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Qualitative and quantitative analysis of hemoglobin variants by capillary isoelectric focusing

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Abstract

We developed two capillary isoelectric focusing (CIEF) assays, in narrow pH gradients, with the aim of routinely separating and quantitating normal and abnormal hemoglobins (Hbs): a one-step CIEF assay where residual electroosmotic flow mobilizes the proteins during focalization, and a two-step CIEF assay where focused Hbs are mobilized by low pressure by maintaining high-voltage. The resolution of 0.10 pH unit obtained with the one-step assay allowed the separation of the Hbs A, F, S and C; but Hb A_2, which represents about 2–3% of whole Hb, could not be quantitated. The better resolution of 0.02 pH unit obtained with the two-step assay allowed the separation of some Hb variants of very close isoelectric points. The reproducibility of retention times was satisfactory (C.V.$<$5%). Moreover, in this configuration quantitation of Hb A_2, Hb F and Hb S led to a standard deviation of less than 5%, allowing the diagnosis of thalassemias. The one-step assay could be useful only for the detection of abnormal variants, while the two-step assay could be applied to the routine analysis of Hbs, with quantitation of minor fractions and presumptive identification of variants. © 1998 Elsevier Science B.V.

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1. Introduction

Hemoglobins (Hbs), the main component in red blood cells, are polypeptide tetramers consisting of two pairs of unlike globin chains to each of which a heme group is bound. Many congenital and acquired disorders are associated to abnormal levels of normal globin chains (thalassemias) and/or production of variant globin chains [1]. Thus, accurate identification and quantitation of normal and abnormal Hb variants are of great clinical interest. Different electrophoretic approaches, including electrophoresis at alkaline pH on cellulose acetate, at acidic pH on citrate agar and isoelectric focusing (IEF), as well as high-performance liquid chromatography (HPLC), immunological assays, structural analysis and genotypic methods are used for Hb investigations [2].

Capillary isoelectric focusing (CIEF) first reported by Hjertén and Zhu [3] combines the advantages of IEF which emerges as a remarkable resolving method for Hb analysis and of high-performance liquid cation-exchange chromatography which offers automation and direct quantitation with full computerization. CIEF methods can be differentiated by the mobilization which can be performed either after focalization by both chemical and pressure methods (two-step CIEF) [3] or which can occur simultaneously with focalization (one-step CIEF) [4]. Two-step CIEF with chemical mobilization [5,6] as well

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as one-step CIEF [7] were previously applied to qualitative Hb analysis; however only two-step CIEF with pressure mobilization was applied to both qualitative and quantitative Hb analysis [8]. The aim of the present work was to develop two CIEF assays, one-step and two-step, on a unique coated capillary and to compare their performances for routine qualitative and quantitative investigations of Hbs.

2. Experimental

2.1. Apparatus

The capillary electrophoresis (CE) system was a P/ACE 5000 equipped with ultraviolet–visible detection set at 415 nm and System Gold software, version 8.1 from Beckman Instruments (Fullerton, CA, USA). The separations were performed on a 37 cm (30 cm to detector)×50 μm I.D. neutral-coated capillary from Beckman.

2.2. Chemicals

All chemicals used were of analytical-reagent grade. N,N,N′,N′-Tetramethylethylenediamine (TEMED) was obtained from Bio-Rad (Anaheim, CA, USA) and carrier ampholytes, Pharmalyte pH 6–8 and pH 7–9, from Pharmacia (Uppsala, Sweden). All other chemicals were from Sigma (St. Louis, MO, USA).

2.3. Biological samples

Adult and newborn samples were routinely collected at the maternity ward of Hôpital Saint-Antoine (AP-HP, Paris, France) and received in the laboratory for Hb analysis. Blood was drawn by venipuncture using EDTA-containing tubes. Whole blood samples (30 μl) were lysed by addition of 1 ml hemolyzing solution (5 m*M* KCN). Then the lysate was centrifuged for 3 min at 12 000 *g* and the clear supernatant was directly used for CIEF. A pathological control (Lyphochek hemoglobin A_2 level 2 from Bio-Rad) containing Hbs A_2, S, F and A [isoelectric point (p*I*): 7.4, 7.21, 7.05 and 6.98, respectively] was stored and used according to the manufacturer's instructions.

2.4. Capillary isoelectric focusing

For the one-step CIEF assay, experiments were performed in the reverse polarity mode with 20 m*M* NaOH as catholyte at the capillary inlet and 10 m*M* H_3PO_4 as anolyte at the outlet. The capillary was filled (138 kPa, 3 min) with the sample mixed to the 5% ampholyte pH 6–8 solution in 0.2% hydroxypropylmethylcellulose (HPMC) 4000 cP at 20 g/l supplemented with 0.75% TEMED to prevent sample focusing past the detector window. Application of the electrical field (7 kV) simultaneously focused and mobilized the hemoglobins.

For the two-step CIEF assay, experiments were performed in the normal polarity mode with 20 m*M* H_3PO_4 as anolyte at the inlet and 20 m*M* NaOH as catholyte at the outlet. The capillary was filled (138 kPa, 3 min) with the 2% ampholyte solution (pH 6–8 for 75% and pH 7–9 for 25%) in 0.4% methylcellulose (MC) 1500 cP at 20 g/l followed by a sodium hydroxide backflush (138 kPa, 0.1 min) to limit the focusing area to the capillary section anodal to the detection window. The sample was loaded by low-pressure injection (3.5 kPa, 15 s), focused for 3 min at 30 kV and then mobilized by low-pressure by maintaining a high voltage (25 kV).

The reagents must be changed every twenty runs, which corresponds to the capacity of the apparatus, or after 18 h at room temperature.

