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AN AUTOMATIC METHOD FOR DETERMINATION OF GLYCOSYLATED HEMOGLOBINS USING LOW-PRESSURE LIQUID CHROMATOGRAPHY

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SUMMARY

Several studies have revealed a correlation between blood levels of glucose and hemoglobin A_{1c} (HbA_{1c}), a minor form of hemoglobin (Hb) present at elevated concentrations in patients with diabetes mellitus. To facilitate a clinical study of the level of circulating HbA_{1c} we have developed an automatic chromatographic system. An efficient separation of HbA_{1c} from HbA_0 and other rapid hemoglobins (HbA_{1a}, HbA_{1b}) was achieved on Bio-Rex-70 columns using three buffers. This system allows the daily analysis of 40 samples. The mean level of HbA_{1c} in normal subjects was $5.4 \pm 0.4\%$. The method also detects the presence of elevated levels of HbF and the most frequent forms of abnormal hemoglobin (HbS, HbC).

INTRODUCTION

The determination of the level of glycosylated hemoglobins (Hbs) provides a follow-up of diabetes mellitus [1]. The original chromatographic method by Trivelli et al. [2] is too time consuming to be widely used in clinical analysis. Several attempts have been made to set up a rapid, low-cost and reliable procedure to assay glycosylated Hbs. A method for the radioimmunoassay of HbA_{1c} proposed by David et al. [3] has not been developed on a routine basis. Recently, an affinity technique using the capacity of boronate to form complexes with 1,2-*cis*-diol has been described by Mallia et al. [4]. This technique measures the total glycosylated fractions, including those of HbA_0. Its routine use implies a precise knowledge of assayed fractions. Several groups have reported HbA_{1c} assay by gel scanning after isoelectric focusing (IEF) [5—9] with an improved separation of HbA_{1c} from HbA_0; nevertheless, the method has not yet been used for routine experiments on a large scale. Rapid chromatographic methods, especially those using microcolumns, have been developed [10—16]. Some of them propose the measurement of the total glycosylated Hbs

designated as HbA_1. Unfortunately, the preparation in the laboratory of microcolumns needed for HbA_1 determination is excessively time consuming. Commercially available microcolumns are highly temperature dependent and require careful attention [17, 18]. Therefore several groups have been prompted to develop other types of fast and automatic chromatographic methods for estimation of glycosylated Hbs using liquid chromatography under high [19, 20] or medium range [21] pressure.

This article presents the results obtained with the previously described [22] automatic peptide analyser adapted to the determination of glycosylated Hbs. A three-buffer procedure allowed complete and rapid peak separation of HbA_{1a+1b}, HbA_{1c} and HbA_0, although the pressure remained lower than 4.5 bars. Up to 200 samples could be analysed in one week with, in addition, the detection of abnormal levels of HbF and of several abnormal Hbs.

MATERIALS AND METHODS

Chemicals
The following analytical grade chemicals, $NaH_2PO_4(H_2O)$, $Na_2HPO_4(2H_2O)$, KCN, H_3PO_4, NaOH, chloroform and NaCl were obtained from Merck (Darmstadt, G.F.R.). Bio-Rex-70 resin (200—400 mesh) was supplied by Bio-Rad Labs. (Richmond, CA, U.S.A.).

Preparation of buffer solutions

Solutions	Na_2HPO_4	NaH_2PO_4	KCN	NaCl
1	0.02 M	—	0.008 M	—
2	—	0.04 M	0.008 M	—
3	0.02 M	—	0.008 M	0.1 M
4	—	0.04 M	0.008 M	0.1 M

Buffer A was obtained by mixing solutions 1 and 2 to give a final pH of 6.76 ± 0.01. Buffer B was obtained by mixing solutions 3 and 4 to give a final pH of 6.56 ± 0.01. Buffer C was obtained by adding NaCl up to 0.3 M to buffer A; no adjustment of the pH is required.

