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*Short Communications*

# A Rapid Micro-Scale Method for the Measurement of Haemoglobin $A_{1(a+b+c)}$

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**Summary.** A rapid method is described for the measurement of total glycosylated haemoglobins ($HbA_{1(a+b+c)}$. The procedure utilizes 0.05 ml of blood and takes forty minutes to complete manually. Eighty blood samples can be analysed without automation by one person in a day. Each analysis uses less than 2 mg of potassium cyanide, resulting in a method that is both safe and rapid for routine hospital laboratories. The inter-assay coefficient of variation was 4% and that for intra-assay measurements 3%, over the range 5–20% $HbA_{1(a+b+c)}$. The method confirmed that the level of $HbA_{1(a+b+c)}$ is elevated in imperfectly controlled diabetics. Amongst patients with blood glucose levels of less than 10 mmol/l the mean level of $HbA_{1(a+b+c)}$ was found to be 8.5%; samples from 14 known diabetics gave a mean value of 10.9%, whereas 17 known non-diabetic samples gave a mean value of 8.3%. In the group of samples from 27 diabetic individuals with blood glucose levels above 10 mmol/l the mean level of $HbA_{1(a+b+c)}$ was found to be 13.5%.

**Key words:** Haemoglobin $A_{1(a+b+c)}$, glycosylated haemoglobins, diabetes, diabetic control.

In addition to the two major haemoglobins of the human erythrocyte (HbA and $HbA_2$), several minor components of haemoglobin have been identified [1]. Some of these modified forms of haemoglobin result from the attachment of small molecules to the $\beta$ chain. The reduction of positive charge results in the fast migration of these modified molecules in some of the methods normally used for the electrophoresis and chromatography of haemoglobins. The three fastest moving components are known as $HbA_{1a}$, $HbA_{1b}$, and $HbA_{1c}$. $HbA_{1c}$ has a glucose residue firmly attached to the N terminal end of the $\beta$ chain for the remainder of the life span of the erythrocyte. A two-fold increase in the level of $HbA_{1c}$ has been reported in diabetics [2]. Similarly the levels of $HbA_{1a}$ and $HbA_{1b}$ are raised in diabetics [3]. It has been suggested that the measurement of these three haemoglobins would be valuable for the assessment of past increases in blood glucose [4]. Previous methods for the measurement of $HbA_{1a}$, $HbA_{1b}$ and $HbA_{1c}$ by ion-exchange chromatography have been long (up to 24 h) and used considerable quantities of potassium cyanide per sample. Such procedures have not been practicable for routine hospital laboratory screening, so that a rapid safe method is required for the benefits of this estimation to be available for diabetic care generally.

## Methods

Venous blood samples collected for glucose analysis into fluoride-oxalate tubes were used. Blood (0.05 ml) was mixed with 1.0 ml NaCl (0.9 g/100ml) and 0.5 ml of Buffer A (5.20 g $NaH_2PO_4.2H_2O$; 1.20 g $Na_2HPO_4$; 0.1 g KCN; 0.5 g KCl; dissolved in 1 litre of water and adjusted to pH 6.74). After centrifugation at 2000 g for 5 min the supernatant was removed and 0.5 ml of Buffer A was added. After mixing, the cells were lysed either by brief sonication or by a single freezing (−20° C) and thawing. The preparation of lysates was carried out within 24 hours of sample collection. Aliquots of whole blood and lysates were stored at 4° C and −20° C respectively to assess the stability of $HbA_{1(a+b+c)}$.

Bio-Rex 70 cation exchanger, sodium form, 100–200 mesh (Bio-Rad Laboratories) was equilibrated with Buffer A to a pH of 6.74, and poured

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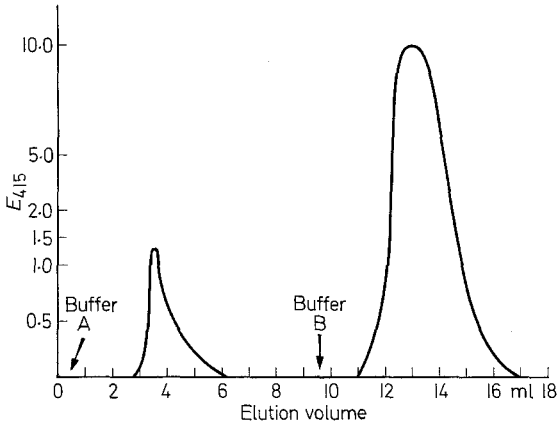


**Fig. 1.** Elution profile of haemoglobin (measured at 415nm) from a Bio-Rex 70 column, showing the complete separation of $HbA_{1(a+b+c)}$ from the major haemoglobin fraction

