

A semi-micro colorimetric method for estimating glycosylated haemoglobin

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A simple, rapid, precise and inexpensive colorimetric method is described, for estimating glycosylated haemoglobin (HbA1) using a drop of blood obtained from a finger prick. Values obtained, correlated very well with those of column chromatography ($P < 0.001$). The regression formula derived could be used to express the values in the conventional way as the percentage of total haemoglobin. Analysis of samples from 100 non-diabetics, 200 overt diabetics and 100 subjects, with impaired glucose tolerance gave distinct ranges for each group, without any overlapping between groups. The method measures the stable, ketoamine-linked hexose, without requiring pretreatment of the samples. Hence, the values can be used as an objective index of long-term blood glucose control, in the management of diabetic patients. The feasibility of using this method, for estimating HbA1 in blood samples, stored as dried spots, on a special type of filter paper, offers the possibility of mailing blood samples, before visiting a clinic. Further, the method should be more acceptable to all patients particularly children.

Glycosylated haemoglobin (HbA1) assay has gained special importance in the assessment of glycaemic control in diabetic patients. It is widely accepted as an objective, time-averaged index of blood glucose control over the preceding 6 to 8 wk. Currently, there are a variety of chromatographic electrophoretic and colorimetric methods that are being used for the estimation of HbA1^{1,2}. Each method has some drawbacks and technical limitations which include measurement of the aldimine-linked labile precursor pre A₁C along with the ketoamine-linked stable form of HbA1c¹. Methods suggested for the removal of the labile pre A1c are laborious, expensive and time consuming³. As a result, the HbA1

values are often not available at the time of the clinical assessment of a diabetic patient.

We describe here a simple, inexpensive, and precise colorimetric method for estimating HbA1 in a drop of blood collected from a finger prick. We have also investigated the feasibility of estimating HbA1 in blood spots collected and dried on a filter paper by comparing the HbA1 values with those obtained from freshly collected blood samples.

Material & Methods

Blood samples were collected using EDTA as the anticoagulant from 200 overt diabetics and 100 subjects with impaired

glucose tolerance (IGT) attending the Diabetes Research Centre and M.V. Hospital for Diabetes, Madras. Control samples were obtained from 100 non-diabetic, healthy, volunteers among the house staff.

Dried blood samples : Blood was spotted on 2 spots covering a circular area of 1.5 cm diameter on a special type of filter paper (Grade 2992 Schleicher and Scheull, FRG) pretreated with a solution of glucose oxidase and dried at room temperature. The filter papers with the blood spots were air dried and stored at room temperature (25°C) for two weeks before estimating HbA1.

Estimation of HbA1 in fresh blood : The method used to estimate HbA1 was the same as described elsewhere⁴ with modifications as given below :

100 μ l of whole blood, collected in EDTA, was added to 0.9 ml of distilled water in a screw cap glass tube (Kimax 13 \times 108 mm), mixed, and allowed to stand for 5 min for complete lysis of the red blood cells. 100 μ l of this diluted blood was used, for the estimation of haemoglobin, by cyanmethaemoglobin method. 1.0 ml of 40 per cent TCA was added to the remaining solution, mixed, and centrifuged at 1500 \times g for 10 min. The supernatant was drawn out and discarded. To the precipitate in the tube, 2 ml of 2.5 M orthophosphoric acid was added. The tubes were capped tightly with teflon lined caps, and placed in a hot air oven at 100°C for 1 h. The tubes were taken out and cooled in ice-cold water. 1 ml of 40 per cent TCA was added to precipitate the protein, and the tubes were centrifuged at 1500 \times g for 15 min. 2 ml of the clear supernatant was placed in a glass

tube (10 \times 75 mm) containing 0.8 ml of a 50 mM solution of TBA, mixed, and incubated at 40°C for 30 min. The tubes were cooled to room temperature, and the absorbance was read at 443 nm in a spectrophotometer (ECIL, India). Reagent blanks were run simultaneously, by adding 0.8 ml of TBA to 2 ml of a mixture of 40 per cent TCA and 2.5 M orthophosphoric acid (1 : 2 by volume). Absorbance obtained for blanks were subtracted from the sample readings. The values were expressed as nmol 5-hydroxymethyl furfural (HMF) released per mg of haemoglobin (Hb) by using the millimolar extinction co-efficient of the adduct formed between HMF and TBA⁵.

