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NEW LESS TEMPERATURE-SENSITIVE MICROCHROMATOGRAPHIC METHOD FOR THE SEPARATION AND QUANTITATION OF GLYCOSYLATED HEMOGLOBINS USING A NON-CYANIDE BUFFER SYSTEM

E. BISSÉ and E.C. ABRAHAM*

Department of Cell and Molecular Biology, Medical College of Georgia, Augusta, GA 30912 (U.S.A.)

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SUMMARY

This paper describes a new microchromatographic method on Bio-Rex 70 ion-exchanger, which enables the isolation and quantitation of the minor components hemoglobin A_{Ia+b} and hemoglobin A_{Ic}. The method relies on the equilibration of the resin in a polyphosphate buffer with a pH closer to the pK_a of the carboxylic group of the resin, and on the adjustment of the sample pH at 5. This induces linear pH and ionic strength gradient during the elution of the hemoglobin components. The method is little affected by temperature between 20 and 30°C, by the presence of the aldimine Schiff base, and is not expected to be greatly influenced by moderate fluctuations in pH and ionic strength of the buffers used. There was good correlation between the values obtained by the new micromethod and four procedures currently used, namely high-performance liquid chromatography (r = 0.96), the Trivelli Bio-Rex 70 macromethod (r = 0.99), bioaffinity chromatography (r = 0.93), and Isolab hemoglobin A_I kit (r = 0.91). The method is reproducible, the inter-assay and the intra-assay correlation coefficients did not exceed 4.2%. The mean value for hemoglobin A_{Ic} was 4.6 ± 0.35% for eleven non-diabetics and 8.9 ± 2.6% for twenty-one diabetics.

INTRODUCTION

In recent years the determination of glycosylated hemoglobins has been accomplished by a wide variety of methods. These methods include colorimetric testing [1, 2], macrocolumn chromatography [3], high-performance liquid chromatography (HPLC) [4—7], microcolumn chromatography [8—10], electrophoresis [10—12], isoelectric focusing [13, 14], and bioaffinity chromatography [15, 16]. These procedures are all affected in different ways by potential interferences and analytical variables. The colorimetric method

suffers to be non-stoichiometric, requires rigorous assay conditions, and is time-consuming. The chromatography on ion-exchange and electrophoresis measures together the Schiff base with the stable glycohemoglobin (Hb A_{Ic}). The cation-exchange microcolumns widely used are sensitive to small temperature and ionic strength variations [10]. Moreover, the ion-exchange chromatographic methods generally utilize cyanide-containing buffers. In an attempt to minimize the inherent difficulties in often used ion-exchange chromatography, we developed a new method with non-cyanide buffers with a working pH near the pK_a of the carboxylate groups of the ion-exchanger. We adopted a sample preparation method which allowed simultaneous elimination of the Schiff base [5, 17]. To routinely assess the performance of the method, we studied the temperature sensitivity of the columns and assessed the clinical and research applicability by comparing the new technique with other established techniques, namely HPLC, macrochromatography (the Trivelli method), and bioaffinity chromatography.

EXPERIMENTAL

Resin preparation

A 30-g amount of Bio-Rex 70 ion exchanger (200—400 mesh) from Bio-Rad Labs. were dispersed in 400 ml distilled water and degassed for 15 min under vacuum. The supernatant was discarded, and the resin was suspended again in 400 ml water. The pH of the suspension was then set to 6.1 with phosphoric acid (85%) under thorough stirring. After discarding the water, the slurry was equilibrated in developer 1 until the pH of the supernatant was 6.38.

Microcolumn preparation

Plastic columns, 8.5 cm × 7 mm, which were obtained from P. Berger Lab. (Neuchâtel, Switzerland) have a wide top of about 2.5 cm long which serves as a reservoir for the developer. A polyethylene frit at the bottom serves as the support for the resin. A 50% suspension of the resin was transferred into the column until the packed resin reached 4.5 cm (it was about 1.8 g of the slurry).

