEMOGLOBIN AND THE ESTABLISHMENT OF REFERENCE VALUES FOR SRI LANKA

E. H. KARUNANAYAKE AND N. V. CHANDRASEKHARAN
Department of Biochemistry, Faculty of Medicine, University of Colombo, Colombo 8, Sri Lanka.

(Date of receipt : 21.01.85)
(Date of acceptance : 17.02.86)

Abstract : The importance of the measurement of glycosylated haemoglobin as a quantitative index reflecting metabolic control in diabetics during the preceeding four to six weeks is now well established. The suitability of the colorimetric method of Fluckiger and Winterhalter as a technique adaptable for developing countries has been evaluated. Incubation with oxalic acid for one hour indicated good precision with coefficient of variation within assay of 3.19 (n=20) and between assay of 2.8% (n=15) for normals and 1.8% for diabetics. β–D–fructose was also used in addition to 5–hydroxy methyl furfuraldehyde (HMF) as standards. This gives a measure of efficiency of the reaction. The non-specific blank value for all samples were a constant. Whole blood samples could be stored at room temperature (30 ± 3°C) for 5 days and for nine days at 4°C. Haemolysates were stable at 4°C for 5 days and for 30 days at −20°C. A good correlation coefficient of r = 0.92 was obtained with a microcolumn test kit (n=20, $p < 0.001$). The normal mean (±SD) for glycosylated haemoglobin (HbA_1) for Sri Lanka was found to be 5.85% ± 0.79 for males (n=70) and 5.88% ± 0.79 for females (n=30). A significant difference was not observed between males and females. The overall mean for normals was 5.9% ± 0.79 (or 0.39% ± 0.05 μM HMF/g Hb). Mean for non-insulin dependent diabetics was 10.83% ± 3 (or 0.68 ± 0.20 μM HMF/g Hb). The mean %HbA_1 for pregnant women (n=15) in the 3rd trimester of pregnancy was 7.0% ± 0.7 The colorimetric method meets many of the criteria for an ideal laboratory test. It proves to be the most suitable method for the measurement of glycosylated haemoglobin in developing countries.

1. Introduction

The minor haemoglobins (also referred to as fast haemoglobins[2] – HbA_1) accounting for about 8% of total haemoglobin in blood arise from non-enzymatic post translational modifications of haemoglobin A (HbA) (Figure 1). HbA_{1c} constitutes the major fraction.

HbA_{1c} is formed from HbA by the chemical condensation of a molecule of glucose specifically with the NH_2–terminal of the β–chain of HBA.[4] It is a slow process occuring during the entire life span of the erythrocyte[5] and is dependent on *in vivo* concentration of glucose. HbA_{1c} levels are therefore elevated in diabetics.

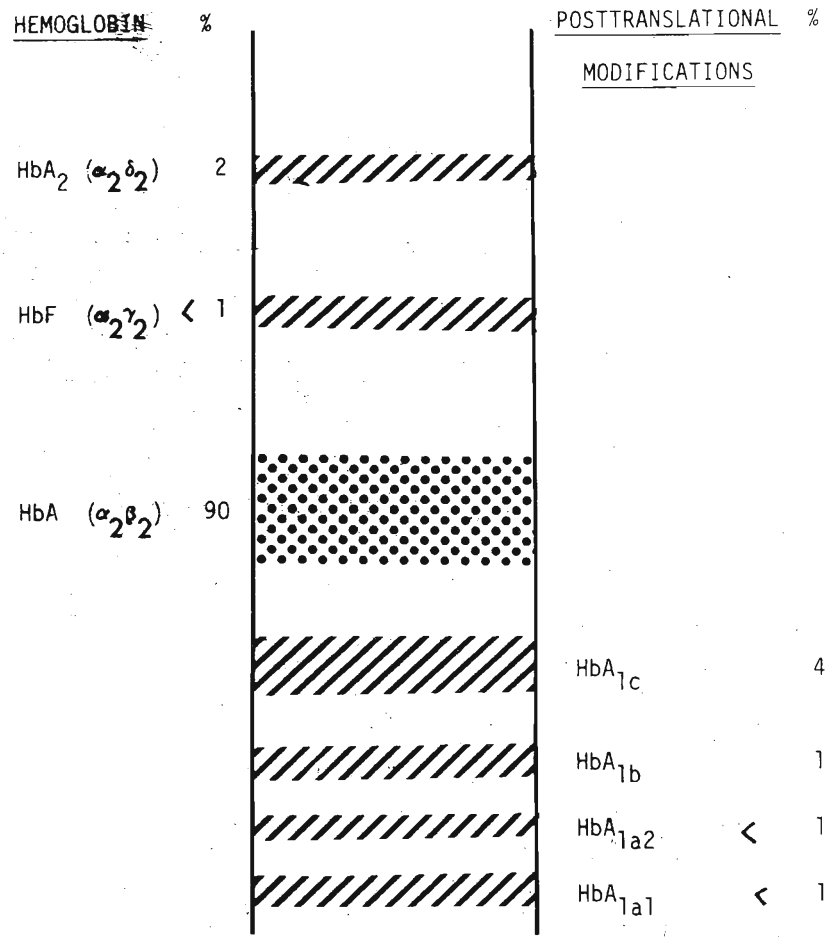

Figure 1. Electronic pattern of haemoglobin components in normal red blood cells.

The measurement of HbA_{1c} approximately reflects the time averaged serum glucose concentration in the preceeding four to six weeks[17] and hence gives a precise reflection of metabolic control in diabetes over a long period of time.

Methods described for the measurement of glycosylated haemoglobin include electrophoresis,[19] ion exchange chromatography,[25] high pressure liquid chromatography (HPLC),[8] affinity chromatography,[18] immunoassay,[16] fluorometry[13] and colorimetry.[11]

Prior to the present investigation no studies have been carried out in Sri Lanka on glycosylated haemoglobin. We have therefore evaluated a suitable colorimetric technique originally described by Fluckiger & Winterhalter[11] and subsequently modified by Worth *et al*[28] and established the reference values for Sri Lanka.

In this method the carbohydrate moeity is cleaved by acid hydrolysis to yield 5–hydroxy methyl furfuraldehyde (5–HMF) which is subsequently complexed with thiobarbituric acid. The resulting chromogen has a maximal absorbance at 443 nm.

The colorimetric method determines all glycosylated haemoglobins. Ideally HbA_{1c} should be the fraction that should be quantitated. However, the determination of total glycosylated haemoglobin has been shown to correlate well with the measurement of HbA_1[28] and HbA_{1c}[22] by column chromatography.

2. Materials and Methods

All reagents used were of analytical grade. Spectrophotometer readings were obtained on a Pye Unicam UV/VIS spectrophotometer (Model SP6 – 450 UV/VIS). Bench centrifuge was used for all centrifugation purposes. All experiments were carried out at room temperature ($30^{\circ}C \pm 3^{\circ}$) unless otherwise specified.

2.1 Standard haemoglobin solutions

Standard haemoglobin solutions were prepared according to Van Kampen and Zijlstra[26] (Hemiglobincyanide method) and standardised according to Cannan.[7] These prepared solutions were stored in brown vials at $4^{\circ}C$ after standardising with commercially available haemoglobin standards (Acuglobin–Ortho diagnostic systems U.S.A.).

2.2 Collection of Blood Samples

All blood samples (5 ml) were obtained into heparinised vacutainers. Normoglycaemic samples for males (average age 26.1 ± 5.86, n = 70) and females (average age 24.01 ± 5.6, n = 30) were collected from the Central Blood Bank, General Hospital, Colombo. Blood samples from diabetic patients of both sexes (average age 47 ± 13.5, n = 107) were obtained from the General Hospital, Colombo.