To express the values of HbA1, as per cent total Hb, separate aliquots of the blood samples used for the colorimetric method, were chromatographed on commercially available cation exchange minicolumns (Biorad, Richmond, California). Assays were performed in duplicate and values obtained, were corrected for temperature variation, according to the manufacturers temperature correction chart, and by using calibrators, provided in the kit. Linear regression analysis was done to determine the correlation between the values obtained by the colorimetric and column chromatographic methods.

Estimation of HbA1 from dried blood spots : The blood spots were cut out and placed in a screw cap glass tube (Kimax) containing 1 ml of distilled water. The tubes were allowed to stand for 1 h at room temperature for complete elution of the blood. The filter paper was removed and the haemolysate obtained was processed for the estimation of HbA1 as described earlier.

Plasma glucose was measured by the orthotoluidine method⁶.

Linear correlations were calculated by the least squares' method. Statistical analysis was done by Student's 't' test.

Results

Correlation with column chromatography : A good correlation was found (Fig. 1) between results obtained in the colorimetric assay and those of the mini-column chromatography ($r=0.82$; $y=2.9x+2.42$; $n=50$; $P<0.001$).

Non-diabetic subjects : A comparison of the results obtained in non-diabetic subjects with those of overt clinical diabetics and IGT subjects is shown in Table-I.

In the colorimetric assay, values for HbA1 in non-diabetic subjects ($n=100$) ranged between 1.2 to 1.85 nmol of HMF formed per mg of total haemoglobin with a mean (\pm SD) of 1.4 ± 0.15 nmol HMF/mg Hb. Using the regression formula shown in Fig. 1, this corresponds to a HbA1 range of 5.9 to 8.0 per cent with a mean (\pm SD) of 6.5 ± 0.7 per cent.

Overt diabetics and IGT subjects : Values for overt diabetic patients ($n=200$) ranged between 2.3 to 3.5 nmol HMF/mg Hb, (HbA1=9-13%), with a mean (\pm SD) of 3.0 ± 0.22 nmol HMF/mg Hb, which corresponds to a HbA1 value of 11.1 ± 0.8 per cent. There was no overlapping of values between non-diabetic subjects and overt diabetic patients. However,