Preparation of the column resin
The resin (50 g of Bio-Rex-70, 200—400 mesh) was washed three times with 1 l of distilled water during which fine particles were discarded, then washed with 500 ml of 0.5 N NaOH and rinsed with water. The pH of the slurry was adjusted to pH 2.2 with H_3PO_4 (85%). After removal of the supernatant the resin was suspended in solution 1. Then NaOH (10 N) was added to bring the final pH to 6.76.

The resin suspension was poured in the column (6.25 mm × 15 cm) to give a 5—6 cm bed height, packing the Bio-Rex-70 resin (200—400 mesh) by pumping buffer C at a flow-rate of 17 ml/h at room temperature. The column should be conditioned by operating it during ten buffer cycles prior to any sample analysis. The back-pressure should not exceed 4.5 bars.

Equipment

This consisted of two Accu-Flow pumps (Beckman, Palo Alto, CA, U.S.A.), several pneumatic valves (CAV 3031, PA 875, SR, R 6031 V6), adjustable columns (6.25 mm × 15 cm) and connectors from Chromatronix (Berkeley, CA, U.S.A.), a Mikrorapid Centrifuge from Hettich (Tüttlingen, G.F.R.), centrifuge tubes from Eppendorf (Hamburg, G.F.R.) and an Ismatec peristaltic pump (Zürich, Switzerland). A sample distributing rack TD 15 T3 was supplied by Gilson (Villiers le Bel, France) and colorimeters (fitted with 418-nm and 570-nm filters) by Technicon (Dublin, Ireland). All connections were made with PTFE tubing (0.8 × 1.50 mm and 0.5 × 1.50 mm) (Habia, Montmirail, France). A Dynamaster recorder with two channels and point-by-point recording was obtained from Elliot Automation (Vichy, France). The integrator (ICAP 10) was made by Delsi (Suresnes, France). The programmer was constructed from Crouzet components (Valence, France) by Touzart & Matignon (Paris, France).

Samples

Normal blood samples were obtained from normal blood donors free of any medication who were tested for normoglycemia, normal mean reticulo-cytes and normal hematocrit, absence of any hemoglobinopathy and no persistence of fetal hemoglobin using IEF separation [23] and by dosage of HbF according to the method of Betke et al. [24].

Chromatographic system

A sample injector was added to the chromatographic system already described by Blouquit et al. [22]. Samples were injected on the column from the specimens in the sampler rack by means of a 20-μl loop. The filling of this loop was controlled by the peristaltic pump. Sample injection on the column was done by commutation of V_1 and V_2, the two three-way pneumatic valves shown in Fig. 1. This system was equipped with two columns, C_1 and C_2, one being equilibrated with buffer A while the other was running. Column selection was done through V_3 and V_4, the two four-way pneumatic valves (Fig. 1). The flow-rate of the pumps was 17 ml/h. Buffer selection was done through R, a six-way rotating valve (Fig. 1) under the following program. (1) Sample injection. (2) Buffer A: elution of the fast migrating fractions, i.e. HbA_{1a1}, HbA_{1a2}, HbA_{1b}, during 4 min. (3) Buffer B: elution of HbA_{1c} during 12 min. (4) Buffer C: elution of the remaining HbA_0 and HbA_2 fractions during 16 min. (5) Buffer A: rinsing the loop sampler for 2 min. The complete cycle lasts 36 min. The chromatography was performed at ambient temperature between 20°C and 25°C; no noticeable variations were observed.