2.3 Determination of Haemoglobin

Samples of blood (5 ml) collected into heparinised vacutainers were centrifuged (1500g, 10 min) and the plasma removed. The red blood cells were washed twice in an equal volume of physiological saline (0.154M). The cells were haemolysed by the addition of one or two volumes of distilled water and carbon tetrachloride (0.25 vol). The mixture was vortexed for 5 min and centrifuged (1500g, 15 min). The clear haemolysate was collected and the haemoglobin concentration of the haemolysate was determined by the method of Van Kampen and Zijlstra[26] by adding haemolysate (20 μl) to hemiglobincyanide reagent (5 ml). After at least 3 min the optical density was measured at 540 nm and the haemoglobin concentration (g/l) calculated by the use of already prepared standards or O.D. x 377.Blank used was distilled water. Samples were then accurately adjusted to 50 g/l total haemoglobin concentration by the addition of distilled water.

2.4 Determination of glycosylated haemoglobin

Oxalic acid (1 ml, 0.3M) was added to an aliquot (2 ml) of adjusted haemolysate, mixed and placed immediately in a boiling water bath at 100°C for exactly 60 min. Evaporation was minimised by placing glass marbles on each test tube. After incubation samples were cooled in cold water for 2 min and deproteinisation carried out by the addition of trichloroacetic acid (1 ml, 40% w/v). The tubes were vortexed (30 seconds) and then centrifuged (1500g, 15 min). Thiobarbituric acid (0.5 ml, 0.05M) was added to the clear supernatant (2 ml), mixed and incubated (40°C, 60 min). The colour developed was read at 443 nm.

Included in each assay were a blank using distilled water instead of haemolysate, aqueous standards of 5–HMF (0.01 mM/L to 0.05 mM/L), aqueous standards of fructose (1 mM/L to 4mM/L) and diabetic and normal pooled haemolysates previously stored at −20°C.

2.5 Correlation with column method

Twenty samples of blood (8 normals and 12 diabetic) were assayed both by the ion-exchange column procedure and by the colorimetric method.

Commercial microcolumns were obtained from Bio-rad laboratories, Richmond, California.

2.6 Effect of storage of samples

Whole blood, washed packed erythrocytes, and haemolysates (50 g/l) were stored at various temperatures and assayed periodically. Determinations were performed in triplicate.

2.7 Washing for the removal of glucose

Aliquots (2 ml) of 20 haemolysates (all diabetics), the haemoglobin concentration of which were previously adjusted (50 g/l) were analysed for the presence of glucose by the glucose oxidase method.[15]

2.8 Effect of Haemoglobin concentration on colour development

Pooled normal haemolysates were adjusted to total haemoglobin concentrations varying from 15 g/l to 100 g/l. These were assayed and the colour developed read at 443 nm. Determinations were performed in triplicate.

2.9 Effect of Incubation time with 0.3 M oxalic acid on colour development

Aliquots (2 ml) of normal pooled haemolysate (50 g/l) were incubated with oxalic acid (2 ml, 0.3 M) and periodically samples were removed from the incubation chamber and assayed. Determinations were performed in duplicate.

2.10 Stablility of prepared haemoglobin standards

Haemoglobin standards were prepared[7] and standardised with commercially available Acuglobin (Orthodiagnostic systems, USA). The standards were stored in coloured vials at 4°C. The optical density at 540 nm was determined at various time intervals, ranging over six months.

2.11 Variation in the total haemoglobin adjustment to 50 g/l

Unadjusted haemolysate was obtained after washing of the red cells. An aliquot (20 μl) of the haemolysate was added to hemiglobincyanide reagent (5 ml) and the colour developed was read at 540 nm after at least 3 minutes. Twenty such determinations were performed.

2.12 Effect of concentration of oxalic acid on colour development

The effect of the molarity of oxalic acid on colour development was investigated on pooled normal haemolysates (50 g/l). Determinations were performed in triplicate.

2.13 Effect of incubation time with thiobarbituric

Twenty aliquots (2 ml) of supernatant (obtained after digestion with oxalic acid) were incubated with thiobarbituric acid (0.5 ml, 0.05 M) at 40°C. The optical density at 443 nm were measured at various time intervals.

2.14 Standards

Both 5–HMF and β–D–fructose in appropriate concentrations were used as standards in duplicate. Fructose standards were used to measure the efficiency of the reaction.

2.15 Effect of heating 5–HMF with 0.3 M oxalic acid at 100°C

Varying concentrations of 5–HMF (2 ml) were heated with oxalic acid (1 ml, 0.3 M) in a water bath (100°C, 1 hour) and the normal assay was performed. To identical varying concentrations of 5–HMF were added oxalic acid (1 ml, 0.3 M) but was not heated (100°C, 1 hour) and the normal assay performed. The colour developed was read at 443 nm.

2.16 Recovery of Added 5–HMF

Normal and diabetic pooled haemolysates were adjusted to 100 g/l total haemoglobin concentration. To the haemolysate (1.0 ml) was added varying concentrations of 5–HMF (1 ml), so that the final concentration of haemoglobin was 50 g/l. The haemolysates were then assayed. Determinations were done in triplicate.

2.17 Intra assay coefficient of variation

Multiple (n=20) analysis of blood obtained from one donor was carried out in a single assay.

2.18 Inter assay coefficient of variation

Pooled haemolysate from normal and diabetic subjects stored at −20°C were assayed in 15 consecutive runs during a period of a month. Also included were aliquots of standard fructose (4 mM). All results were obtained in duplicate.

2.19 Blank values and non-specific colour production

Haemolysate (50 ml) was prepared and haemoglobin concentration adjusted to 50 g/l. Aliquots were then analysed as described below.

a) i. Five aliquots (2 ml) were assayed as described previously.
   ii. The non-specific colour produced was determined by adding distilled water (0.5 ml) instead of thiobarbituric acid to the supernatant.
   iii. Five aliquots (2 ml) were assayed as described except that water (1 ml) was added instead of oxalic acid (1 ml).
   iv. Five aliquots (2 ml) were assayed as described except that the samples were not heated at 100°C.

b) Adjusted haemolysate (30 ml) was ultrafiltered under centrifugation (2000 g, 30 min) using Centricon Microconcentrator Membranes (purchased from Amicon Corporation) to obtain a protein free ultrafilterate.
   i. Two aliquots (2 ml) of the ultrafilterate were assayed as for glycosylated haemoglobin.
   ii. The non-specific colour produced by the ultrafilterate was determined by adding distilled water (0.5 ml) instead of thiobarbituric acid.

2.20 Validation of modified method

Haemolysates (50 g/l) from diabetics and normals were mixed in varying proportions to obtain a final volume of 2 ml. The different combinations were now assayed and colour developed read at 443 nm.

3. Results and Discussion

The importance of the measurement of glycosylated haemoglobin for the evaluation of long term glycaemic control is apparent from studies carried out so far. Numerous methods for its quantitation have been made available since its importance had been recognised. A cheap but sensitive and reproducible method that could be standardised between laboratories is the colorimetric method originally described by Fluckiger and Winterhalter[11] and subsequently modified by Worth *et al.*[28] However, very few attempts have been made to standardize the method so that results could be compared from all laboratories.

This investigation centred around the evaluation of a number of critical factors of the colorimetric technique, and its usefulness as a routine assay. The study on the effect of storage of samples on glycosylated haemoglobin content prior to assay showed that whole blood could be stored over a week at 4°C and 5 days at room temperature (30 ± 3°C). Worth *et al.*[28] have reported it to be stable for 2 weeks at room temperature and 4°C.