Buffer preparation

The composition of the developers 1, 2, and 3 are listed in Table I. Developer 1 was prepared by dissolving 7.58 g (54.8 m*M*) of monobasic sodium phosphate, 1.96 g (13.8 m*M*) of dibasic sodium phosphate, and 0.5 g (8.56 m*M*) of sodium chloride in 900 ml of distilled water. The final pH was adjusted to 6.5 by titrating with a potassium phosphate solution (34.84 g/l K_2HPO_4 and 79.9 g/l $K_3PO_4 \cdot 3H_2O$). Distilled water was added to a final volume of 1 l, then the ionic strength of the buffer was measured. The only difference between developer 1 and developer 2 was that developer 2 contained 2.5 g/l sodium chloride instead of 0.5 g/l sodium chloride for developer 1. The ionic strength (I) measured in mS or $m\Omega^{-1}$ of developer 2 was 7 and of developer 1 was 5. Developer 3 was prepared with 54.8 m*M* monobasic potassium phosphate and 13.8 m*M* dibasic potassium phosphate and contained 1 g/l sodium chloride. The ionic strength of this developer was also 7 in mS.

TABLE I

BUFFERS USED FOR SEPARATION OF GLYCOHEMOGLOBINS

The pH of all the buffers was set to 6.5 with a potassium phosphate solution (34.84 g/l K_2HPO_4 and 79.9 g/l $K_3PO_4 \cdot 3H_2O$).

	$NaH_2PO_4 \cdot H_2O$ (mM)	Na_2HPO_4 (mM)	KH_2PO_4 (mM)	K_2HPO_4 (mM)	NaCl (g/l)	I* (mΩ^{-1})	pH
Developer 1	54.8	13.8			0.5	5	6.5
Developer 2	54.8	13.8			2.5	7	6.5
Developer 3			54.8	13.8	1.0	7	6.5

*I is the ionic strength of the buffer in mS or mΩ^{-1} measured on a conductivity meter (Radiometer, Copenhagen) after pH adjustment.

Potassium biphthalate buffer, 0.05 M, pH 5.0

The pH of 50 ml of a 0.1 *M* potassium biphthalate (20.42 g/l) was set to 5.0 with 0.1 *M* sodium hydroxide, then distilled water was added to a final volume of 100 ml (final pH = 5.0).

Sample preparation

Whole blood anticoagulated with ethylenediamine tetraacetate (EDTA) was centrifuged to separate the cells from plasma. Of the packed cells 1 vol. was lysed with an equal volume of distilled water, and complete lysis was obtained using a vortex mixer. Cellular debris was settled by centrifugation at 1000 *g*. The hemolysate was diluted with 1.5 vols. of biphthalate (0.05 *M* potassium biphthalate), pH 5.0. The sample was then spinned using Eppendorf tubes to settle the remaining proteins precipitated by lowering the pH of the sample.

Chromatographic separation and quantitation of the Hb A_{Ic} and Hb A_{Ia+b}

A 50-μl hemolysate prepared as described above was applied to the micro-column and allowed to be absorbed in the top of the column. A 100-μl aliquot of the elution buffer (developer 2) was added to achieve a complete transfer of the sample into the resin.

The Hb A_{Ia+b} fraction was collected by passing 1.8 ml of developer 2 through the column. The second fraction containing Hb A_{Ic} was eluted with 2 ml of the same buffer. The remaining hemoglobins were then eluted with 2 ml of 0.6 *M* sodium chloride and diluted by adding 20 ml distilled water. The absorbances of the fractions were read at 415 nm, and the % Hb A_{Ic} and Hb A_{Ia+b} percentages calculated as follows:

$$\text{Percentage Hb } A_{Ic} = \frac{E_2}{0.9\,E_1 + E_2 + 11\,E_3} \times 100$$

$$\text{Percentage Hb } A_{Ia+b} = \frac{0.9\,E_1}{0.9\,E_1 + E_2 + 11\,E_3} \times 100$$

where E_1 = absorption of the Hb A_{Ia+b} fraction, E_2 = absorption of the Hb A_{Ic} fraction, and E_3 = absorption of other hemoglobin.

