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Novel approach for the analysis of glycated hemoglobin using capillary isoelectric focusing with chemical mobilization

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Abstract

In this work, a novel CIEF methodology for the analysis of the glycated hemoglobin, HbA_{1c}, in dimethylpolysiloxane coated fused-silica capillaries (DB-1, 50 μm I.D., 27 cm, 0.20 μm coating thickness), using a narrow pH ampholyte mixture (4% pH 6–8:pH 3–10, 10:1, v/v) in 0.30% methylcellulose, was developed. In the focusing procedure, a 0.100-mol l^{-1} phosphoric acid solution was used as anolyte and a 0.040-mol l^{-1} NaOH solution was used as catholyte. During method development, two types of mobilization of the focused hemoglobins were tested: pressure and chemical mobilization. Chemical mobilization performed better, allowing the complete baseline resolution of the hemoglobin of interest, HbA_{1c}, from its adjacent peak, HbA, in less than 8 min. In the chemical mobilization procedure, the catholyte was replaced by a 0.040-mol l^{-1} NaOH solution containing 0.080 mol l^{-1} NaCl. The proposed methodology was applied to the analysis of 31 hemolysate samples and validated with respect to the selectivity, inter-assay and intra-assay precision (both migration time and hemoglobin percentage concentration). In addition, HbA_{1c} determinations were compared for the CIEF method and a chromatographic standardized procedure using cation-exchanger columns (Variant™, Bio-Rad), adopted in a local clinical laboratory, showing excellent correlation ($r^2=0.872$, $n=31$). The slope was found to be statistically equal to one but the intercept differed from zero. Also the Bland-Altman plot indicates bias, implying that the CIEF method yields HbA_{1c} concentration higher than the reference method. The separation of the hemoglobins HbA, HbA_2, HbF and HbA_{1c} and the variants HbS and HbC was also demonstrated (8 min run). The resolving power of the proposed CIEF method allowed baseline resolution of hemoglobins with a p*I* difference as small as ca. 0.03, as it is the case for the pairs HbC/HbA_2 and HbA/HbA_{1c}.

Keywords: Hemoglobin, glycated

1. Introduction

Diabetes Mellitus is considered a class of diseases that exhibits as a sole characteristic intolerance to glucose [1]. This condition results from a deficiency in the secretion or action of a pancreatic hormone, the insulin, causing severe metabolic disorders. Typically, high levels of glucose are found in blood, which can promote the glycosilation of hemoglobin, the oxygen carrier protein found in red blood cells. Glycated hemoglobin, HbA_{1c}, is the reaction product of glucose and the N-terminal valine of the β-chain of hemoglobin. Considering that this reaction is nonenzymatic and the red blood cells are completely

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permeable to glucose, the quantity of HbA_{1c} formed is directly proportional to the average plasma glucose concentration that the red blood cells have been exposed to during their 120-day life span. Therefore, for clinical diagnostic purposes and most importantly for monitoring the metabolic status of patients with *diabetes mellitus*, the determination of HbA_{1c} has been strongly recommended.

Various electrophoretic approaches including agar gel electrophoresis in acidic medium and isoelectric focusing electrophoresis in polyacrylamide or agarose gels, as well as liquid chromatography using ion-exchange or affinity as separation mechanisms, immunological assays and colorimetric methods have all been used to investigate glycated hemoglobins [1]. The high resolving power of the electrophoretic methods, especially those based on isoelectric focusing, makes them still widely used in clinical sets. However, the difficulty of automation and inaccurate quantitation of minor hemoglobin constituents are among their major shortcomings. High-performance liquid chromatography (HPLC) is nowadays considered a sensitive, specific, and reproducible alternative to electrophoresis and its use has been significantly increased in clinical laboratories, especially with the development of rapid and well-resolving cation-exchange methods, applied to fully automated analyzers.

Capillary isoelectric focusing (CIEF) is a mode of capillary electrophoresis contemplated in the literature for the analysis of switterionic analytes of clinical importance [2]. It combines the remarkable resolving power of the yet laborious isoelectric focusing methods with the automation demand of clinical laboratories and should be a promising alternative for the unequivocal identification and analysis of a large number of hemoglobins and their variants.