Haemolysates were found to be stable for only 30 days at −20°C. It has also been reported to be stable upto 70 days.[23] Pecararo[21] although has commented about the stability of frozen samples but has failed to indicate the temperature at which it was stored. According to Worth *et al.*[28] haemolysates were stable upto 6 months at −70°C. In the absence of deep freezing facilities in most laboratories in Sri Lanka, it is advisable not to attempt to store the haemolysates, but whole blood can be stored upto 6 days and assayed in one batch. Washed packed cells were stable at room temperature for only 3 days and is consistent with the results of Fischer *et al.*[10] At 4°C and −20°C the washed packed cells were stable only upto 9 days. The stability of whole blood and haemolysates are of great practical importance for a routine assay.

Initial dialysis has been carried out prior to the assay.[20,23] This is a serious setback of the assay because of the time factor involved. However, the assay of the haemolysate for glucose by the glucose oxidase method in the present study did not reveal any detectable levels of glucose. It therefore seems unnecessary to include a prior dialysis step.

The precision of the method is dependent on the initial accurate adjustment of haemoglobin concentration to 50 g/l. Worth *et al.*[28] have reported a conversion factor of 377 by which the optical density at 540 nm is multipled to give the haemoglobin concentration of unadjusted haemolysate in g/l. The accuracy of this was checked using standard Acuglobin vials (Ortho diagnostic systems, USA) available commercially and was found to be in fair agreement. However, as the pH of the hemiglobincyanide reagent affects the optical density, it should be adjusted accurately. Using the haemoglobin standards prepared in our laboratory, the coefficient of variation was found to be 2.8% (n=20). Results on the stability of prepared haemoglobin standards which were stored for over 6 months and assayed periodically were acceptable.

The study on the effect of haemoglobin concentration on colour development at 443 nm indicated a linear response upto 75 g/l (Figure 2) followed by flattening of the curve above this concentration. This is consistent with the findings previously reported.[28] A linear response between haemoglobin concentration (5 g/l to 80 g/l) has also been reported.[10] Apparent non-linearity in colour development has also been reported.[20,27] Pecoraro[21] observed a linear relationship upto 20 g/l. From the results obtained in the present studies an optimum haemoglobin concentration of 50 g/l as proposed by Worth *et al.*[28] which gives adequate colour development and ensures linearity was selected for the assay.

The study of the effect of oxalic acid concentration on colour development showed (Figure 3) a linear response upto 0.3 M and a fall off above this concentration. Based on our investigation we have used an

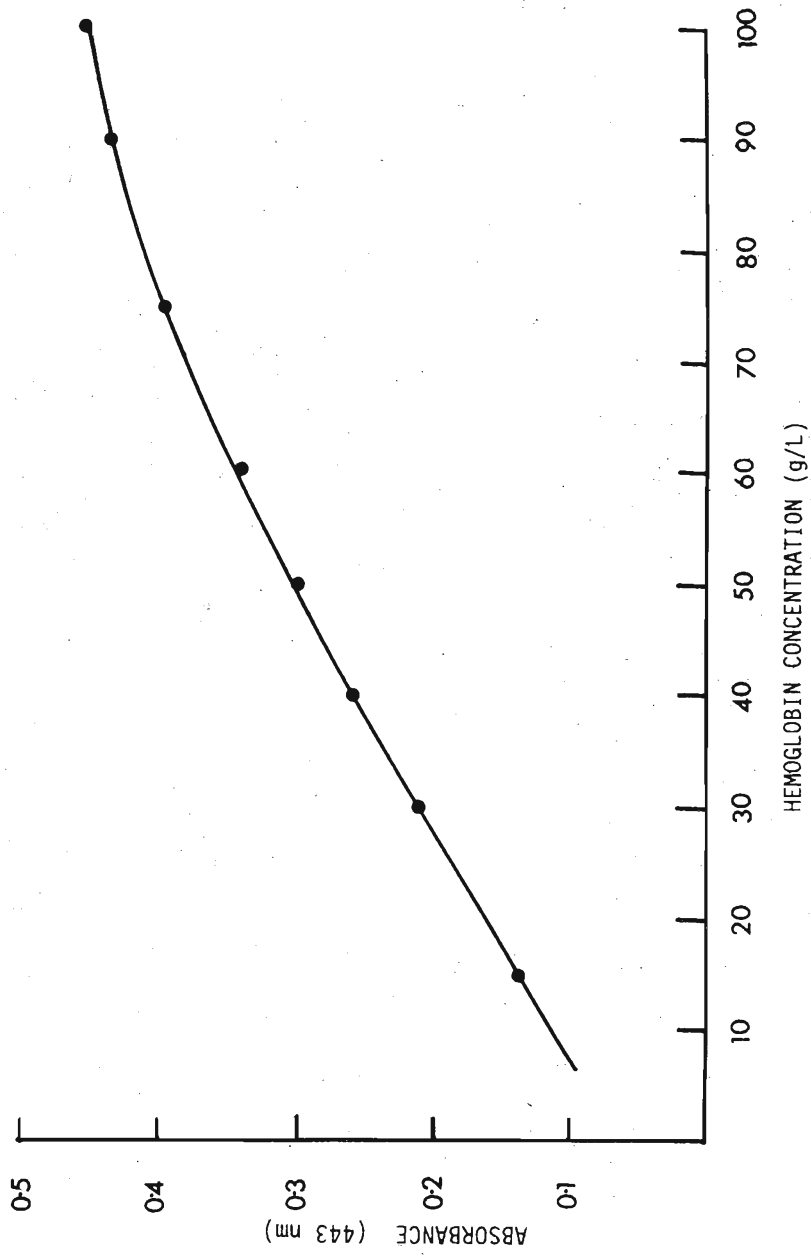

Figure 2. Effect of haemoglobin concentration on colour development.

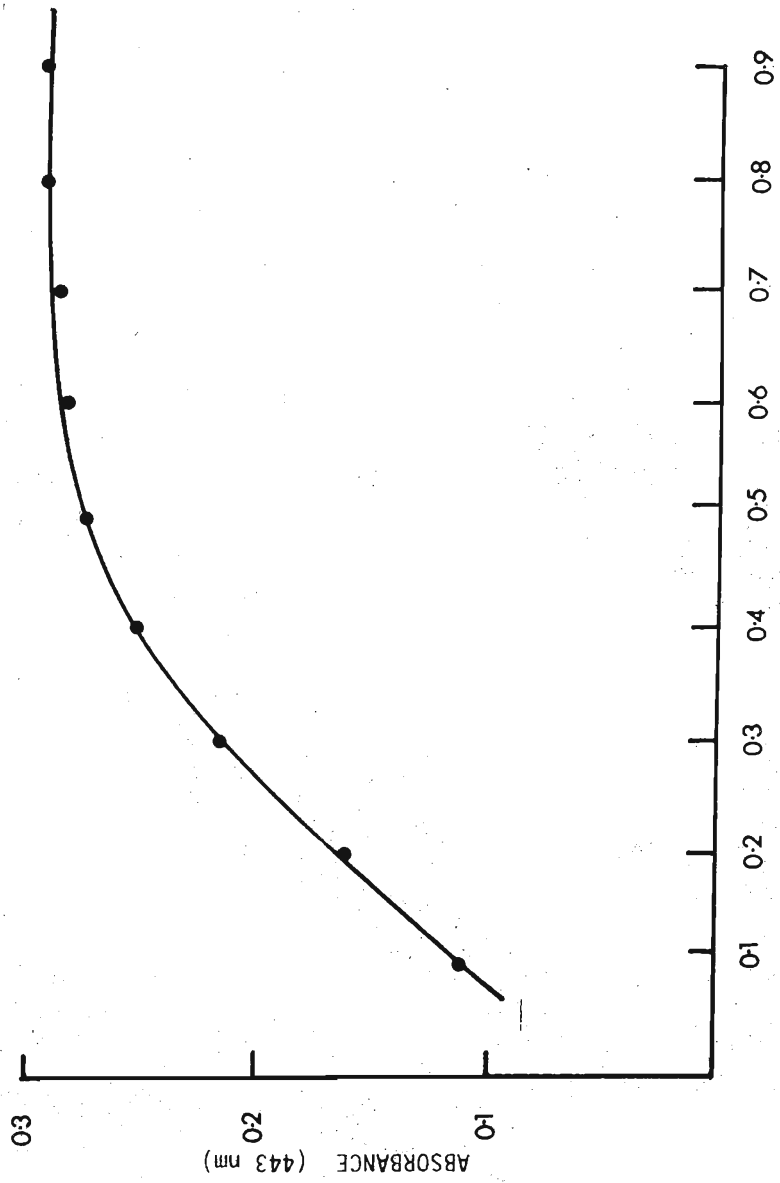

Figure 3. Effect of oxalic acid concentration on colour development.