Additional methods

HPLC. A cation-exchange HPLC column of 25 cm × 4.1 mm (Synchropak CM 300 column from Synchrom, Linden, IN, U.S.A.), 10 μm particle size, was used. The chromatographic system was similar to that previously described [7]. Analyses were carried out at a flow-rate of 1 ml/min at room temperature and the pressure of the column was 70 bar. Detector was set at 405 nm (405-nm filter). The column was preceded by a guard column (5 cm × 4.1 mm) packed with a similar resin (CSC packing material also from Synchrom). Equilibration of the column was carried out for 20 min with 85% of developer A (30 m*M* Bis-Tris—1.5 m*M* potassium cyanide, pH 6.4) and 15% of developer B (30 m*M* Bis-Tris—1.5 m*M* potassium cyanide—0.15 *M* sodium acetate, pH 6.4). After application of 20 μl hemolysate (200 μg Hb) the separation of the minor Hb components was achieved by setting a linear gradient of sodium acetate (15—80% of developer B, 110 min).

Macrocolumn method. This method was carried out according to Trivelli et al. [3] by applying 30 mg hemolysate to a 25 × 1 cm column of Bio-Rex 70 and developing isocratically with developer 6 with a flow-rate of 15 ml/h. After Hb A_{Ic} was eluted, the remaining hemoglobins (Hb A_0 and Hb A_2) were eluted with a high phosphate developer.

Commercially available microcolumn methods. Hb A_I was also quantitated by cation-exchange microcolumns from Isolab (Akron, OH, U.S.A.). The total glycosylated hemoglobin (GHb) was quantitated by the bioaffinity chromatographic technique utilizing Glyc-Affin System also from Isolab. In both instances the methods recommended by the manufacturer were strictly followed.

RESULTS

Temperature sensitivity

The effect of the temperature on the new microcolumn method has been studied between 20 and 35°C, using both normal and diabetic samples (Fig. 1).

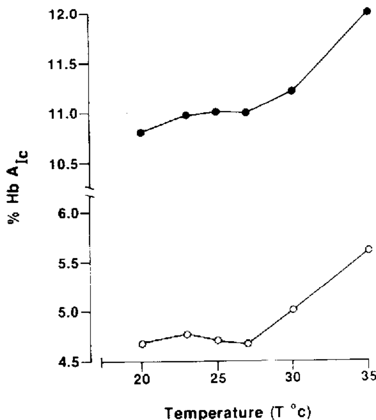


Fig. 1. Effect of the temperature on the new microcolumn method. ○, Normal; ●, diabetic.

Fluctuation in a temperature from 20 to 30°C produced no significant change in the values obtained. The coefficient of variation (C.V.) was 3% and 3.8% in normal and in diabetic samples, respectively. Raising the temperature above 30°C increased the Hb A_{Ic} values more than 10% compared to the values obtained at 20°C.

Elimination of the labile glycosylated hemoglobin

To determine the efficiency of the new procedure to remove the Schiff base during the sample preparation, erythrocytes from normal subjects were incubated with 20 m*M*, 50 m*M*, and 100 m*M* of glucose for 3 h at 37°C. A portion of each incubated sample was treated with 0.05 *M* biphthalate buffer, pH 5.0 (15 min at 37°C). Both treated and non-treated samples were analyzed by HPLC and the results were compared with those obtained by the new micromethod. The mean increase of pre-Hb A_{Ic} after incubation of erythrocytes with 100 m*M* glucose was 7.3 ± 0.3%. This increment was reversed in the new microcolumn method because the biphthalate treatment step was incorporated in the sample preparation for the microcolumn method.