Righetti et al. described a CIEF method for the analysis of HbA_{1c} using polyacryloylaminopropanol coated fused-silica capillaries, testing two protocols for chemical mobilization of analytes [3]. The method was applied to the analysis of a large number of samples and the results were contrasted with clinically accepted routine techniques such as a slab gel electrophoresis (Helena REP Glyco gel system), performing accordingly. Mario et al. contrasted in terms of performance characteristics, precision and quantitation ability, two potentially suitable methods for routine implementation in the clinical analysis of HbA_{1c}: a CIEF method with polyacrylamide coated capillary in narrow pH gradient, using pressure mobilization, and a HPLC method with a weak cation-exchanger column [4]. In other work, Mario and collaborators optimized the CIEF methodologies, comparing a one-step method with reverse polarity and a two-step pressure mobilization approach [5]. Despite the convenience of the one-step method, in which focalization and mobilization are performed concomitantly, the two-step method performed slightly better in terms of resolution for HbA_{1c}. Hempe and Craver described the analysis of normal hemoglobins and variants using dimethylpolysiloxane coated capillaries and pressure mobilization [6]. The quantitation of HbA_{1c} was performed by this method and by its revised form thereafter [7]. This last work provides an extensive compilation of the use of CIEF for human hemoglobin analysis aiming at diagnostics and control. Jenkins and Ratnaike developed a one-step method for the CIEF analysis of hemoglobins using a polyacryloylaminoethoxyethanol coated capillary, with an overall analysis time of less than 13 min [8]. One distinct characteristic in favor of this method is the endurance of the coated capillaries (over 100 runs per capillary). More recently, Gerritsma et al. evaluated the use of a proprietary kit (Analis, Belgium) for the determination of HbA_{1c}. The protocol comprises proprietary buffers, which promote a dynamic double coat to the capillary wall, presenting an impressively fast analysis, approximately 3.2 min for HbA_{1c} [9].

Except for the Analis proprietary kit, none of the above cited methods provided a complete baseline resolution of the HbA_{1c} peak from its adjacent peak, usually HbA. In addition, the overall analysis time, comprising focalization and mobilization steps, varied from 13 to 28 min, which might not be considered entirely appropriate for routine clinical purposes. In this work, a CIEF method for the analysis of glycated hemoglobin is proposed using well-defined and readily available reagents and materials. In the proposed methodology, chemical mobilization of the focused hemoglobins is performed, as opposed to pressure mobilization. Separation of several hemoglobins and variants (HbA, HbA_2, HbF, HbA_{1c}, HbS and HbC) is demonstrated with baseline resolution

for all peaks, in quite reasonable analysis time. In addition, a few method validation parameters are established and a correlation study between the proposed methodology and a standard chromatographic method for HbA_{1c} (Variant™, Bio-Rad) is illustrated.

2. Experimental

2.1. Equipment

All experiments were conducted in a capillary electrophoresis system (model P/ACE 5510, Beckman Coulter Instruments, Fullerton, CA, USA) equipped with a diode array detector set at 415 nm (specific for the heme group) and a temperature control device set at 25 °C. The data acquisition and treatment software was supplied by the manufacturer (Beckman P/ACE System Gold® Software).

2.2. Capillary conditioning

Dimethylpolysiloxane coated fused-silica capillaries (DB-1, J&W Scientific, Folsom, CA, USA) with dimensions of 27 cm total length, 20 cm effective length, 50 μm I.D. and 0.20 μm coating thickness were used. At the beginning of the day, the capillary was conditioned by a flush of methanol followed by deionized water (138 kPa for 10 min, each flush). In between runs, the capillary was just replenished with fresh ampholyte working solution (2 min flush). At the end of the day, the capillary was washed with deionized water, methanol, and dried by a flush of nitrogen (138 kPa, 5 min each flush).