optimum oxalic acid concentration of 0.3 M which gives a linear response as well as adequate colour at 443 nm. This is in agreement with Worth *et al.*[28]

An important factor around which much discussion has been centred is the incubation period with oxalic acid and the end-point in colour development. A long period is necessary to achieve an end-point in colour development. Gabbay *et al.*[12] used a 5 h incubation period and Fischer *et al.*[10] used a 4.5 h incubation period. The latter has quoted data to show that 87.5% of the maximum colour development can be expected at 4.5 h. However, both Gabbay and Fischer have not commented about the end-point in colour development. Pecoraro *et al.*[21] incubated initially for 3 h, and subsequently changed it to 5 h. Subramaniam *et al.*[24] used acetic acid and heated for 16 h before reaching end-point in colour development.

The reaction is non-stoichiometric and our study indicated that even after 6 h no end-point in colour development could be achieved, although it tends to stabilize between 6½ and 7 hrs (Figure 4). A linear increase in colour development was observed upto 3 hours followed by a subsequent fall off in the rate. Based on our studies, we have used an incubation period of 1 h. Since the rate of colour development is slow, a small error in timing will have a negligible effect on optical density at 443 nm and hence will not sacrifice the precision of the assay. However, quick cooling at the end of exactly 1 h incubation followed by immediate deproteinisation (which arrests the release of 5–HMF) with trichloro-acetic acid is recommended.

In the final colour development by incubating the deproteinized supernatant and thiobarbituric acid, our studies indicated the need for a minimum of 45 min incubation time for maximum colour development (Figure 5). Hence it is recommended that an incubation period of 60 minutes be used. Abraham *et al.*[1] and Saibene[23] have incubated for 40 minutes which may be inadequate at times. It is also apparent (Figure 5) that the rate of colour development was extremely rapid in the first 10 minutes followed by a slow gradual increase from there on until 45 minutes. It was also found that the maximum colour developed was stable for 2½ h and therefore readings could be taken within this period.

A major criticism of the colorimetric assay is the occurence of high blank values. The sample blank was obtained by replacing haemolysate with distilled water. The value for this has always been low or zero and hence negligible. Even before the addition of thiobarbituric acid for final colour development a significant (11.32% of total colour development) amount of non-specific colour development was observed (Table 1). This was a constant for all haemolysates. This value was therefore substracted from the final optical density at 443 nm.

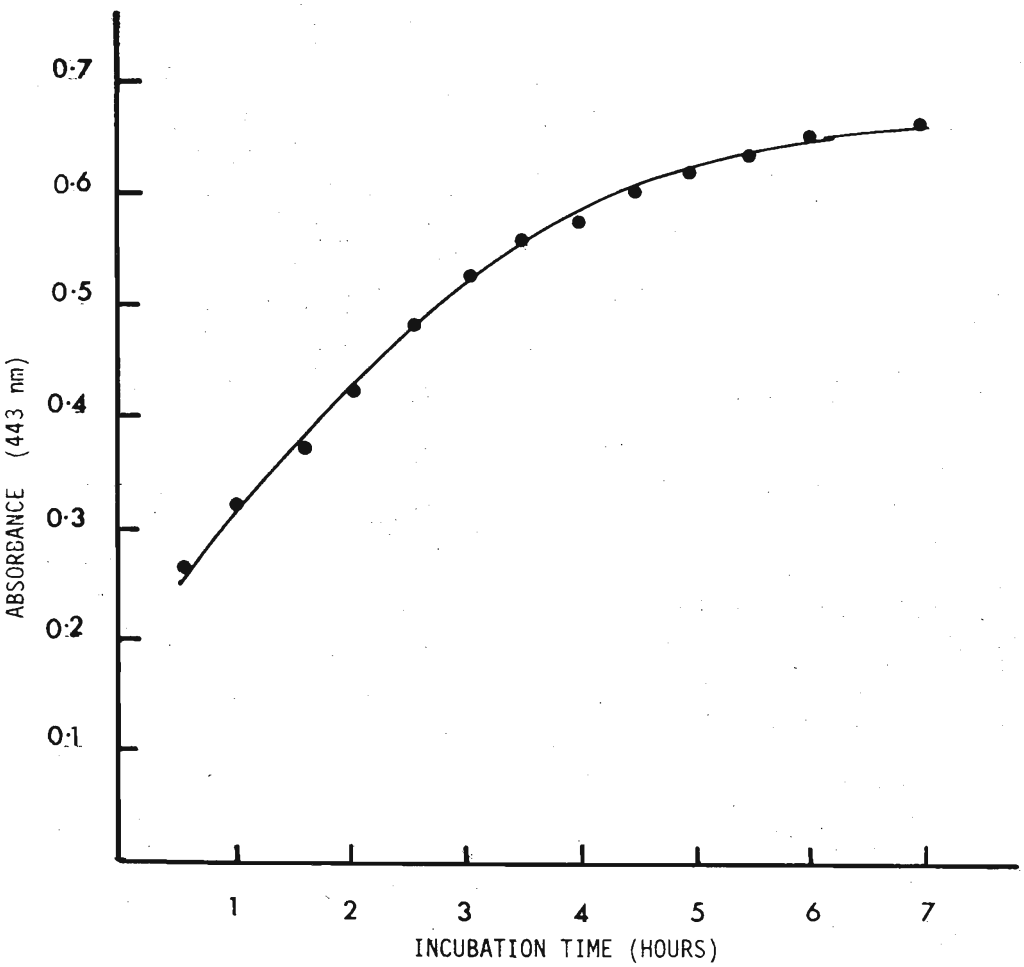

Figure 4. Effect of incubation time with oxalic acid (0.3M) on the colour development.