pH and ionic strength

The variation of pH and ionic strength in the solution leaving the column

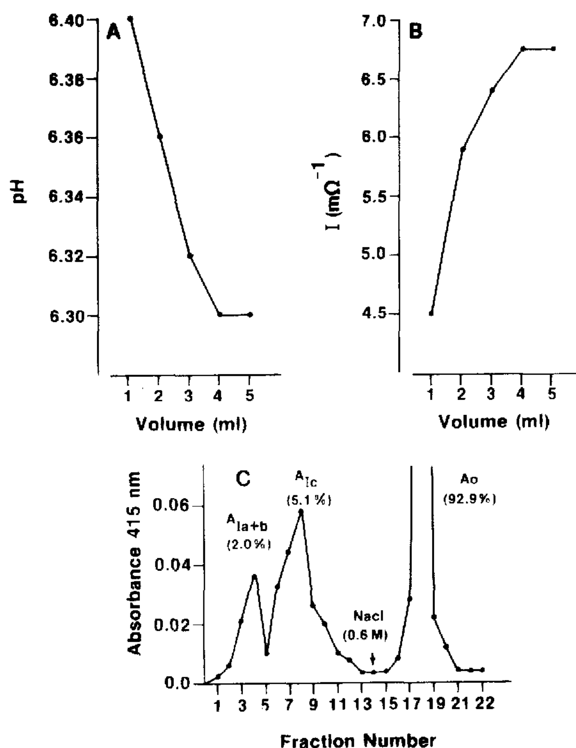


Fig. 2. Variation of pH (A) and ionic strength (B) in the solution leaving the microcolumn during the elution of the hemoglobin fractions with developers containing both sodium and potassium (C). Normal sample (1 mg Hb) and developer 2 were used. Bio-Rex 70 resin was equilibrated in developer 1. Fraction volume: 0.4 ml.

is illustrated in Fig. 2A and B. Under the elution conditions described above, 1-ml fractions were collected, the pH and the ionic strength of each fraction were checked. During the elution of hemoglobin components the pH in the column dropped 0.1 unit, while there was an increase in ionic strength of 48% (2.2 $m\Omega^{-1}$) compared to the initial value. The pH and the ionic strength on a fully equilibrated column are 6.40 and 4.50 $m\Omega^{-1}$, respectively.

Potassium phosphate effect

To estimate the effect of phosphate salts we substituted developer 2 with developer 3 (containing 1 g/l sodium chloride, I = 7 $m\Omega^{-1}$, pH 6.5) for the elution of the first fraction. The Hb A_{Ia+b} fraction still eluted at the buffer front in about 2 ml, while the Hb A_{Ic} was retained longer on the column and 3 ml of the buffer were needed for complete elution. Hb A_{Ia+b} and Hb A_{Ic} were retained even longer when Bio-Rex 70 resin was equilibrated with developer 3 that contained no sodium chloride (I = 5.5 $m\Omega^{-1}$, pH 6.5). As shown in Fig. 3C, Hb A_{Ia+b} was in this instance eluted with 2.3 ml of developer 3 while 5 ml of the same buffer were needed for complete elution of Hb A_{Ic}. During the elution of hemoglobins the pH slightly dropped about 0.03 unit, and the ionic strength increased for about 33% (1.5 $m\Omega^{-1}$) compared to the initial value. Thus, equilibration and development of the micro-column with a buffer containing only potassium phosphate (no sodium phos-

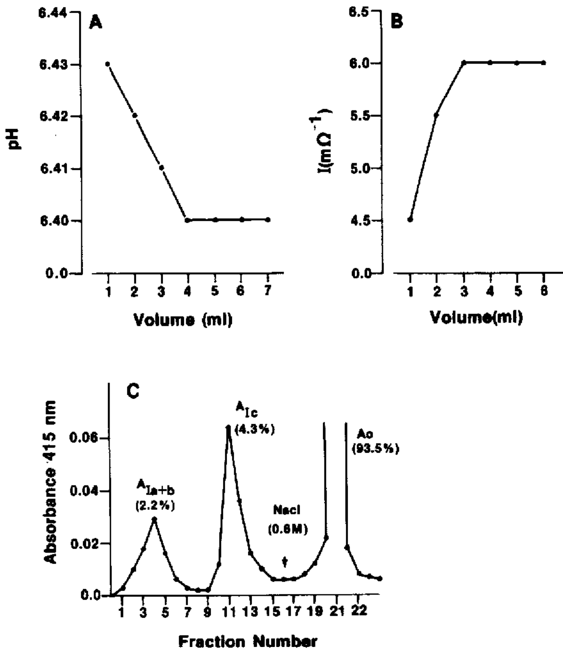


Fig. 3. Variation of pH (A) and ionic strength (B) during the separation of hemoglobins by microchromatography on Bio-Rex 70 with developers containing no sodium and only potassium (C). Sample: 1 mg hemolysate from a normal person. Bio-Rex 70 resin was equilibrated with developer 3 which contained no sodium chloride (I = $m\Omega^{-1}$, pH 6.5). Developer 3 containing 0.96 g/l potassium chloride instead of sodium chloride was used for the elution of the hemoglobins (I = 7 $m\Omega^{-1}$, pH 6.5). Fraction volume: 0.4 ml.

phate) increases the retention time. The separation of Hb A_{Ic} from Hb A_{Ia+b} was also increased as the ionic strength of developer 2 was adjusted with potassium chloride instead of sodium chloride.