Calculation

The absorbance of the eluate was measured at two wavelengths, 418 nm for HbA_{1c} and 570 nm for HbA_0. An integrator interfaced between the colorimeter (570 nm) and the recorder allowed a precise estimation of the percentage of HbA_{1c}. Moreover, it was possible to check the integration count
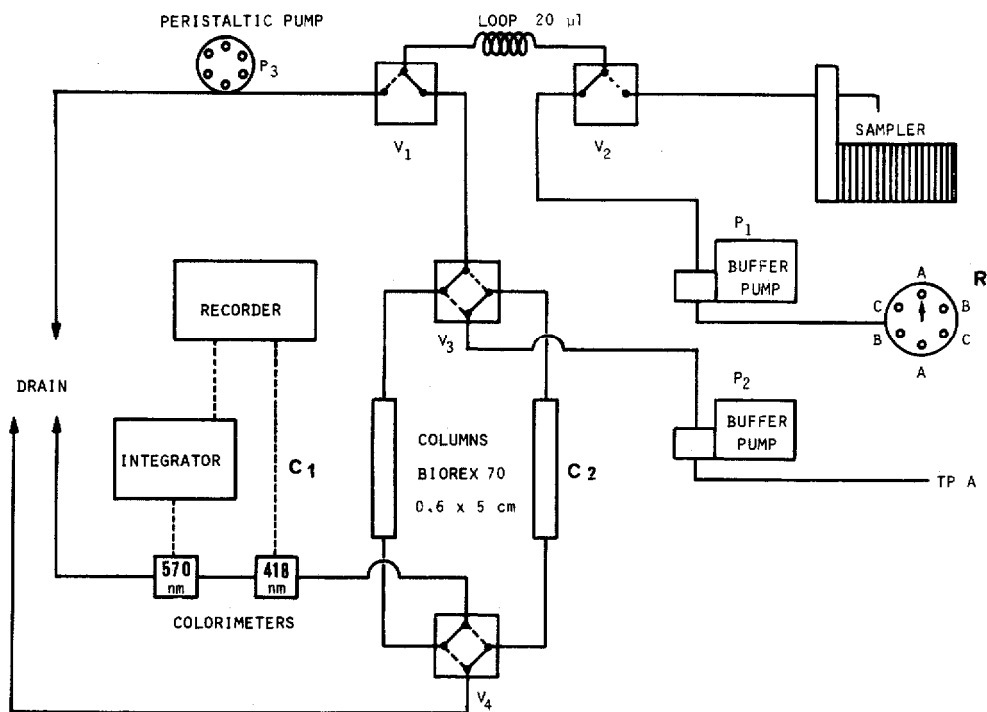
Fig. 1. Flow diagram of the chromatographic system for the determination of glycosylated hemoglobins. The sampler rack, two three-way valves (V_1, V_2) a six-way valve (R), two four-way valves (V_3, V_4), a peristaltic pump (P_3) and the buffer pumps (P_1, P_2) are controlled by a punched-card programmer.

by a manual estimation of the fraction with the formula

$$\frac{\text{Area HbA}_{1c} \ (418 \ \text{nm})}{\text{Area HbA}_{1c} \ (418 \ \text{nm}) + \text{Area HbA}_0 \ (570 \ \text{nm})} \times 10^{*}$$

Preparation of the hemolysates

Blood was withdrawn into an anticoagulant solution and stored at 4°C until use. A 1-ml aliquot of blood was washed twice with isotonic salt solution. During these washings care was taken, not to remove any red blood cells, since those present in the upper layer of the pellet were principally constituted of reticulocytes, low in HbA_{1c}. Their loss might alter the assay resulting in an artificial increase in old red cells rich in HbA_{1c}. The red blood cell pellet was hemolyzed by the addition of 600 μl of distilled water and 300 μl of chloroform. This mixture was shaken for 30 sec and then centrifuged at 4°C during 30 min at 8000 g. The supernatant was then diluted five times with buffer A and stored at 4°C for no more than 48 h. The final concentration in Hb was 10—20 g/l.

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*The ratio $\epsilon_{418}/\epsilon_{570}$ (where ϵ = molar absorptivity) of the Hb measured with the filters used has been found to be very close to 10.