2.3. Reagents and solutions

All reagents were of analytical grade, the solvents were of chromatographic purity and the water was purified by deionization (Milli-Q, Millipore, Bedford, MA, USA). The hemolyzing reagent used during sample preparation consisted of 0.05 mol l^{-1} EDTA and 0.010 mol l^{-1} KCN. Two ampholyte starting solutions in the pH range 6–8 and 3–10 (Amersham Pharmacia Biotech, Uppsala, Sweden) and a 4% methylcellulose stock solution (Applied Biosystems, Foster City, CA, USA) were used. Working ampholyte solutions composed of 4% (pH 6–8:pH 3–10, 10:1, v/v) in 0.30% methylcellulose were prepared. During the focalization procedure, a 0.100-mol l^{-1} phosphoric acid solution was used as anolyte and a 0.040-mol l^{-1} NaOH solution was used as catholyte. During the chemical mobilization procedure, the catholyte was replaced by a 0.040-mol l^{-1} NaOH solution containing 0.080 mol l^{-1} NaCl.

2.4. Sample preparation

Whole blood samples centrifuged and 15 μl of the deposited red cells were collected and transferred into a vial containing 200 μl of the hemolyzing reagent. The vial was then mixed in a vortex and the resulting hemolysate was stored at −20 °C until analysis in the day after blood collection.

2.5. CIEF method

After conditioning, the capillary was filled with the working ampholyte solution (135 kPa, 4 min). The hemolysate was then injected hydrodynamically (3.5 kPa, 10 s) and the focalization step proceeded with the anode at the injection port (30 kV) during 5 min. After this period, the catholyte solution was replaced by that containing sodium chloride and the chemical mobilization proceeded for an additional 3 min (30 kV).

2.6. Chromatographic reference method

The VARIANT™ Hemoglobin A_{1c} program, intended for use in the Bio-Rad VARIANT Hemoglobin testing system (Bio-Rad, Hercules, CA, USA), for the determination of HbA_{1c} in human whole blood, has been certified by The National Glycohemoglobin Standardization Program and provides as a reagent kit, elution buffers, calibrator, controls and hemoglobin primers. The Variant Hemoglobin A_{1c} Program utilizes the principles of ion-exchange chromatography. Prepared hemolysate samples are automatically injected into the analytical flow path and applied to the cation-exchanger column. The separated hemoglobin then passes through the flow cell of the filter photometer, where changes in the absorbance (415 nm) are measured. The

chromatographic analyses of 31 samples were conducted in a local clinical laboratory (Centro de Patologia Clínica Campana, São Paulo, SP, Brazil) with the same samples used to test the proposed CIEF methodology.

3. Results and discussion

3.1. Method development

Preliminary experiments for the analysis of glycated hemoglobin followed the method described by Hempe et al., which utilizes pressure in the mobilization procedure [7]. Even though minor modifications in the concentration of methylcellulose and in the composition of the ampholyte mixture were attempted, baseline resolution between HbA and HbA_{1c} was never achieved. The best condition employing pressure mobilization for the modified method is depicted in Fig. 1. The peak identified as HbA_3 in the electropherogram of Fig. 1 has been attributed to an adduct of HbA associated with poor sample storage [7]. Formation of HbA_3 can be avoided by storage of the hemolysate at −20 °C, immediately after preparation.

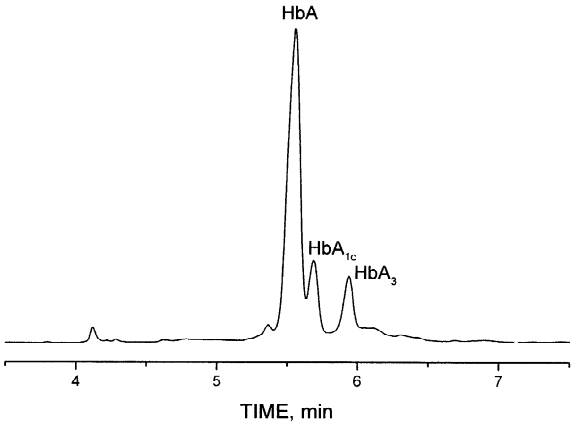

Fig. 1. Analysis of a typical hemolysate sample using CIEF in a coated capillary with pressure mobilization. Capillary: DB-1, 50 μm I.D., 27 cm total length, 0.20 μm coating thickness, flushed with methanol (1 min), water (0.5 min) and ampholyte working solution (1 min) in between runs (138 kPa in all cases). Catholyte solution: 0.040 mol l^{-1} NaOH. Anolyte solution: 0.100 mol l^{-1} phosphoric acid in 0.40% methylcellulose. Working ampholyte solution: 4% (pH 6–8:pH 3–10, 10:1, v/v) in 0.40% methylcellulose. Hydrodynamic sample injection: 3.5 kPa by 10 s. Focalization step: 30 kV during 5 min. Pressure mobilization step: 30 kV and 3.5 kPa pressure, applied simultaneously.