Fraction identification

The hemoglobin fractions were collected, pooled, concentrated, and 25 μg Hb of each fraction were injected on the HPLC column. Fraction identity was based on retention time characteristics and comparison with an elution profile of a normal blood sample. As shown in Fig. 2C, the new micromethod separated the red cell hemolysates into three components, the first one the so-called Hb A_{Ia+b}, the second one Hb A_{Ic}, and the third one the other hemoglobins. The purity of each fraction was above 90%. No significant cross-contamination of the hemoglobin components was evident.

Column load

The method was accurate over the Hb concentration range studied here (0.5—2 mg Hb per column). The C.V. for the data of this loading range was 3.0% (n = 8).

Correlation

Diabetic (n = 21) and normal (n = 11) samples have been analysed by the new microchromatographic method. The results obtained were correlated with those obtained with three different methods: HPLC (Fig. 4), cation-exchange microchromatography (Isolab kit) (Fig. 5), and bioaffinity chromatography (Fig. 6). The mean values for normals and diabetics obtained by those four methods are listed in Table II. HPLC and the new method correlated well (r = 0.96). A correlation coefficient of 0.91 was obtained for the new method versus Isolab kit, 0.93 for the new method versus the bioaffinity method. The new method was also compared to the classical standard Trivelli method [3], and Fig. 7 shows the relationship obtained (r = 0.99). Correla-

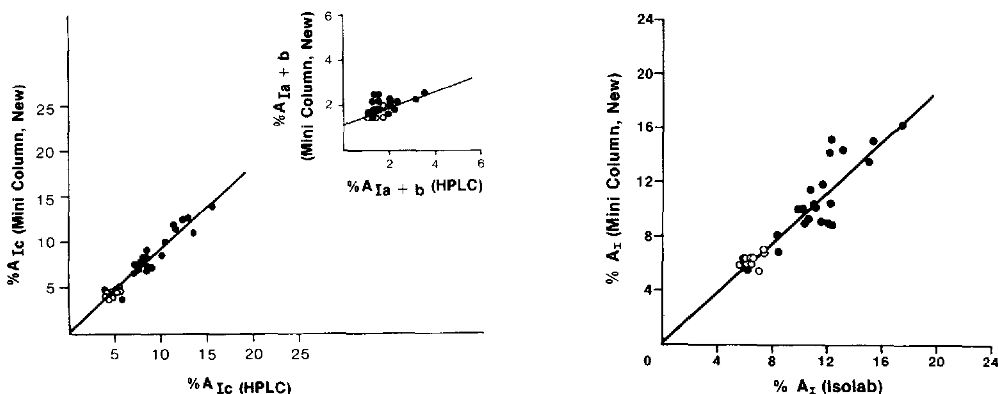
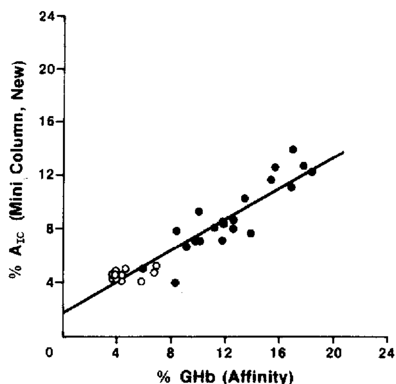


Fig. 4. Correlation between Hb A_{Ic} values determined by the microcolumn and values given by HPLC ($y = 0.91x + 0.39$; $r = 0.96$). Inset is a comparision of values for Hb A_{Ia+b} determined by HPLC and microcolumn ($y = 0.37x + 1.28$; $r = 0.65$). ○, Normal; •, diabetic.