RESULTS

Storage effect

The level of glycosylated Hbs was correlated with the age of the red blood cells (RBCs) [25], the time during which these RBCs were in contact with glucose [26] and perhaps with still unknown factors. For all these reasons special attention was given to the choice of the anticoagulant medium and to the conditions of storage of the samples. Blood from a normal individual was withdrawn into three different anticoagulant media, citric acid—dextrose (ACD), EDTA and heparin. No significant difference in the HbA_{1c} level was observed amongst these three samples. Since the oldest RBCs, the richest in HbA_{1c}, are the most susceptible to hemolysis, ACD was selected as anticoagulant. This medium was the most satisfactory in preventing hemolysis in stored samples. RBCs of normal volunteers and of diabetic patients were stored at $4°C$ for up to eight days. The levels of HbA_{1c}, as well as that of HbA_1 (A_{1a} + A_{1b} + A_{1c}) were not significantly modified during the eight days storage on ACD either in normal or diabetic individuals. These results were in accordance with already reported HbA_1 data using Bio-Rad microcolumns [17]. The HbA_{1c} level was also determined on fresh hemolysates diluted with five volumes of buffer A and stored at room temperature in the sample rack of the analyser. Stable levels of HbA_{1c} were observed over a period of 36 h.

Chromatographic patterns

Examples of chromatographic patterns obtained with hemolysates from a normal control and from a patient with diabetes mellitus are shown in Fig. 2. The separation pattern of the different peaks is comparable to that obtained with the original method of Trivelli et al. [2] and with that obtained with the high-performance liquid chromatographic (HPLC) techniques [19, 20]. In contrast to Cole et al. [20], we did not observe detectable deflection of the baseline after the introduction of the second buffer, as has been noted when performing the chromatography without sample introduction.

