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The Chromatographic Separation of Glycosylated Haemoglobins: A Comparison between Macro- and Micromethods

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Summary: The microchromatographic determination of "fast haemoglobins" is currently used in long-term control of diabetes: its use is made easier by commercially available kits, that are impaired by some analytical variables. For a good standardization of these kits a comparison with an accurate method of analysis is necessary. The macrochromatographic column allows the determination of each fast component with a good precision, and reveals the great complexity of human haemoglobin. Comparison of the two methods shows that the most appropriate temperature of elution for the kit is 20 °C and that the increase in the fraction of "fast haemoglobins" in diabetic samples is only related to the HbA_{1c} component.

Chromatographische Trennung glykosylierter Hämoglobine: Vergleich zwischen Makro- und Mikromethode

Zusammenfassung: Die Mikromethode zur chromatographischen Bestimmung der „schnellen Hämoglobine" ist heute in der Langzeitkontrolle des Diabetes üblich. Ihr Gebrauch wird durch käufliche Reagenziensätze erleichtert, jedoch durch einige analytische Variable beeinträchtigt. Für eine gute Standardisierung dieser Reagenziensätze ist der Vergleich mit einer Analysenmethode hoher Richtigkeit erforderlich. Die Makromethode erlaubt die Bestimmung jeder der schnellen Komponenten mit guter Präzision und offenbart die große Komplexität des menschlichen Hämoglobins. Der Methodenvergleich zeigt, daß die geeignetste Temperatur zur Elution bei der Mikromethode 20 °C ist und der Anstieg der schnellen Hämoglobine bei Diabetikern nur die HbA_{1c}-Komponente betrifft.

Introduction

Several methods of quick analysis of glycosylated haemoglobins are proposed (1) and presently the most used is a microchromatographic one (2), aided by the use of commercially available kits (BioRad, Helena Lab., Isolab). It is well known that these kits, which allow the determination of all "fast haemoglobins" without discrimination of the single components, are affected by some analytical variables (3,4,5). For control and comparison we have used an analytical macrochromatographic method, a variation of that used by *McDonald* et al. (6) for preparative purposes. In fact this is one of the most accurate techniques currently available, because it allows the simultaneous determination of the percentage of each chromatographically separable component.

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Materials and Methods

Sample preparation

Venous blood was collected in EDTA tubes from diabetic and normoglycaemic volunteers and processed in the same day of collection. After washing the erythrocytes (4 times), lysis was performed with distilled water (30 min at room temperature); after a salting out step (NaCl about 0.4 mol/l, 4 °C, 10 min) under mild stirring, centrifugation (15000 *g*, 4 °C, 30 min) was performed. The supernatant, after dialysis against distilled water, was submitted to a second centrifugation (60000 *g*, 4 °C, 60 min). Samples with a fraction of HbF greater than 0.01 (7) were not submitted to the subsequent chromatography.

Macrochromatographic method

For the macrochromatography on Bio-Rex 70 (BioRad; 200–400 mesh) a sample of about 150 mg of Hb (3.0 ml), in the carbon monoxide form, dialysed against the column buffer, was loaded onto a 2 cm^2 × 50 cm column. Flow rate was 35 ml/h and the fraction volume 3.5 ml; elution temperature was 4 °C. Hb components were eluted with a K phosphate buffer 0.05 mol/l (see Results, fig. 1). The content of each component was computed as described by *Schroeder & Huisman* (2), and is given as fractions.

Microchromatographic method

We selected the commercially available BioRad kit because of our experience with this system. The technique suggested by BioRad was used, with temperature variation through an original multichambers system, and with the application of two washing with saline, prior to haemolysis, to avoid triglyceride contamination (3).

Reagents

All reagents were pure grade, purchased from Merck, Calbiochem, Ega Chemie.

Results and Discussion

Macrochromatographic method

A classical elution pattern of a diabetic blood sample is represented in figure 1; normoglycaemic and diabetic haemoglobins differ only quantitatively in the fractions of the components, and not in their position.

The component fractions determined by this method are reliable, as demonstrated by the interassay statistical data reported in table 1b (an intraassay analysis is difficult until a significative number of analyses are performed).