Fig. 5. Correlation between Hb A_I values obtained by the new micromethod and by the Hb A_I Isolab kit ($y = 0.93x + 0.15$; $r = 0.91$). ○, Normal; •, diabetic.



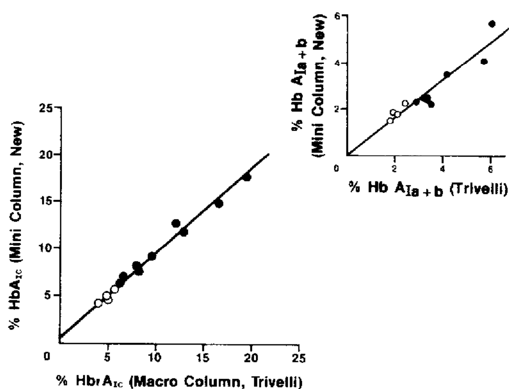


Fig. 6. Correlation between Hb A_{Ic} values obtained by the new micromethod and the GHb (bioaffinity) ($y = 0.58x + 1.70$; $r = 0.93$). ○, Normal; ●, diabetic.

Fig. 7. Comparison of A_{Ic} levels determined by the new microcolumn and the classical Trivelli macromethod [3] ($y = 0.86x + 0.86$; $r = 0.99$). Inset is a comparison of values for Hb A_{Ia+b} determined by the Trivelli macromethod and the new micromethod ($y = 0.79x + 0.04$; $r = 0.94$). ○, Normal; ●, diabetic.

TABLE II

LEVELS OF GLYCOHEMOGLOBINS IN NORMAL (n = 11) AND DIABETIC PATIENTS (n = 21)

Method		Level (mean ± S.D.)			
		Hb A_{Ic}	Hb A_{Ia+b}	Hb A_{I}	GHb
Microcolumn (new)	Diabetic	9.0 ± 2.6	2.0 ± 0.4	10.8 ± 2.8	
	Normal	4.6 ± 0.4	1.6 ± 0.2	6.2 ± 0.3	
HPLC	Diabetic	9.2 ± 2.6	1.7 ± 0.7	10.9 ± 3	
	Normal	4.7 ± 0.5	1.3 ± 0.2	6.0 ± 0.5	
Microcolumn (Isolab)	Diabetic	—	—	11.5 ± 2.4	
	Normal	—	—	6.4 ± 0.5	
Bioaffinity chromatography (Isolab)	Diabetic	—	—	—	12.4 ± 3.3
	Normal	—	—	—	4.7 ± 1.1

tion of the Hb A_{Ia+b} values by the new method with the values obtained by the Trivelli method was also excellent; however, the correlation with the HPLC method was decidedly poor.

Precision

The intra-assay C.V. was 2.5% and 3% for normal and diabetic samples, respectively (n = 6). The inter-assay was calculated from six daily assays of samples diluted in 0.05 *M* potassium biphthalate buffer, pH 5.0, and stored at 4°C (see *Sample preparation*). The between-run imprecision did not exceed 4% (C.V.) for the normal samples and 5.5% (C.V.) for the diabetic samples.

Glycosylated hemoglobin levels

As shown in Table II the mean values of Hb A_{Ic} in normal persons were 4.6 ± 0.35% (percentage Hb A_{Ic} ± S.D.). The values ranged from 4 to 5.2% in eleven normal persons. Hb A_{Ic} in twenty-one diabetic patients ranged from

6.6 to 14%, mean 8.9 ± 2.6% (percentage Hb A_{Ic} ± S.D.). The mean percentages of Hb A_{Ia+b} were 1.6 and 2.0% for normal and diabetic, respectively.

DISCUSSION

Schroeder and Holmquist [18] first introduced the use of Bio-Rex 70 for the separation of fast hemoglobins by applying sodium phosphate gradient elution. Trivelli et al. [3], on the other hand, introduced the use of a high phosphate buffer to elute the remaining hemoglobin after elution of fast components, thus utilized a step-wise elution device. This method is very widely used with or without modifications. In some instances, the column size has been reduced, potassium cyanide concentration reduced and some steps (dialysis of sample, temperature control) were omitted. The commercially available kits are also based on the method of Trivelli et al. [3]. In spite of several modifications and improvements, the determination of glycohemoglobin on ion-exchange requires rigorous control of pH, ionic strength, and temperature. We have improved the Bio-Rex 70 microchromatographic technique. The new method is little affected by temperatures between 20 and 30°C (Fig. 1). The Schiff base is removed during the sample preparation, and the Hb A_{Ic} and Hb A_{Ia+b} fractions are isolated in 30 min using microcolumns.