During pressure mobilization, a mechanical force acts upon the solution, generating a parabolic flow profile, in the same manner as it occurs in liquid chromatographic systems. Loss of resolution of bands previously focused during the focalization procedure has been attributed to pressure driven flows. During chemical mobilization, performed by altering the composition of the catholyte solution by the addition of an inert salt, the solution does not suffer the effect of a mechanical force. Consequently, the high-resolution profile achieved during the focalization step is transferred into the mobilization step resulting in well-defined focused bands.

A new approach was then proposed by incorporating a chemical mobilization procedure in the already tested pressure mobilization based method (Fig. 1). Even though an increase in the ampholyte concentration is known to improve peak resolution by diminishing protein adsorption to the capillary wall, a variation of 2–4% did not contribute to any improvement in the separation quality. A 4% ampholyte working solution in methylcellulose was then employed due to satisfactory performance during the new proposed mobilization procedure. The concentration of methylcellulose in the ampholyte working solution was also tested in the 0.30–0.40% interval. There was no observable effect on resolution, however, the less viscous 0.30% solution made the capillary filling and flushing in between runs easier and more efficient, being adopted thereafter. It is worthy mentioning that capillary conditioning in between runs with methanol prior to the flush with ampholyte working solution produced a large variation of migration times. Flushing with only the ampholyte working solution seemed to be much more effective and reproducible as demonstrated by the statistical results compiled in Section 3.2. Another particularity of the method is that viscosity of the anolyte and catholyte does not have to match, i.e. the anolyte does not need to be prepared in methylcelullose since this solution is not pulled into the capillary during the chemical mobilization procedure.

Fig. 2 presents the electropherogram of a

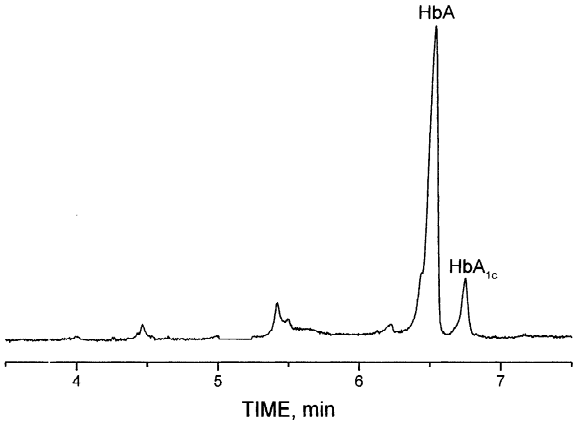

Fig. 2. Analysis of a typical hemolysate sample by the proposed CIEF methodology in a coated capillary using chemical mobilization. Capillary: DB-1, 50 μm I.D., 27 cm total length, 0.20 μm coating thickness, flushed with ampholyte working solution (138 kPa, 2 min) in between runs. Catholyte solution during focalization: 0.040 mol l^{-1} NaOH. Catholyte solution during mobilization: 0.040 mol l^{-1} NaOH containing 0.080 mol l^{-1} NaCl. Anolyte solution: 0.100 mol l^{-1} phosphoric acid. Working ampholyte solution: 4% (pH 6–8:pH 3–10, 10:1, v/v) in 0.30% methylcellulose. Hydrodynamic sample injection: 3.5 kPa by 10 s. Focalization step: 30 kV during 5 min. Chemical mobilization step: 30 kV during 3 min.

hemolysate sample using the optimized chemical mobilization method. The sample analyzed in Fig. 2 was hemolysed and stored properly right after preparation, therefore, the presence of HbA_3 was not visualized. Most importantly, HbA and HbA_{1c} were baseline resolved, feature not presented when pressure mobilization was employed (Fig. 1).