The macromethod, under the described conditions, gives an accurate picture of "fast components". The HbA_{1b} peak shows, in all the chromatographic experiments, two components, the earlier (HbA_{1b1}) much lower than the second one (HbA_{1b2}). Since a good resolution of these two components was not possible, we have calculated them together. Furthermore a new component is eluted between HbA_{1c} and HbA_0, perhaps HbA_{1d} or HbA_{1e} or an unknown component, which is under study. We prefer not to try an uncertain interpretation, and we have not considered this component separately, since it is not present in the microcolumn pattern. Table 1a shows the correlation coefficients of linear regression for all the components of 21 columns: only HbA_{1a1} and HbA_{1a2} appear to be related. In table 1b the data obtained from normoglycaemic and diabetic samples are reported and they show that only HbA_{1c}, as already known, is the component responsible for the increase of "fast components" observed in diabetics, being the only component related to the plasmatic glucose concentration.

Thus this method seems one of the most accurate for the analysis of "fast components" of haemoglobin (1); nevertheless is time consuming so that it is appropriate only for the standardization of other methods, but not for routine analysis. Thus we decided to use it as a control for the microchromatographic commercial kit (BioRad) used for the quick determination of HbA_1.

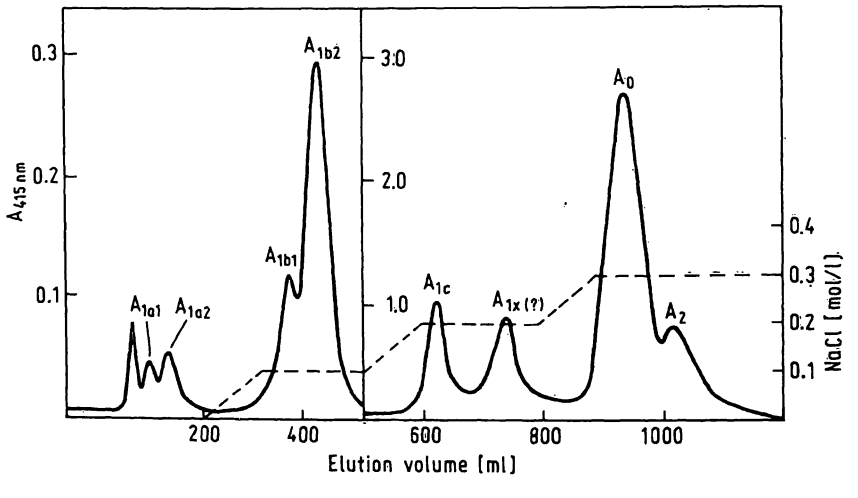

Fig. 1. Elution pattern of diabetic haemoglobin sample on Bio Rex 70 (200–400 mesh). K phosphate buffer 0.05 mol/l pH 6.55 ± 0.01. Dashed line: NaCl concentration (mol/l) in the elution buffer. HbA_{1x} is the component under study (see Results and Discussion).

Tab. 1. a) Crossed correlation coefficients among the components obtained with the macrocolumn (n = 21); the fraction values obtained with the BioRad kit at 20 °C ($Hb_{micro\,(20\,°C)}$) and their correlation to the single values are also included (see bottom line).
b) top – Interassay coefficient of variation for each macrocolumn component and their sum (total).
b) bottom – Mean fraction values for each component and their total for 7 normoglycaemic probands and 14 diabetics. The range is computed as T × S.D., but not for diabetic HbA_{1c} and the sum of values (total), where the differences between the mean and the upper value and the mean and the lower value are reported (see Results and Discussion).

a

	a_1	a_2	b_{1+2}	c
a_2	0.796			
b_{1+2}	0.224	0.141		
c	0.140	0.370	0.401	
$Hb_{micro\,(20\,°C)}$	0.163	0.211	0.490	0.908

b

	a_1	a_2	b_{1+2}	c	total
Interassay CV (%) (N = 7 × 2)	15.8	6.0	3.2	3.6	3.7
Normal $\bar{x} \pm (T \times S.D.)$ (N = 7)	0.0014 + 0.0030 − 0.0014	0.0016 + 0.0027 − 0.0016	0.0107 ± 0.0028	0.0457 ± 0.0202	0.0595 ± 0.0230
Diabetic $\bar{x} \pm (T \times S.D.)$ (N = 14)	0.0018 + 0.0027 − 0.0018	0.0016 + 0.0018 − 0.0016	0.0118 ± 0.0032	0.0897 + 0.0555 − 0.0220	0.1064 + 0.0620 − 0.0240

Microchromatographic method

The relationship (fig. 2) between the sum of HbA_{1a-c} components obtained with the macromethod and that of the fractions obtained with the microcolumn at 23 °C (Hb_{macro} and Hb_{micro} (23 °C) respectively) shows that, at the temperature suggested by BioRad and by some authors (5), the microcolumn overestimates the fraction of "fast components" by about 0.015 in absolute value and by about 0.20 as relative value.