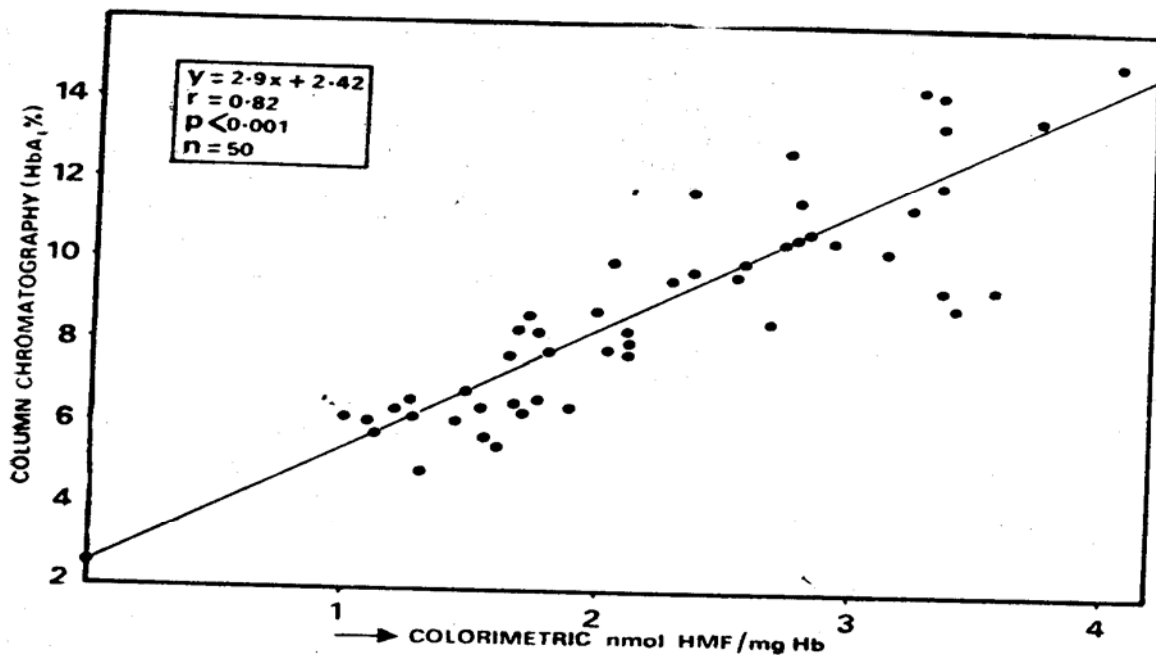


Fig. 1. Correlation of glycosylated haemoglobin levels determined by column chromatography and the colorimetric method.

Table I. Levels of HbA1 in non-diabetic, diabetic and IGT subjects
(Data are mean \pm SD)

Study group	Method of assay		Plasma glucose, (mmol/l)	
	Colorimetric method (nmol HMF/ mg Hb)	Column chromatography (%)	Fasting	Post-prandial
Non-diabetics (n=100)	1.4 \pm 0.15 (1.2—1.85)	6.5 \pm 0.70 (5.9—8)	6.0 \pm 1.2	8.2 \pm 1.6
Overt diabetic patients (n=200)	3.0 \pm 0.22** (2.3—3.5)	11.1 \pm 0.80** (9—13)	10.3 \pm 4.7	14.5 \pm 5.1
Subjects with impaired glucose tolerance (n=100)	2.2 \pm 0.06† (2.0—2.3)	8.8—0.26† (8—9)	6.3 \pm 0.9	9.1 \pm 0.8*

Figures in parentheses indicate the range.

*indicates the 2 h value; ** versus non-diabetic subjects : $P < 0.001$; †versus non-diabetic subjects : $P < 0.01$

patients with impaired glucose tolerance (n=100) as defined by the criteria of WHO had values for HbA1 ranging between 2.0 to 2.3 nmol HMF/mg Hb, (HbA1 8 to 9%) with a mean (\pm SD) of 2.2 \pm 0.06 nmol of HMF/mg Hb, corresponding to a HbA1 value of 8.8 \pm 0.26 per cent.

Method precision : Intra-assay coefficient of variation was 3.2 per cent at 7 per cent HbA1 and 4.0 per cent at 12 per cent HbA1. Inter-assay coefficient of variation was 3.5 per cent at 7 per cent HbA1 and 4.6 per cent at 12 per cent HbA1. These measures include the variability contributed by the step involving the determination of total haemoglobin.

Comparison of results with fresh samples and samples dried on filter paper : In all the ranges of HbA1 tested, viz., 6-8 per cent (non diabetic range), 8-9 per cent

(IGT range), 9-12 per cent and >12 per cent (diabetic range), there was excellent agreement between the values obtained for the fresh samples and those obtained after elution from dried spots on filter paper (Table II). This was further substantiated by the data obtained from a scattergram of the actual HbA1 values obtained for the fresh and dried blood samples (Fig. 2), which indicate an excellent positive correlation ($r=0.97$; $P < 0.001$) between HbA1 concentrations of the fresh and dried blood samples and the regression formula obtained was $y = x + 0.13$ ($y =$ Dried sample), ($x =$ Fresh sample) with a slope of 0.999 and a Y intercept of 0.13.