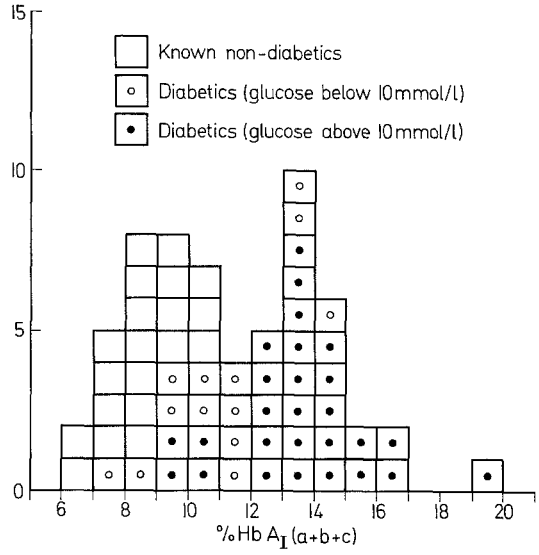


**Fig. 2.** Histogram showing the distribution of $HbA_{1(a+b+c)}$ levels amongst diabetics and non-diabetics

into a small chromatography column 10 cm × 0.7 cm (Bio-Rad 737–1222) to a packed height of approximately 8 cm. The lysate was layered onto the surface of the resin, the column tap opened fully and 9 ml of the eluate collected into a measuring cylinder. The volume of the eluate was adjusted to 10 ml with water. This fraction (F1) contained $HbA_{1(a+b+c)}$. The tap was then closed, and Buffer A was replaced by Buffer B (28.4 g $Na_2HPO_4$; 7.8 g $NaH_2PO_4.2H_2O$; dissolved in 1 litre of water and adjusted to pH 7.40). Further eluate (5 ml) was then collected into a 50 ml measuring cylinder and the volume made up to 50 ml with water. This fraction (F2) contained all the other components of the haemoglobin mixture in the lysate. The absorbance of F1 and F2 was measured at 415 nm and the percentage $HbA_{1(a+b+c)}$ calculated:–

$$\% \ HbA_{1(a+b+c)} = \frac{E_{415}F1 \times 10}{(E_{415}F1 \times 10) + (E_{415}F2 \times 50)} \times 100$$

Using these small chromatography columns with large mesh cation exchanger the chromatographic part of the analysis could be completed manually in less than 20 min. Automation of the absorbance measurements would allow rapid completion of the assay.

The pH of Buffer A and the equilibrated Bio-Rex 70 was found to be critical. A pH of 6.74 appeared to be most suitable, giving complete separation (see Figure 1). At pH 6.78 and above, the major haemoglobins were not retained fully on the column and would contaminate the $HbA_{1(a+b+c)}$ fraction. Conversely at a pH below 6.70, whilst the $HbA_{1a}$ and $HbA_{1b}$ were still eluted at the buffer front, most of the $HbA_{1c}$ was still retained on the column even after 15 ml of Fraction 1 had been eluted. Isoelectric focusing was used to demonstrate the haemoglobin components present in these eluates, and showed no cross contamination.

## Clinical Material

The measurements of $HbA_{1(a+b+c)}$ reported here were made on samples sent for blood glucose estimation to the clinical laboratories in a single day. The identification of known diabetics and non-diabetics was undertaken after completion of all estimations.

## Results

The micro-scale method was shown to be highly reproducible. In an experiment where 10 separate estimations were carried out on a single blood sample with a mean level of 8.49%, the range obtained was 8.26–8.76%. Replicate estimations were found to have been done on 5 patients bled three to five times on one day and the replicates gave coefficients of variation of under 6% (mean 4%). Where measurements were repeated in 10 separate assays the coefficient of variation was 3.5%. Repeat assays on whole blood or lysates stored for up to 7 days showed no significant changes in the levels of $HbA_{1(a+b+c)}$. Lysates prepared in duplicate by sonication and freezing and thawing gave indistinguishable results.

$HbA_{1(a+b+c)}$ levels were measured in 60 patients for whom blood glucose estimations had been carried out. Amongst this group were some known diabetics and non-diabetics, and the distribution of their levels of $HbA_{1(a+b+c)}$ is shown in Figure 2. Two distinct populations are apparent, with an overlap in the region of 11–12%. The diagnostic categories are indicated in this figure to demonstrate the overlap. Patients with a blood glucose of less than 10 mmol/l were found to have a mean level of $HbA_{1(a+b+c)}$ of 8.5%, whilst those with blood glucose levels above 10 mmol/l had values with a mean of 13.8%. All six individuals with $HbA_{1(a+b+c)}$ values of 11% or more were found on enquiry to be diabetic. No change in $HbA_{1(a+b+c)}$ levels were seen in samples from patients whose blood glucose levels changed during the day by up to 15 mmol/l, the values giving the same coefficient of variation as was found in similar samplings where the blood glucose level changed by less than 5 mmol/l.

Correlation coefficients [5] calculated between $HbA_{1(a+b+c)}$ levels and blood glucose levels for the whole group and for all the known diabetics were 0.82 and 0.73 respectively. For the non-diabetics and for the diabetics with blood glucose levels below and above 10 mmol/l the correlation coefficients were 0.4, 0.45 and 0.64 respectively.

## Discussion

The micro-scale chromatographic procedure described in this communication offers a rapid and reliable method for the measurement of $HbA_{1(a+b+c)}$. It could easily be adopted as a routine procedure in hospital laboratories and partially automated. Our results confirm that increased levels are found in imperfectly controlled diabetes mellitus. Also we have shown that the levels of $HbA_{1(a+b+c)}$ are not subject to short term fluctuations and that the values obtained by our method are very similar to those found by previous published methods. Since single 'spot' blood glucose estimations do not adequately reflect diabetic control it is not surprising that the correlation with $HbA_{1(a+b+c)}$ was not higher. The value of $HbA_{1(a+b+c)}$ estimations lies in the assessment of overall elevation of blood glucose levels in the recent past, since extensive glucose sampling cannot easily be achieved. This assay makes possible the routine use of $HbA_{1(a+b+c)}$ levels for the monitoring of diabetic care.

Recently [6] a method has been published for the estimation of glycosylated haemoglobins using a chromatographic procedure similar to the one described in this paper. The method however required 0.5 ml of blood, $2^1/_2$ h to complete and 70 mg potassium cyanide per sample.

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