3.2. Inter-assay and intra-assay precision

Four hemolysate samples were analyzed in replicates of six, in four different days [10]. Results of concentration and migration time of HbA_{1c} (Tables 1 and 2) were treated by means of one-way analysis of variance (ANOVA). Tables 3 and 4 summarize the results of variance and *F* statistics. ANOVA results demonstrated that the HbA_{1c} concentration of the four samples, measured in four different days in different weeks were not statistically different at 95% confidence level. Tests of ANOVA took 4 weeks to be completed. Since the concentrations of HbA_{1c} did not vary, it follows that hemolysate samples can be stored at least for 1 month.

Migration times did not show equivalent results in different days, despite of the small variance in a single day (Table 4). This might be related to the capillary storage procedure, which might contribute to the alteration of the capillary surface, loss of coating and also the ineffectiveness of the capillary conditioning prior to analysis in recovering the capillary properties. However, despite the drift in migration time, the correct identification of HbA_{1c} or any other hemoglobin of interest can be easily done by means of a plot of isoelectric point versus migration time (such as that demonstrated in Section 3.4).

Table 1
Concentration of glycated hemoglobin used in the ANOVA calculations of Table 3. Four hemolysate samples were analyzed in replicates of six, during 4 days in different weeks

Sample	% HbA_{1c}			
	1° day	2° day	3° day	4° day
01	6.4	6.1	6.3	6.9
	6.7	7.1	5.3	5.8
	6.5	7.1	6.0	8.2
	8.0	7.3	5.5	6.3
	7.2	6.4	5.8	6.3
	6.9	6.4	7.2	6.3
02	9.9	9.6	10.0	9.5
	9.8	10.6	10.6	11.8
	9.6	10.4	9.8	11.7
	9.7	10.5	9.7	10.5
	10.3	9.8	9.2	10.6
	10.0	7.5	10.4	10.5
03	12.5	12.8	14.2	11.3
	13.9	14.1	12.8	12.0
	11.6	12.9	13.2	11.4
	12.5	13.4	12.3	12.5
	11.5	10.8	13.3	12.1
	13.5	13.6	12.2	11.6
04	7.8	6.2	6.9	7.7
	7.1	6.5	6.9	7.9
	7.2	6.8	7.1	6.4
	6.5	6.8	7.1	6.7
	7.4	7.3	7.8	6.5
	6.1	7.5	7.8	8.2

Table 2
Migration time of glycated hemoglobin used in the ANOVA calculations of Table 4. Four hemolysate samples were analyzed in replicates of six, during 4 days in different weeks

Sample	Migration time (min)			
	1° day	2° day	3° day	4° day
01	7.12	7.03	7.18	7.15
	7.14	6.94	7.15	7.20
	7.13	6.94	7.28	7.15
	7.10	7.02	7.10	7.17
	7.09	7.25	7.10	7.12
	7.12	6.95	7.23	7.12
02	7.13	7.01	7.32	7.24
	7.04	6.95	7.32	7.05
	7.06	6.84	7.32	7.01
	7.02	6.92	7.22	7.21
	6.94	7.05	7.29	7.07
	7.10	7.39	7.17	7.07
03	7.02	7.33	7.47	7.01
	6.98	7.09	7.37	7.13
	6.93	7.19	7.09	7.00
	6.94	7.11	7.02	6.99
	6.94	7.32	7.02	6.91
	6.91	7.06	7.05	6.93
04	6.97	6.94	6.88	6.97
	6.98	6.92	6.90	6.99
	6.98	6.94	6.90	6.97
	6.97	6.90	6.88	7.04
	6.96	6.91	6.90	7.05
	6.96	6.92	6.88	7.09

3.3. Correlation

Thirty-one hemolysate samples were analyzed by both the proposed CIEF and the reference chromatographic methods. The correlation between the two sets of results is demonstrated in Fig. 3. In Fig. 3A, student *t*-test was used to verify whether the slope and intercept differ from 1 and zero, respectively [11]. The slope was found to be not statistically different from 1 ($t_{calculated}=1.69$, $t_{critical}=2.04$), however the intercept differs from zero ($t_{calculated}=4.52$, $t_{critical}=2.04$), at 95% confidence level. In Fig. 3B, data are presented in a Bland and Altman plot format [12]. These results imply that the proposed CIEF method yields HbA_{1c} concentrations greater than those provided by the reference method. In order to implement the proposed CIEF methodology in a clinical laboratory, a complementary study with a larger number of samples to determine the reference range of HbA_{1c} for non-diabetics and diabetic patients should be conducted to assure reliable diagnostics.