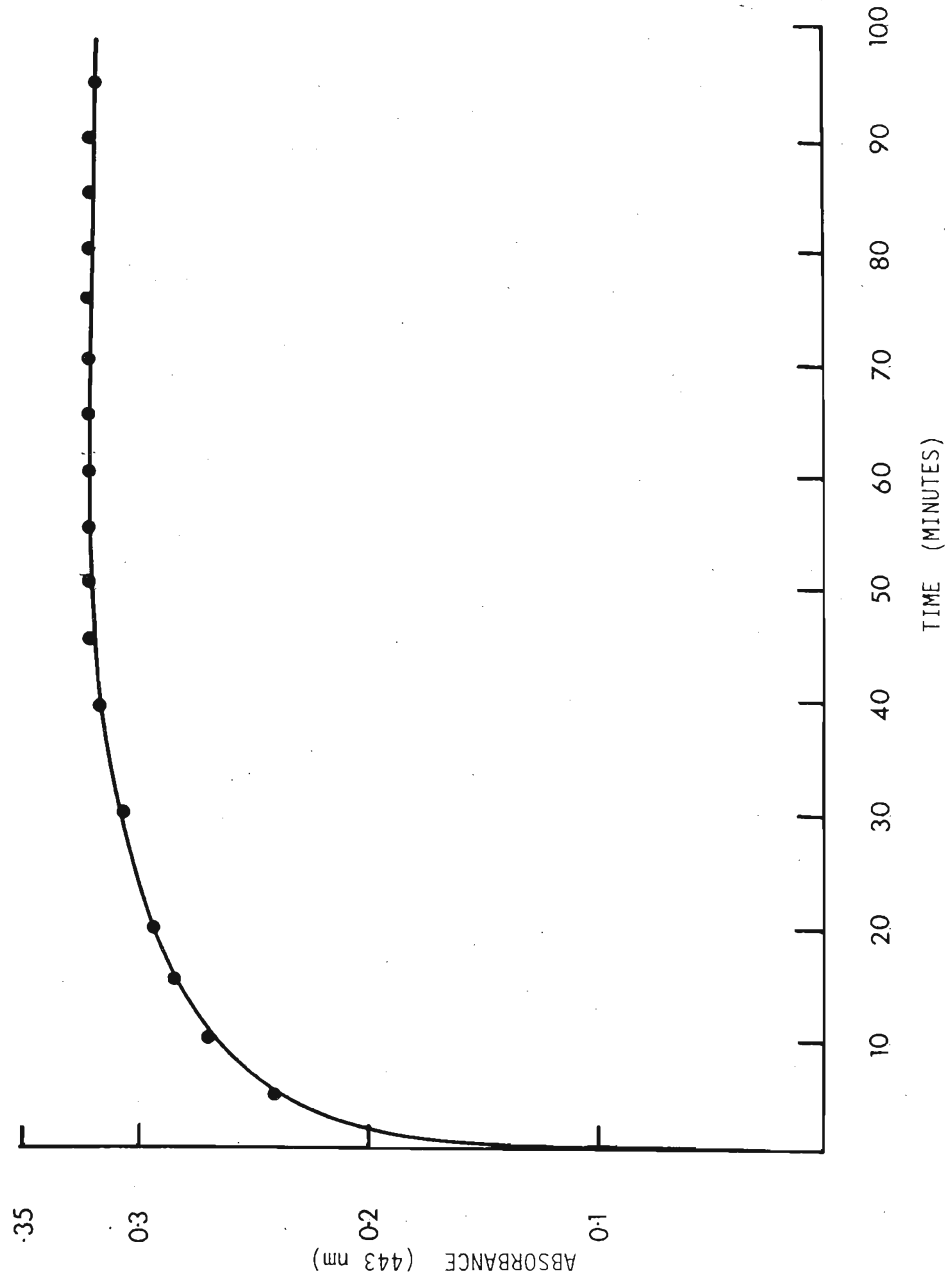

Figure 5. Effect of incubation time with thiobarbituric acid on colour development.

Table 1

	Procedure	O.D. at 443 nm	% of maximal O.D. at 443nm
a. (i)	Normal assay (n=5)	0.265	100%
(ii)	Non specific colour (n=5) produced at 443 nm	0.03	11.32%
(iii)	Normal assay but H_2O instead of oxalic acid (n=5)	0.157	59%
	Non specific colour	0.015	5.6%
(iv)	Normal assay but no heating	0.06	22.64%
	Non specific colour	0	0
b.	With haemoglobin free filterate		
(i)	Normal assay	0.05	18.86%
(ii)	Non specific colour production	0	0

The reagent blank value was obtained by replacing oxalic acid with water. A high blank response was observed (59% of total colour development). Even the haemoglobin free filterate produced significant amounts of colour. Dolhofer and Wieland[9] have used sodium borohydride for the preparation of sample blanks, which is subsequently removed by dialysis. This step was found to be unnecessary as good correlations with microcolumns have been obtained in this study and that of Worth *et al*.[28] and Pecoraro *et al*.[21] omitting this step.

One study of the colorimetric method showed a poor correlation with a column method.[1] But in recent years extremely good correlations have been reported with both the micro and macro columns.[12,21,23,28] The poor correlation may have been due to the lack of control of variable factors in the column method. A good correlation with a commercial microcolumn kit was obtained in this study (r=0.92, n=20). This was used as the basis for the conversion of optical density values at 443 nm to the generally known per cent HbA_1 values. The regression line (Figure 6) intersects the X axis. This is because the colorimetric assay detects glycosylation not only at the N—terminal amino group of the β-chains, but also the substantial glyco-

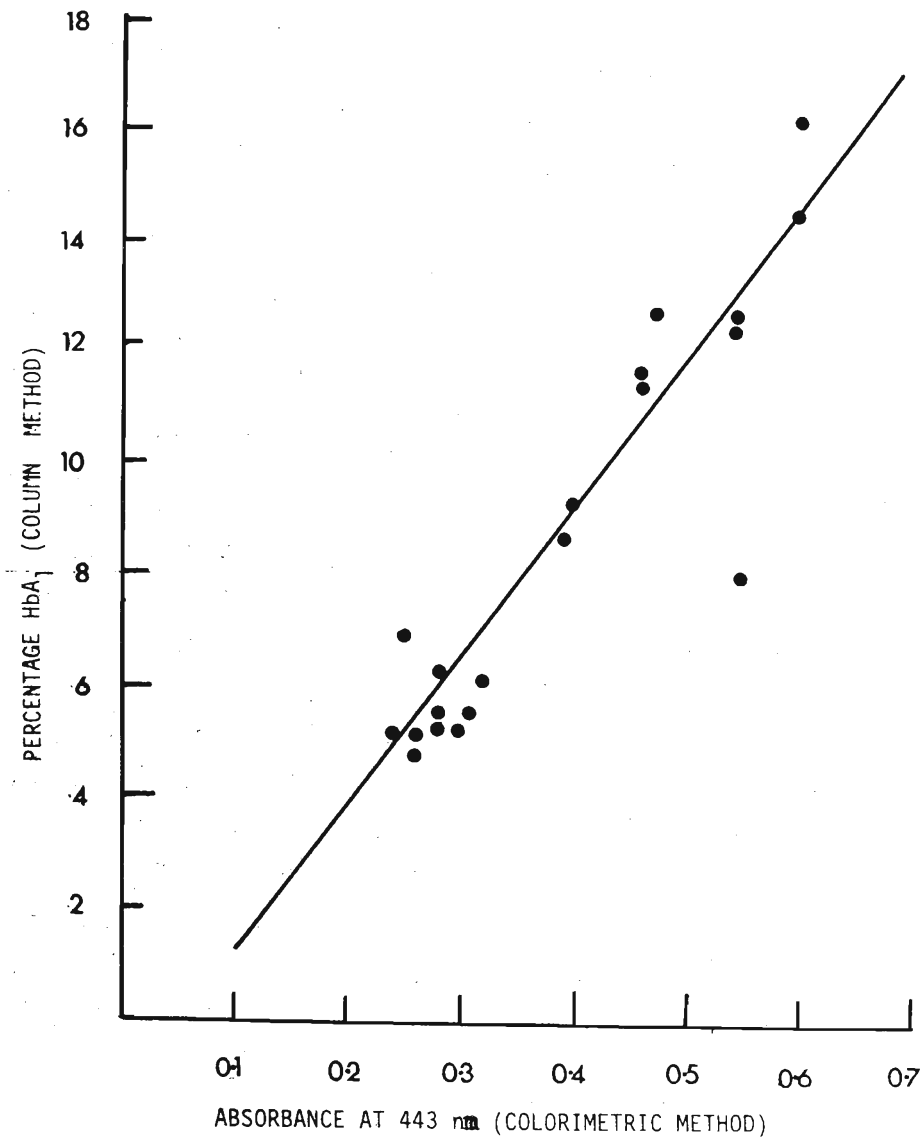

Figure 6. Correlation between HbA_1 determination by the colorimetric method and column method (Bio rad).

sylation of the N–terminal amino groups of the α chains and the ε amino groups of the lysine residues in both α and β chains. Similar observations were made by Saibene *et al.*[23] Intracellular proteins which are glycosylated may also contribute although proportionately very low and hence negligible.