In achieving optimum separation, the characteristics of the Bio-Rex 70 resin were exploited. The pK_a of the carboxylic group of this resin is 6.1 and a change from H^+ to Na^+ induces about 100% increase in the size of the resin and vice versa [4]. To increase the buffering action of the resin and to reduce the particle sizes we set the pH of the resin close to its pK. In this way we reduced the extent of disequilibrium between the resin and the eluent staying closer to the pK. Additionally, the resin was first equilibrated in the buffer with lower ionic strength than the elution buffer, and the sample pH was adjusted to near 5. Under these conditions, therefore, we induced change in pH (0.1 pH unit) and ionic strength (2.2 $m\Omega^{-1}$) during the chromatography (Fig. 2A and B). As expected, a pH gradient was formed in the column during the elution, and the ionic strength of the eluate increased with decreasing of the pH (Fig. 2A and B). This is similar to the observation made by Sluyterman and co-workers [19, 20] using the chromatofocusing technique. Thus, the pH and the ionic strength partially compensated each other. This fact explains the small temperature sensitivity observed over the range studied (20—35°C). Moreover, working at a relatively low pH and high ionic strength as compared to the commonly used developer 6 [18, 3] should make this method less susceptable to moderate fluctuation in pH and ionic strength. In contrast with sodium ion [4] the increase of potassium ion in the buffer increased the adsorption of hemoglobin on the resin. The retention of Hb A_{Ic} was increased while the Hb A_{Ia+b} component eluted faster. We took advantage of this potassium ion effect by utilizing developers containing both sodium and potassium phosphate for microchromatography. We found this combination very efficient in glycohemoglobin separation (Fig. 3C).

In addition to the less temperature sensitivity the micromethod described in this paper is characterized by the efficient separation of Hb A_{Ia+b}, Hb

A_{Ic}, and the other hemoglobins. The good correlation between the new micromethod, HPLC (r = 0.96), bioaffinity chromatography (r = 0.91), and the method of Trivelli et al. [3] (r = 0.99) indicated that in the absence of Hb F and abnormal hemoglobins under the described conditions, the new micromethod is selective for Hb A_{Ic} determination. Storing the hemolysate at 4°C in 0.05 M biphthalate buffer, pH 5.0 for up to ten days did not affect the results. The system does not critically depend on the use of pH 5 hemolysates. The use of neutral-pH hemolysates is nearly just as effective. Using pH 5 hemolysates offer, however, some advantages such as the precipitation of the remaining proteins and the elimination of the labile glycohemoglobins. We suggest the preparation of the hemolysate with distilled water if the utilization of the neutral pH is expedient. Furthermore, the use of any detergent for the lysis of red blood cells seems to affect the system. In examining the correlation between the new micromethod and HPLC (Fig. 6) we found a good relationship (r = 0.96) for Hb A_{Ic} values, while the correlation coefficient for corresponding Hb A_{Ia+b} was low (r = 0.65). This is not the result of the insufficient resolution between Hb A_{Ia+b} and Hb A_{Ic} components. The non-heme components (endogenous substances or artifacts) are eluted with Hb A_{Ia+b} fraction on Bio-Rex 70 chromatography. This constitutes the major factor of the fluctuation in the Hb A_{Ia+b} values determined by this method. Moreover, it is often mentioned [21—23] that the fluctuation of the Hb A_{Ia+b} values highly influences the determination of Hb A_I. The low correlation coefficient (Fig. 4, inset) seems to indicate that by using ion-exchange hard resin in HPLC the non-heme components are absorbed on the resin and not eluted with Hb A_{Ia+b} fraction. The Hb A_{Ia+b} values, therefore, tend to be low compared with those obtained by the methods using Bio-Rex 70 resin.

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