3.4. Determination of isoelectric point

A mixture of hemolysate samples containing hemoglobins HbS, HbC, HbF, HbA, HbA_2 and HbA_{1c} was analyzed by the proposed method and the electropherogram is illustrated in Fig. 4. Taking into consideration literature data of isoelectric points for the above mentioned hemoglobins [7], and their corresponding migration times, a calibration curve was built (Fig. 5). This type of curve allows for the identification of newer hemoglobin variants as well as to establish the identity of known hemoglobins in a given sample. It is also worthy commenting on the resolving power of CIEF: a p*I* difference as small as ca. 0.03 is enough to promote baseline resolution, as it is the case of the HbC/HbA_2 and HbA/HbA_{1c}. The later pair presents an additional experimental difficulty because HbA is much more abundant hemoglobin, occurring in an average concentration of 90% whereas HbA_{1c} occurs at concentrations

Table 3
Results of the ANOVA calculation for the concentration of glycated hemoglobin depicted in Table 1

Sample	Average % HbA_{1c}				Variance				*F*
	1° day	2° day	3° day	4° day	1° day	2° day	3° day	4° day	
01	7.0	6.7	6.0	6.6	0.4	0.2	0.5	0.7	2.21
02	9.9	9.7	10.0	10.7	0.1	1.4	0.2	0.8	2.00
03	12.6	12.9	13.0	11.8	0.9	1.3	0.5	0.2	2.30
04	7.0	6.8	7.3	7.2	0.3	0.2	0.2	0.6	0.66

Obs: *F* critical=3.09 at 95% confidence level.

Table 4
Results of the ANOVA calculation for the migration time of glycated hemoglobin depicted in Table 2

Sample	Migration time (min)				Variance				*F*
	1° day	2° day	3° day	4° day	1° day	2° day	3° day	4° day	
01	7.12	7.02	7.17	7.15	0.0003	0.01	0.005	0.0009	5.22
02	7.05	7.03	7.27	7.11	0.004	0.04	0.004	0.009	5.51
03	6.95	7.18	7.17	7.00	0.002	0.01	0.04	0.006	5.53
04	6.97	6.92	6.89	7.02	0.0005	0.0002	0.0001	0.002	26.4

Obs: *F* critical=3.09 at 95% confidence level.