Included in the assay in addition to standard 5–HMF, were standard fructose solutions as recommended by Goldstein.[14] The optical density developed at 443 nm from the fructose solutions gives a good measure of the efficiency of the reaction. The amount of 5–HMF formed from fructose is dependent on the time of heating and oxalic acid concentration. Hence the colour developed from fructose standards can be converted to 5–HMF values from the standard graph for 5–HMF and results expressed as an index of 5–HMF. Furthermore the possible errors arising from variations in heating time (1 h) and concentration of oxalic acid etc all of which contribute to inter assay variations could be minimised.

The present studies indicate that HMF is not destroyed on heating with 0.3 M oxalic acid and also there is no loss of added 5–HMF (Recovery = 100% ± 0.5) (Figure 7). This is consistent with the previous reports.[28] However, Goldstein *et al.*[14] have observed partial destruction of 5–HMF. Both standard curves (5–HMF and β–D–fructose) were linear in the range of concentrations used (Figures 7 and 8). As expected a linear increase in colour development was observed with increasing percentage diabetic haemolysate (Figure 9).

The intra assay coefficient of variation was 3.19% and the inter assay coefficient of variation obtained from 15 consecutive runs was 2.8% for normals and 1.8% for diabetics and 2.1% using a single fructose standard.

Three indices could be used for reporting glycosylated haemoglobin values. The reference range for glycosylated haemoglobin (HbA_1) for Sri Lankan population (average age 25.5, n=100) is 4.26 to 7.4 per cent (Mean ± 2SD) with a mean of 5.86 per cent. In terms of micromoles of HMF liberated, the range is 0.29 to 0.48 μM HMF/g Hb. The reference values could also be given in terms of micromoles of fructose according to which the reference range for Sri Lanka is 17.02 to 27.73 μM fructose/g Hb with a mean of 22.37 μM fructose/g Hb (Figure 10).

There was no significant difference between males and females. The mean for non-insulin dependent diabetics (Average age 47, n=107) was 10.83% HbA_1 or 0.68 μM HMF/g Hb or 39.4 μM fructose/g Hb.

The reference values in the present studies for Sri Lanka are in close agreement with those quoted for other countries.

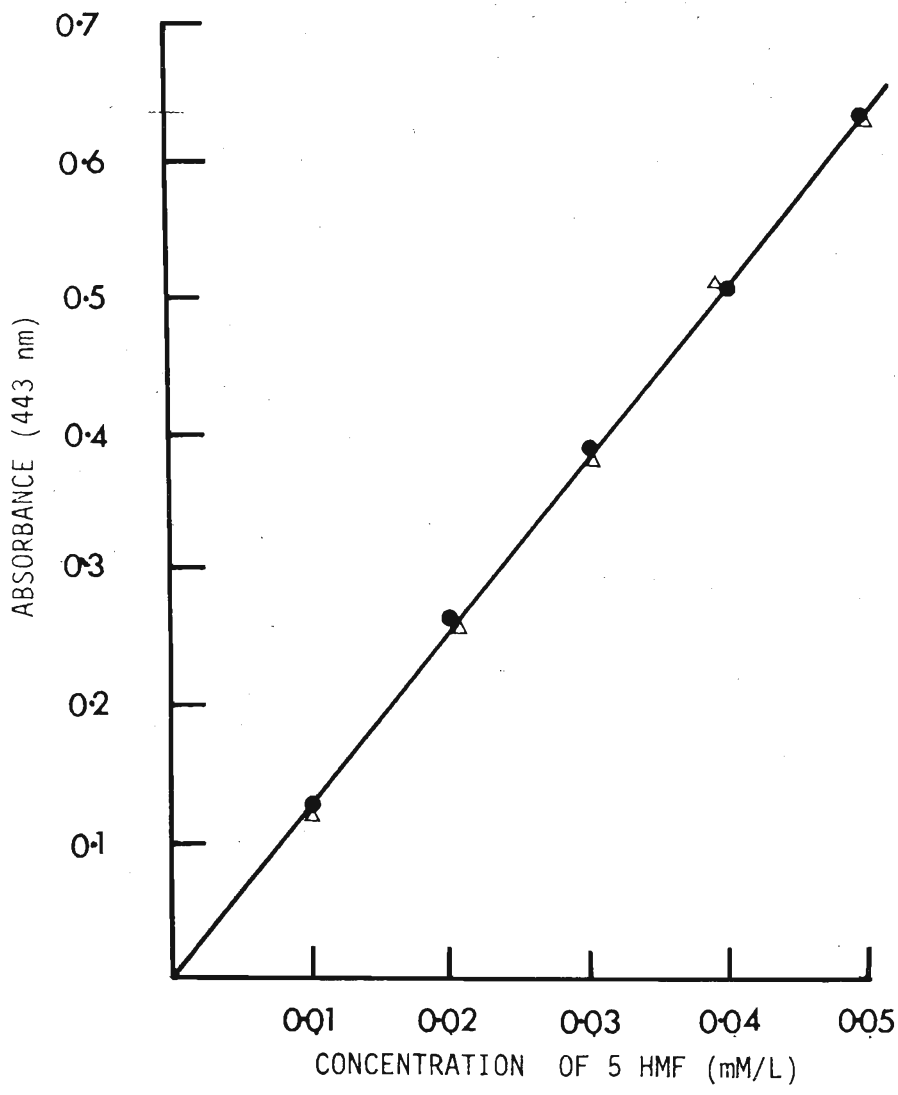

Figure 7. Standard curve for 5–HMF
- ● Standards carried right through the assay
- △ Standards carried right through the assay without heating.