Discussion

The colorimetric methods, based on the measurement of 5-hydroxymethyl furfural, liberated from the ketoamine-linked

Table II. Comparison of the HbA₁ values of the fresh and dried blood samples
(Data are mean \pm SD)

HbA ₁ range (%)	Glycosylated haemoglobin (%)	
	Fresh samples	Dried on filter paper
6-8 (n=11)	7.5 \pm 0.3	7.8 \pm 0.7
8-9 (n=26)	8.6 \pm 0.3	8.6 \pm 0.4
9-12 (n=22)	10.7 \pm 0.5	10.9 \pm 0.7
> 12 (n=9)	13.2 \pm 1.2	13.4 \pm 1.0

Samples were assayed by the colorimetric method and the results expressed as % of total haemoglobin using the linear regression formula $Y = 2.9x + 2.42$

hexose by mild acid hydrolysis, is the only method which measures the stable glycosylated haemoglobin⁷. This obviates the need for any pretreatment of the sample or the haemolysate and thus it should be the method of choice, for laboratories that require precise, inexpensive means of assessing long term glycaemic control, without interference from the fast changing labile precursor pre-A_{1c}.

The well known major drawbacks of the colorimetric assays such as the long duration of hydrolysis and the problems encountered in the standardization procedure⁷ have been overcome in the assay reported here by us.

The conditions described for hydrolysis (1 h at 100°C) in the present method

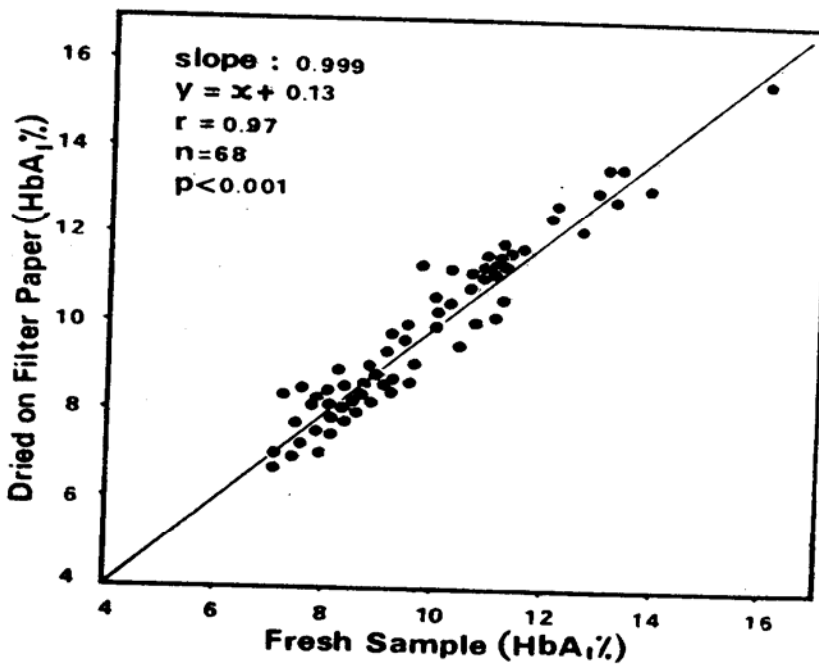


Fig. 2. Relation between glycosylated haemoglobin concentration measured in the fresh blood samples and those of the same samples dried on filter paper.

have been found⁴ to be ideal for the optimal liberation of HMF. The problems encountered while using pure HMF as the standard each time have been overcome by using the millimolar extinction coefficient of the HMF-TBA adduct as reported elsewhere⁵. This would imply that every laboratory adopting this procedure should determine the range for non-diabetic and diabetic populations.

Our results indicate that, with this assay, a clear distinction could be made between values for non-diabetic and diabetic subjects with no overlapping between the two. Further, subjects with IGT, did show a marginal, but distinct increase, over the non-diabetic range, which has not been reported earlier using other methods of HbA1 determination.

The good correlation observed between the results obtained in the colorimetric assay, and column chromatography, also facilitates expression of the results as percentage total Hb which is compatible with the conventional manner of expressing HbA1 values.

The colorimetric assay described here is simple, relatively rapid, and needs no special equipment. A large number of samples can be handled simultaneously. The material cost of the test is low, and hence, ideally suited for routine use, even by small, non-specialized laboratories.

The excellent agreement observed between results obtained for the fresh blood samples, with those of the dried samples on filter paper throws open the possibility of mailing the blood samples by patients living in remote places. The

filter paper technique avoids venepuncture and requires only a few drops of blood. Hence the technique may be expected to have a high degree of patient acceptance, and also prove to be ideal for a children as also for epidemiological studies on diabetes in large populations.

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