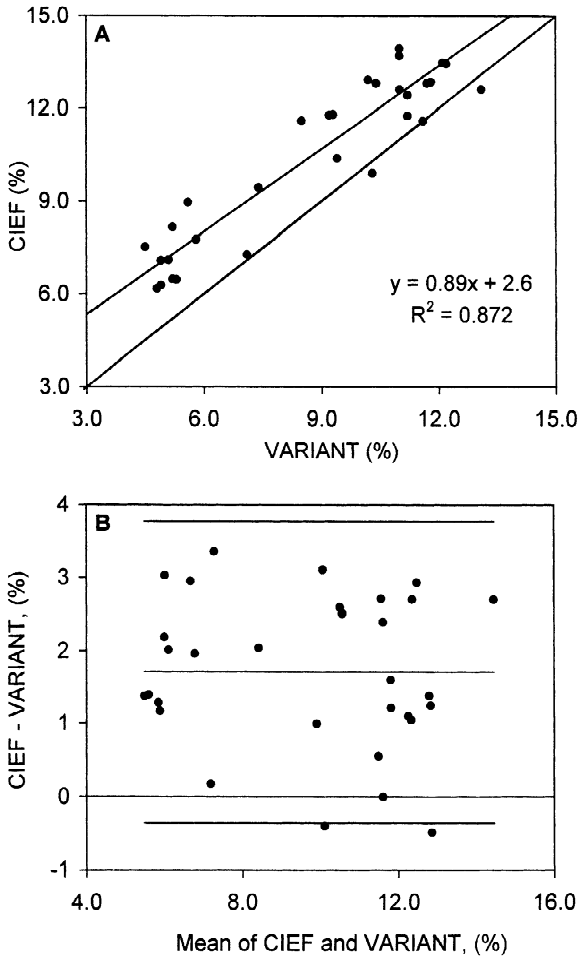

Fig. 3. Correlation study of the proposed CIEF method and the chromatographic reference method (Variant™, Bio-Rad). (A) Scatter plot and (B) Bland-Altman plot. Lines in (A) indicate the regression line and slope 1 line. Lines in (B) indicate average and ±1.96 standard deviation limits.

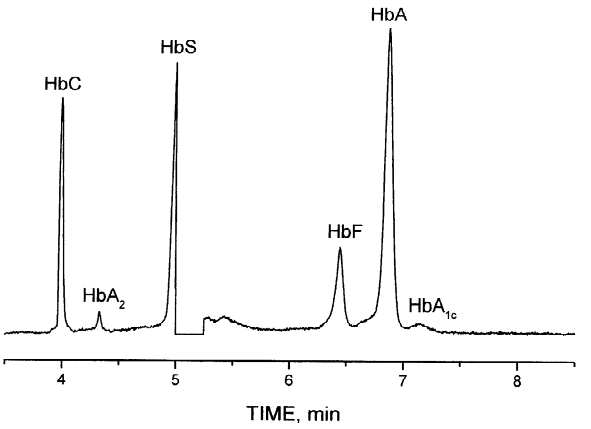

Fig. 4. Analysis of a mixture of hemoglobins by the proposed CIEF methodology. Conditions as in Fig. 2. Isoelectric points: HbC (7.445); HbA_2 (7.412); HbS (7.210); HbF (7.060); HbA (6.972) and HbA_{1c} (6.935) [7].

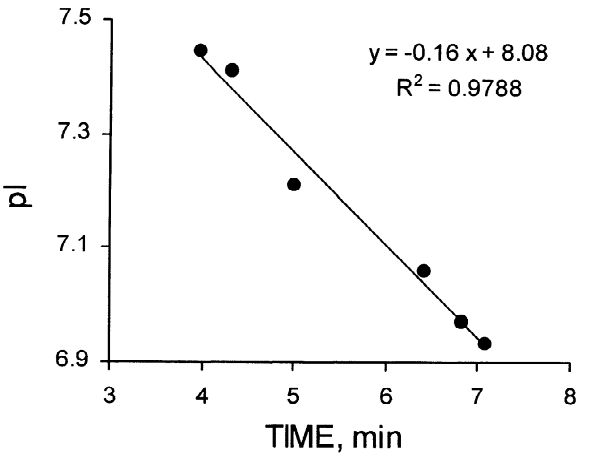

Fig. 5. Calibration curve of isoelectric point versus migration time for normal hemoglobins and variants.

lower than 10% in normal subjects. If the ratio of concentrations approaches one, it is possible to resolve hemoglobins that differ in p*I* by even less than 0.03, as suggested by the peak spacing of HbC and HbA_2 with 0.4 min apart.

By inspecting the calibration curve of Fig. 5 it is readily observed that hemoglobin HbS p*I* value lacks fitting. That is happening because HbS presented a migration time of 5 min, which corresponds to the time that the power supply is turned off for the replacement of the catholyte vial to initiate chemical mobilization. If HbS is the hemoglobin of interest, this lack of fitting can be easily overcome by increasing the focalization time before mobilization starts.

4. Conclusion

A novel CIEF methodology for the analysis of glycated hemoglobin was proposed in which the use of chemical mobilization has been proven to be crucial for the baseline resolution of closely related hemoglobins. The proposed CIEF methodology stands out from the methods in the literature due to its simplicity, rapidness (8 min run), reliability, availability of materials and reagents and easy to implement and transfer to clinical laboratories. The results demonstrated that the method can be applied not only to HbA_{1c} but also to other hemoglobins and variants, such as HbA, HbA_2, HbF, HbS and HbC, as well as to the p*I* detemination of new hemoglobin variants.

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