A comparison between the results obtained by manual planimetry of the curves and by a computing integrator was made. An excellent regression curve ($r = 0.97$) was obtained over the range of HbA_{1c} concentration of clinical interest (4—20%).

The reproducibility of our chromatographic method was tested by comparing fifteen runs of two blood samples, one from a normal volunteer and the other from a diabetic patient. The mean HbA_{1c} level in the normal hemolysate was 5.8 ± 0.19% with a coefficient of variation (C.V.) of 3.2%. The mean HbA_{1c} level in the patient's hemolysate was 14.5 ± 0.23%, C.V. 1.5%.

Values from normal and diabetic subjects

Seventy-four normal control individuals ranging in age from 22 to 51 years were studied. HbA_{1c} levels ranged from 4.9% to 6.2%. The mean HbA_{1c} level was 5.4 ± 0.4%, C.V. 7.4%. The present method was used routinely in our laboratory to determine the HbA_{1c} level of a large number of patients, most of whom were suffering from diabetes. During this study approximately
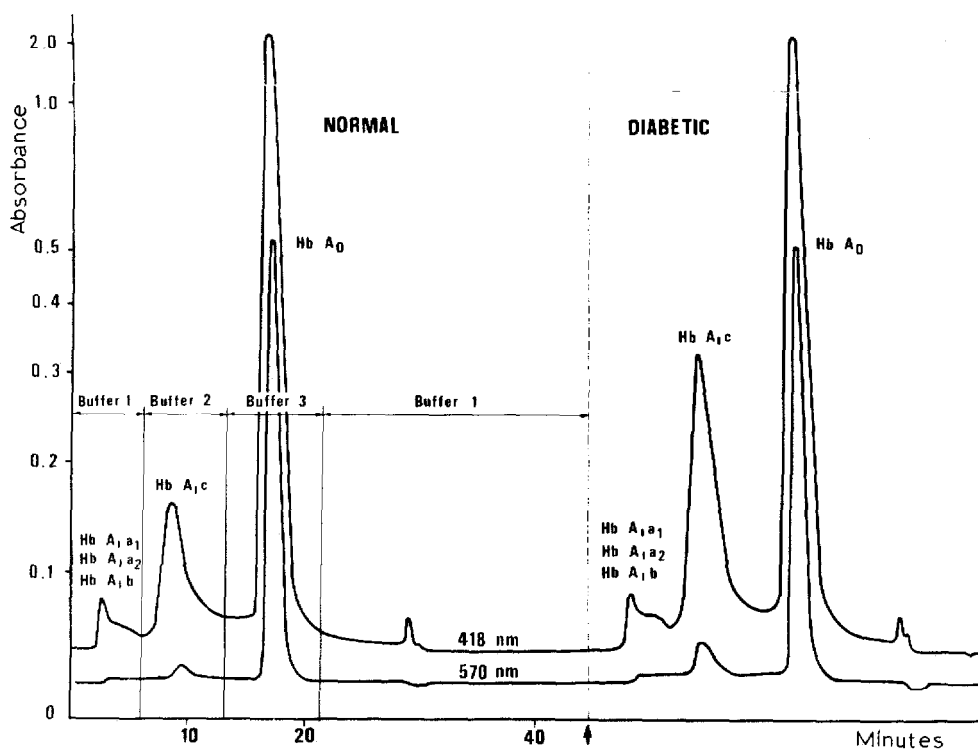
Fig. 2. Typical chromatographic separations of glycosylated hemoglobins using the buffer system described under Materials and methods. Chromatographic profile on the left side is typical of a normal hemolysate, and on the right side of a diabetic patient. The injection of the diabetic blood sample is indicated by the arrow. HbA_{1c} detection was effected at 418 nm, and of HbA_0 at 570 nm. The path-length of each colorimeter cuvette was 15 mm.