This fact is evident from an intercept of −0.0122 and from the mean values obtained by the two methods. Because it is known that microcolumn percentages are temperature dependent (3,4,5), we studied the relationship between Hb_{micro} and temperature, and found that 20 °C is the best temperature of microelution for a very good coincidence with the macromethod. Figure 2 shows that lowering the temperature to 20 °C gives a good correlation between the two methods; probably at 23 °C the early part of the

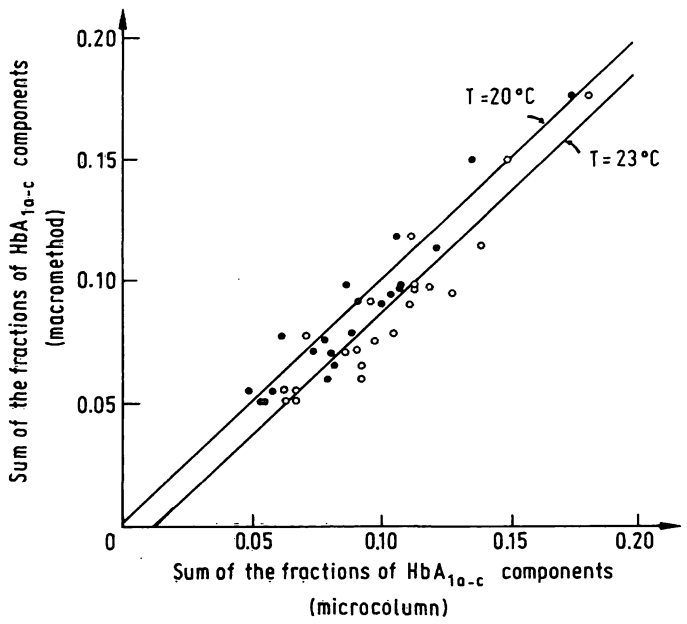

Fig. 2. Linear correlation (least square) between the sum of fractions HbA_{1a-c} obtained with the macrocolumn (Hb_{macro}) and fraction values given by the BioRad kit (Hb_{micro}) at 23 °C (O) and 20 °C (●).

T = 20 °C (n = 21)	T = 23 °C (n = 21)
$Hb_{micro} = 0.983\ Hb_{macro} + 0.0011$	$Hb_{micro} = 0.971\ Hb_{macro} - 0.0122$
r = 0.916	r = 0.914
$\overline{Hb}_{micro} = 0.0852$	$\overline{Hb}_{micro} = 0.0852$
$\overline{Hb}_{macro} = 0.0856$	$\overline{Hb}_{macro} = 0.1005$
$\sigma_{micro} = 0.0299$	$\sigma_{micro} = 0.0299$
$\sigma_{macro} = 0.0285$	$\sigma_{macro} = 0.0293$

HbA_0 peak is eluted at the volume quoted by Bio-Rad. Furthermore, owing to an almost linear relationship between temperature (T) and Hb_{micro} fractions in the range 20–28 °C, the data obtained at higher temperatures (T) can be corrected to the right value with the simple equation

$$\text{fraction } Hb_{micro\ (20\,°C)} = \text{fraction } Hb_{micro\ (T)} + 0.0378 - 0.00191\ T$$

At 20 °C the microcolumn intraassay CV is 2.8% and the interassay CV is 3.2%, which are similar to those observed for the macrocolumn. The mean value obtained at this temperature from 86 normoglycaemic samples is 0.0524 [±0.0248] (mean age 51 [+15; −10]) while that from 106 diabetic samples is 0.0966 [+0.0856; −0.0262] (mean age 53 [+15; −12]). The range of diabetics is obtained from the differences between the mean and the maximal value (upper limit) and the mean and the minimal value (lower limit) of the range; this fact derives from the observation that the diabetic range is not symmetrical, the mean being shifted towards the lowest values of the range; for the same reason this way of computation is applied also to HbA_{1c} and the sum of the fractions (total) from macrocolumns of diabetics reported in table 1b. Finally table 1a shows also the correlation coefficients between $Hb_{micro\ (20\,°C)}$ and each component coming from the macrocolumn: as expected only the HbA_{1c} component shows a good correlation ($r = 0.908$).

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