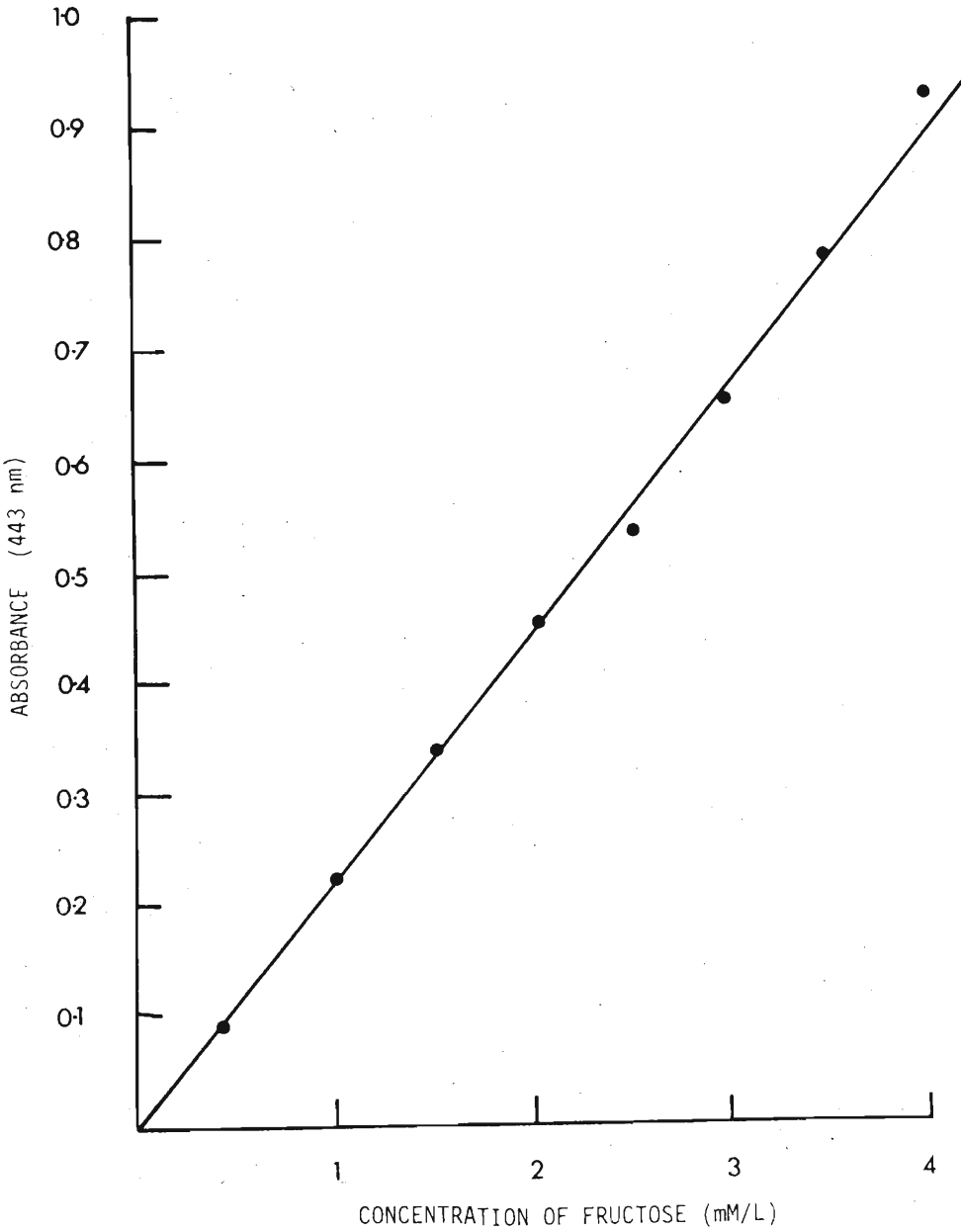

Figure 8. Standard curve for β—D—fructose.

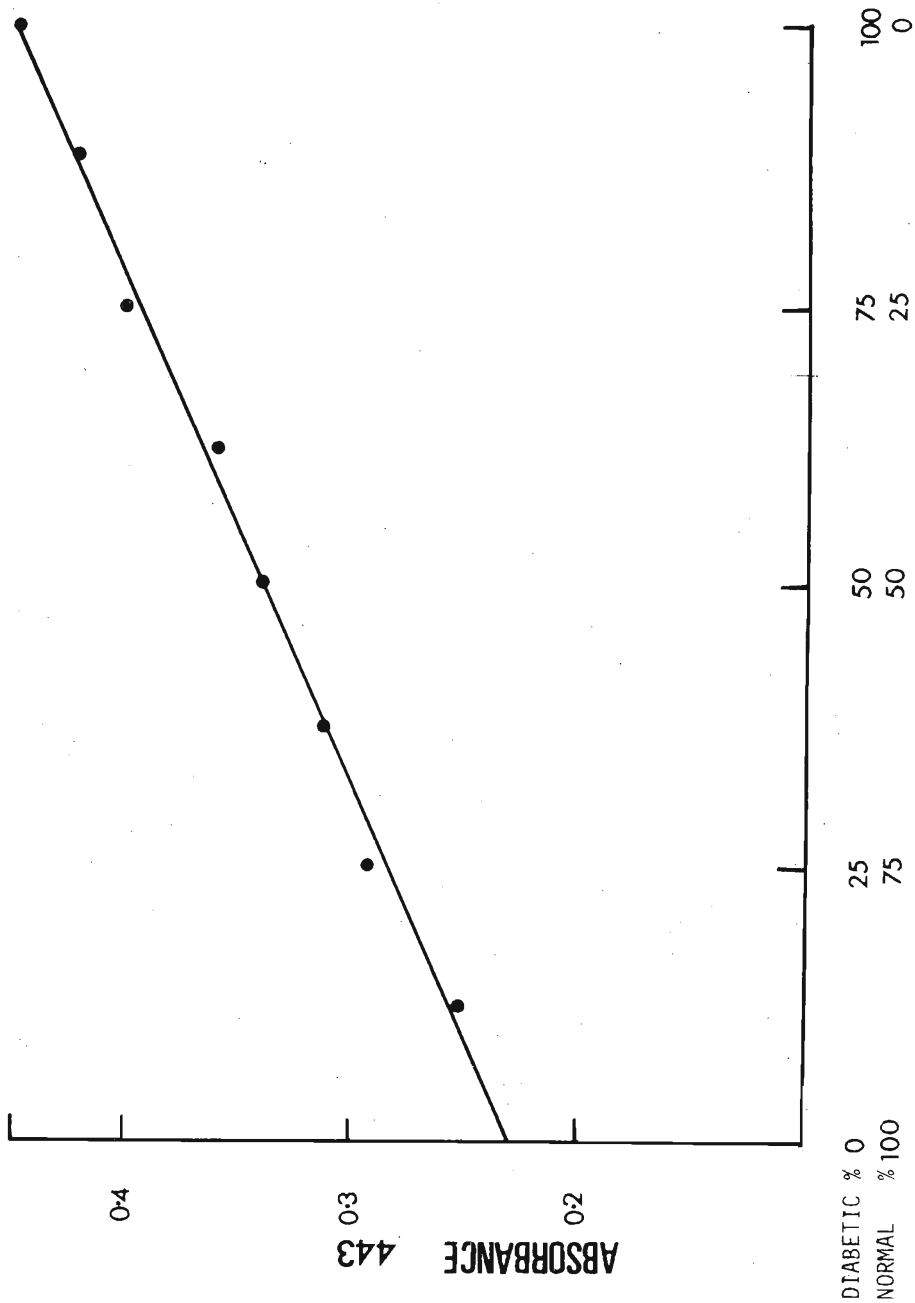

Figure 9. Effect on colour development with increasing percentage diabetic haemolysate.