10,000 samples were studied. The level of HbA_{1c} ranged from 4% to 18%.

Diabetes mellitus and abnormal hemoglobins

In several instances, the chromatographic patterns were modified due to the presence of an abnormal hemoglobin in the hemolysate. Fig. 3 (right side) shows the elution profile given by the hemolysate of a heterozygous HbA/HbS sicklemic patient. In this profile, HbS_0 eluted just after HbA_0. Fig. 3 (left side) shows the elution profile obtained from the hemolysate of a homozygous sicklemic patient. The major peak represents HbS_0, while the peak within the elution volume of HbA_{1c} was mainly constituted by HbF as judged by IEF. Estimation of glycosylated HbS could not be demonstrated in this chromatographic system. Fig. 4 shows the elution profile of the hemolysate from a HbA/HbC heterozygous patient. HbC_0 was eluted after the peaks of HbA_0. In all cases, quantitative evaluation of the level of the glycosylated hemoglobins contained in the hemolysate was questionable [27, 28]. Fig. 5 shows the elution profile produced by an hemolysate containing 5% of HbF, as determined by the method of Betke et al. [24]. HbF was eluted with the peak of HbA_{1c}, but careful inspection of the chromatographic pattern shows the presence of an unusual shoulder on the descending
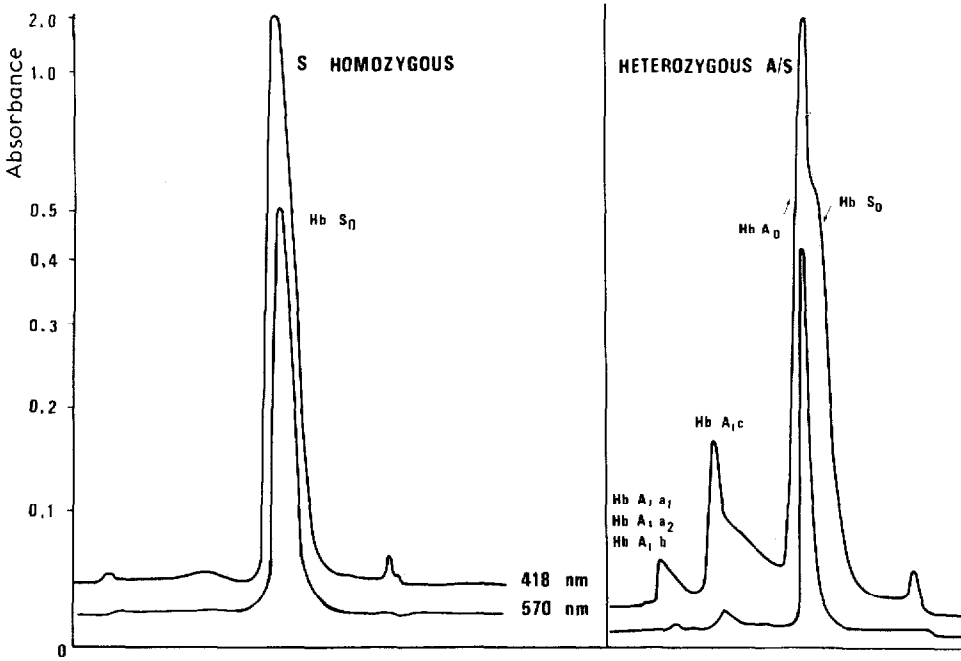
Fig. 3. Right side: a typical elution pattern obtained from the hemolysate of a heterozygous HbA/HbS sicklemic subject. Left side: a typical elution pattern obtained from the hemolysate of an homozygous sicklemic patient.
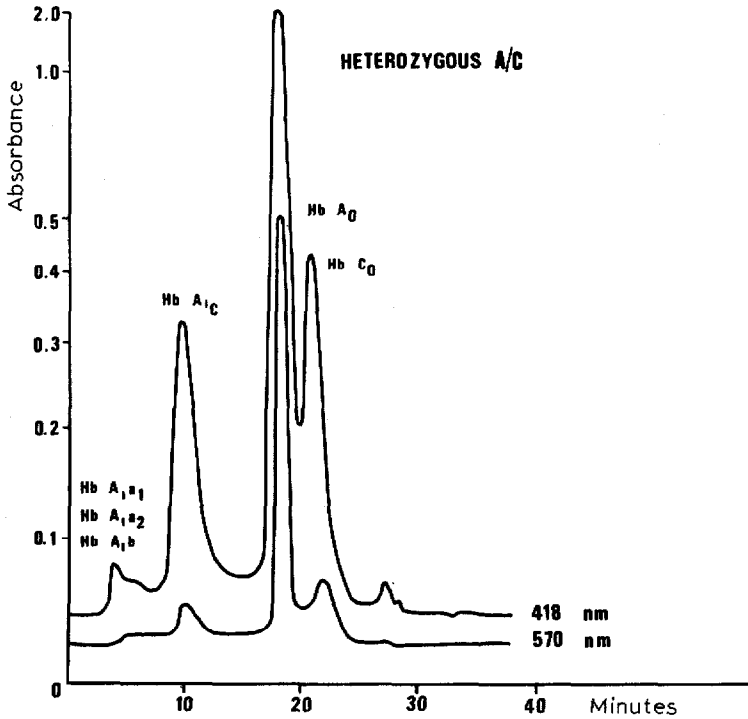

Fig. 4. A typical chromatographic profile obtained from the hemolysate of a HbA/HbC heterozygous subject.
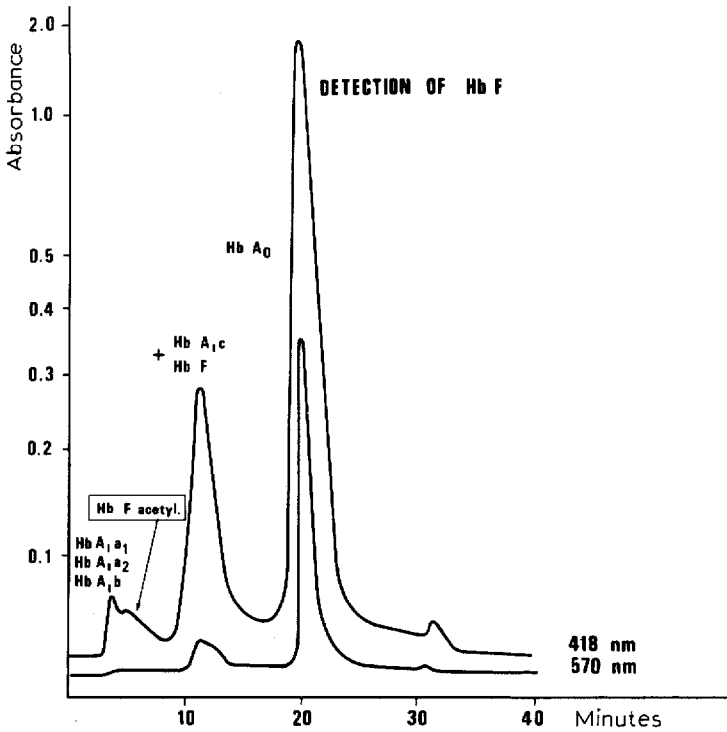
Fig. 5. Chromatographic profile typically seen in hemolysates of patients with HbF persistence. HbF was estimated by the technique of Betke et al. [24] and corresponded to 5% of the total.

side of the HbA_{1a+b} peak which represented the acetylated HbF fraction, the nature of which was definitively demonstrated after IEF analysis of this peak.

DISCUSSION

The method described in this article derives from the original technique for estimation of HbA_{1c} as described by Trivelli et al. [2]. Our method is characterized by its ability to be automated, permitting more than 40 daily tests. The three-step buffer system was used to achieve a complete separation of $HbsA_{1a+1b}$, HbA_{1c} and HbA_0. Its reproducibility was comparable to that obtained with HPLC. Evaluation of the amount of HbA_{1c} can be performed either by manual planimetry or by use of a computing integrator.

The levels of HbA_{1c} in hemolysates stored at room temperature remained stable for at least 36 h. Refrigeration of the sampler did not appear necessary. In contrast to most other authors, we have used ACD in order to prevent to the utmost degree possible hemolysis which might artificially lower the HbA_{1c} level by a selective lysis of the oldest cells rich in HbA_{1c}. We have observed that samples taken in ACD could be stored at 4°C without a significative modification of their HbA_{1c} level for a long period of time. Conversely, one should be careful not to eliminate the youngest RCBs during washing which could modify the overall HbA_{1c} level.

The performance of our system compares favourably to that obtained with either the HPLC method of Cole et al. [20] and of Davis et al. [19], or the method of Wajcman et al. [21]. Nevertheless, it must be emphasized that HPLC resulted in a slightly inferior separation to that obtained with our technique, while it requires expensive equipment and an electronic adjustment of the baseline to minimize graph artefact due to buffer change. The method described by Wajcman et al. gives considerable overlapping of the peaks of HbA_{1a+b} and HbA_{1c} and needs a rigorous monitoring of the pH of the first buffer. The low pressure, which was less than 7 bars, permitted the use of the same column for more than 200 times without repacking.

We found that the introduction of buffer B for the elution of HbA_{1c} greatly improves the resolution of the system by promoting a rapid elution of HbA_{1c} and HbF like in other systems but without eluting HbA_0. However, acetylated HbF was eluted in the descending side of the HbA_{1a+b} peak which permitted the detection of HbF level over 3%. The presence of several abnormal hemoglobins induced modification of the chromatographic profile. HbS was very easy to detect while it produced a shoulder on the descending side of the HbA_0 peak. The presence of HbC in hemolysate was revealed by an extra peak after the HbA_0. Hb Hope [29], which is not rare in the black population, was eluted in our system as a constituent of the HbA_{1c} peak hampering any evaluation of the HbA_{1c} level. These observations imply that a correct evaluation of the amount of HbA_{1c} cannot be done when most of the abnormal hemoglobins among the most frequent are present.

ACKNOWLEDGEMENTS

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