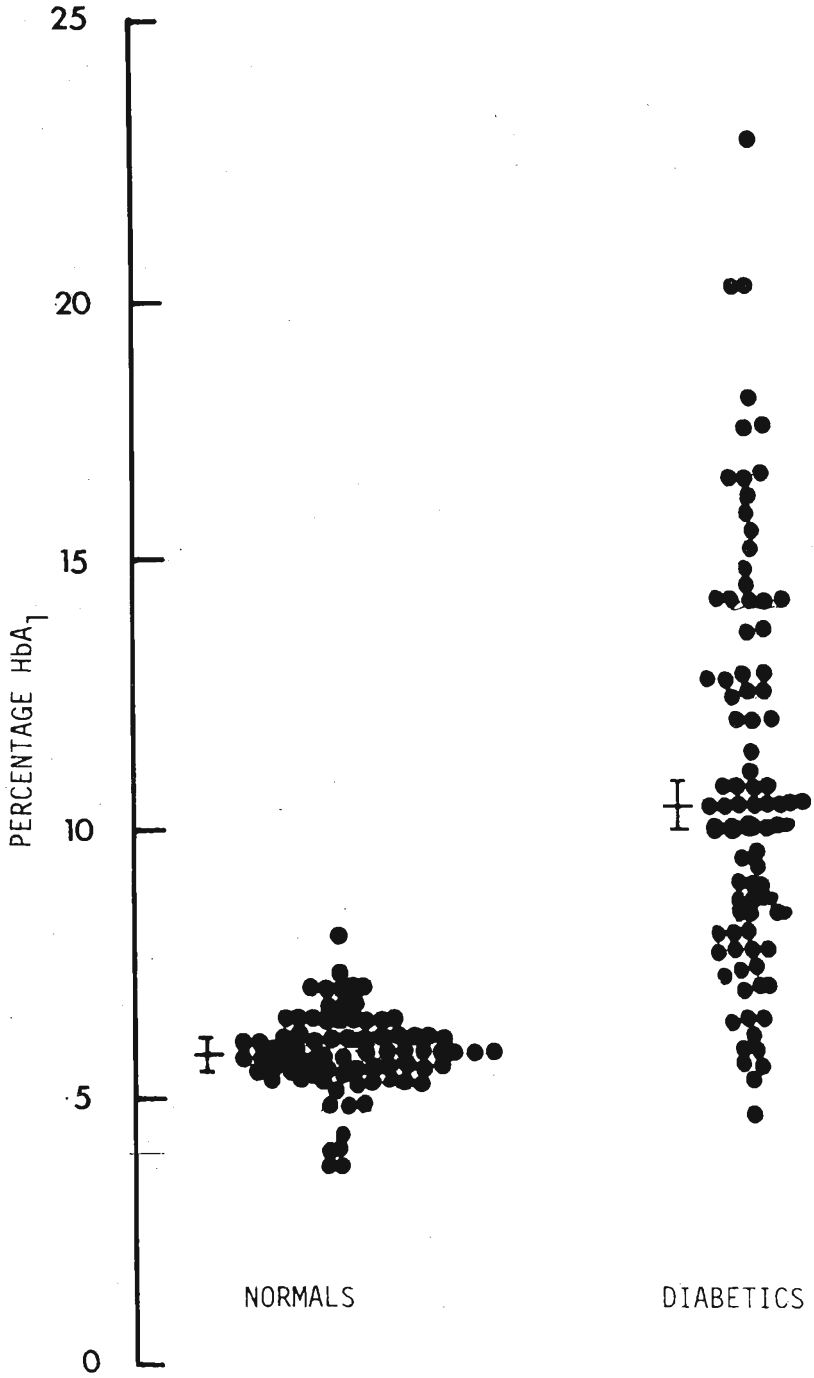

Figure 10. Glycosylated haemoglobin levels in 107 diabetic subjects and 100 normals Mean ± SEM are indicated by the bars.

Presently 30 samples a day are analysed for HbA_1. Average time of assay is approximately 3 hrs.

The colorimetric method for the determination of glycosylated haemoglobin in recent times has received much attention. It meets many of the criteria for an ideal laboratory test. Costwise, it is very cheap. About 2% of the cost for commercial kit methods and needs negligible initial investment. It has a high precision. The method is not laborious and less time consuming than most column methods. This is an important factor for a routine assay. It is easy to standardize between laboratories. The technique does not need specialised equipment and expertise. Therefore even regional hospitals in Sri Lanka can carry out the assay. If necessary the assay could be automated.[6,22] It has no interference from the labile fraction[3,14] and samples could be stored for a fair period before they are assayed.

Apparent disadvantages are the use of cyanide solutions and the lack of glycosylated haemoglobin standards. However, as shown earlier the latter can be overcome by the use of fructose standard solutions and/or pooled normal and diabetic haemolysates previously stored at $-20^{\circ}C$ or better $-70^{\circ}C$. Also the assay does not measure HbA_1 or HbA_{1c} but overall glycosylation. But as definite relationships have been established between the various indices of the colorimetric method and column method, this disadvantage is rectified. Considering the overall factors, the colorimetric method proves to be the most suitable method for the measurement of glycosylated haemoglobin as a routine assay to evaluate long term glycaemic control.

Acknowledgements

We are extremely grateful to Professor Rune Liminga, Head of the International Seminar in Chemistry, Uppsala, for providing the microcolumns.

References

1. ABRAHAM, E. C., HUFF, T. A. & COPE, N. D. (1978). *Diabetes*, **27**: 931 – 7.

2. ALLEN, D. W., SCHROEDER, W. A. & BALOG, J. (1958). *J. Am. Chem. Soc.*, **80**: 1628 – 1634,

3. BOLLI, G., COMPAGNUCCI, P. & CARTECHINO, M. G. *et al.* (1980). *Diabetologia*, **18** : 125 – 130.

4. BUNN, H. F., HANEY, D. N., GABBAY, K. H. & GALLOP, P. (1975). *Biochem. Biophys. Res. Commun.*, **67**: 103.

5. BUNN, H. F., HANEY, D. N., KAMIN, S., GABBAY, K. H. & GALLOP, P. M. (1976). *J. Clin. Invest.*, **57**: 1652.

6. BURRIN, J. M., WORTH, R. & ASHWORTH, L. A. *et al.*(1980). *Clin. Chim. Acta.*, **105**: 45 – 50.

7. CANNAN, R. K. (1958) *Science*, **127**: 1376 – 1378.

8. COLE, R. A., SOELDNER, J. S. & DUNN, P. J. *et al.*(1978). *Metabolism*, **27**: 289 – 301.

9. DOLHOFER, R. & WIELAND, O. H. (1981) *Clin. Chim. Acta.*, **112**: 197 – 204.

10. FISCHER, R. W., DE JONG, C. & VOIGT, E. *et al.*(1980). *Clin Lab. Haemat.*, **2**: 129 – 138.

11. FLUCKIGER, R. & WINTERHALTER, K. H. (1976). *FEBS Letters*, **71**(2): 356 – 360.

12. GABBAY, K. H., SOSENKO, J. M. & BANUCHI, G. A. *et al.* (1979). *Diabetes*, **28**: 337 – 340.

13. GALLOP, P. M., FLUCKIGER, R. & HANNEKEN, A. *et al.*(1981). *Anal. Biochem.* **117**: 427 – 432.

14. GOLDSTEIN, D. E. & PARKER, K. M. *et al.* (1982). *Diabetes*, **31** : 70 – 78.

15. HUGGET, A. St. G. & NIXON, D. A. (1957). *Lancet* **11**: 368 – 370.

16. JAVID, J., PETTIS, P. K., KOENIG, R. J. & ANTHONY, C. (1978) *Br. J. Haemat.*, **38**: 329.

17. KOENIG, R. J., *et al.* (1976). *N. Engl. J. Med.*, **295** : 417 – 420.

18. MALLIA, A. K., HERMANSON, G. T. & KROHN, R. I. *et al.*(1981). *Anal. Letters*, **14**: 649 – 661.

19. MENARD, L., DEMPSEY, M. E. & BLANKSTEIN, L. A. *et al.*(1980). *Clin. Chem.*, **26** : 1598 – 1602.

20. NAYAK, S. S. & PATTABIRAMAN, T. N. (1981). *Clin. Chim. Acta.*, **109**: 267 – 274.

21. PECORARO, R . E., GRAF, R. F. & HALTER, J. B. *et al.*(1979). *Diabetes*, **28**: 1120 – 1125.

22. ROSS, I. S. & GIBSON, P. F. (1979) *Clin. Chim. Acta.*, **98**: 53 – 59.

23. SAIBENE, V., BREMBILLA, L. & BERTELOTTI *et al.*(1979). *Clin. Chim. Acta.*, **93**: 193 – 205.

24. SUBRAMANIAM, C. V., RADHAKRISHNAMURTHY, B. & BERENSON, G. S. (1980) *Clin. Chem.*, **26**: 1683 – 1687.

25. TRIVELLI, A. L., RANNEY, N. T. & LAI, N. (1971). *N. Engl. J. Med.*, **284**: 353.

26. VAN KAMPEN, E. J. & ZIJLSTRA, W. G. (1961). *Clin. Chim. Acta.*, **6**: 538 – 544.

27. WORSLEY, L. (1979) *A. C. B. News Sheet*, **198**: 15.

28. WORTH, R., ASHWORTH, L., BURRIN, J. *et al.*(1978). Personal communication.