For both assays, the Gold software quantifies the data on the basis of peak areas and values are expressed as a percentage of total hemoglobin. Because of the selective absorption of heme at 415 nm, all major and minor detected peaks were attributed to Hbs.

3. Results and discussion

3.1. Resolution

For the separation of Hb variants, a one-step CIEF assay was first described using an uncoated capillary [7]. The insufficient resolution (R_s) obtained in this configuration led us to develop such an assay but on a neutral coated capillary. The resolution was evaluated by the slope of the pH gradient, considering that the best resolution is associated with the lowest

slope. The pH gradient was not directly measurable but it was determined from the retention times of standard Hbs: Hb A_2 (pI=7.42), Hb S (pI=7.21), Hb F (pI=7.05) and Hb A (pI=6.98). We adjusted the TEMED concentration to limit the pH gradient to the capillary area between the detector and the capillary outlet. Among the TEMED concentrations tested, the lowest slope was obtained with 0.75% (Fig. 1). By increasing the concentrations of ampholyte pH 6–8 from 5 to 10% and of HPMC from 0.2 to 0.3%, the resolution was not improved but spikes on the electrophoregrams were more frequent (data not shown). For this reason we kept both lowest concentrations. These spikes could be due to overheating during focalization in relation to high viscosity.

A highly resolving two-step CIEF assay was also described using a dimethylpolysiloxane-coated (DB-1) capillary [4]. In our experience this capillary gave an extremely high variability of retention times so that it could not be used for routine Hb analysis. Thus we developed a new two-step CIEF assay, keeping the physico-chemical conditions described for DB-1 but using the same neutral capillary as for the one-step assay. The resolution, evaluated by the linearity and the slope of the pH gradient, was enhanced when Pharmalyte pH 6–8 and pH 7–9 were mixed (Fig. 2).

Under the conditions described above, the separation of the Hbs F, A, A_{1c} and Hb S (the most common variant in our laboratory) was achieved by both CIEF assays in 15 min. Hb A_2 was not resolved from Hb S in the one-step assay whereas it was separated in the two-step assay (Fig. 3).

When we define the R_s in terms of the difference in pI values of adjacent peaks, assuming that (i) the pH gradient is linear, (ii) these adjacent bands are of equal width and (iii) the solutes are similar in diffusion coefficient, the R_s becomes $(t_2-t_1)/\Delta t$, where t_2 and t_1 are the migration times of the two proteins and Δt the peak width [9]. Thus, based on the separation of Hb F from Hb A, the resolution was about 0.10 pH unit in the one-step assay and of 0.02 pH unit in the two-step assay. Even in the two-step assay the R_s did not reach the value obtained with two-step CIEF on DB-1 [8]. However, the R_s obtained in our two-step assay was better than that obtained with chemical mobilization on linear

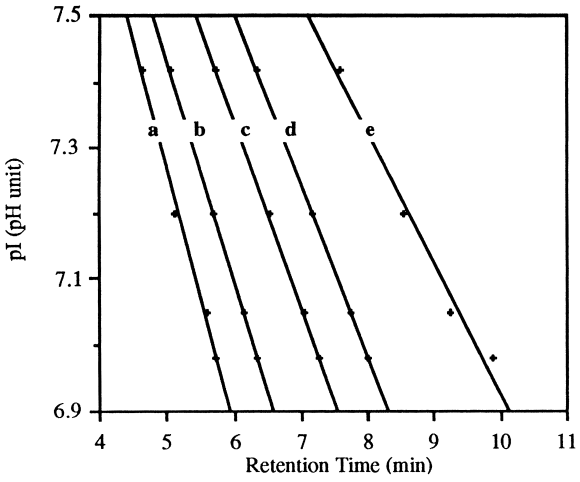

Fig. 1. pH gradient curves of standard hemoglobins (Hb A_2, S, F and A; pI: 7.4, 7.21, 7.05 and 6.98, respectively) by one-step CIEF on a neutral capillary at various TEMED concentrations: (a) TEMED at 0.3% (y=9.268−0.399x, r=0.994), (b) TEMED at 0.4% (y=9.116−0.337x, r=1.000), (c) TEMED at 0.5% (y=9.048−0.284x, r=1.000), (d) TEMED at 0.6% (y=9.075−0.261x, r=1.000), (e) TEMED at 0.75% (y=8.892−0.196x, r=0.981).

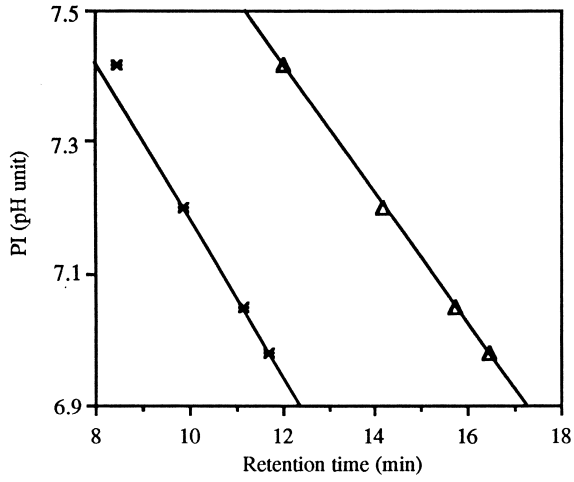

Fig. 2. pH gradient curves of standard hemoglobins (Hb A_2, S, F and A; pI: 7.4, 7.21, 7.05 and 6.98, respectively) by two-step CIEF on a neutral capillary. (*) With the ampholyte solution pH 6–8 at 2% the gradient was not linear for pI above 7.35 (y=8.369−0.119x, r=0.999); (△) with a mixture of ampholyte pH 6–8 (75%) and pH 7–9 (25%) at a final concentration of 2% the linearity was increased at least until pI 7.45 and the slope was decreased.

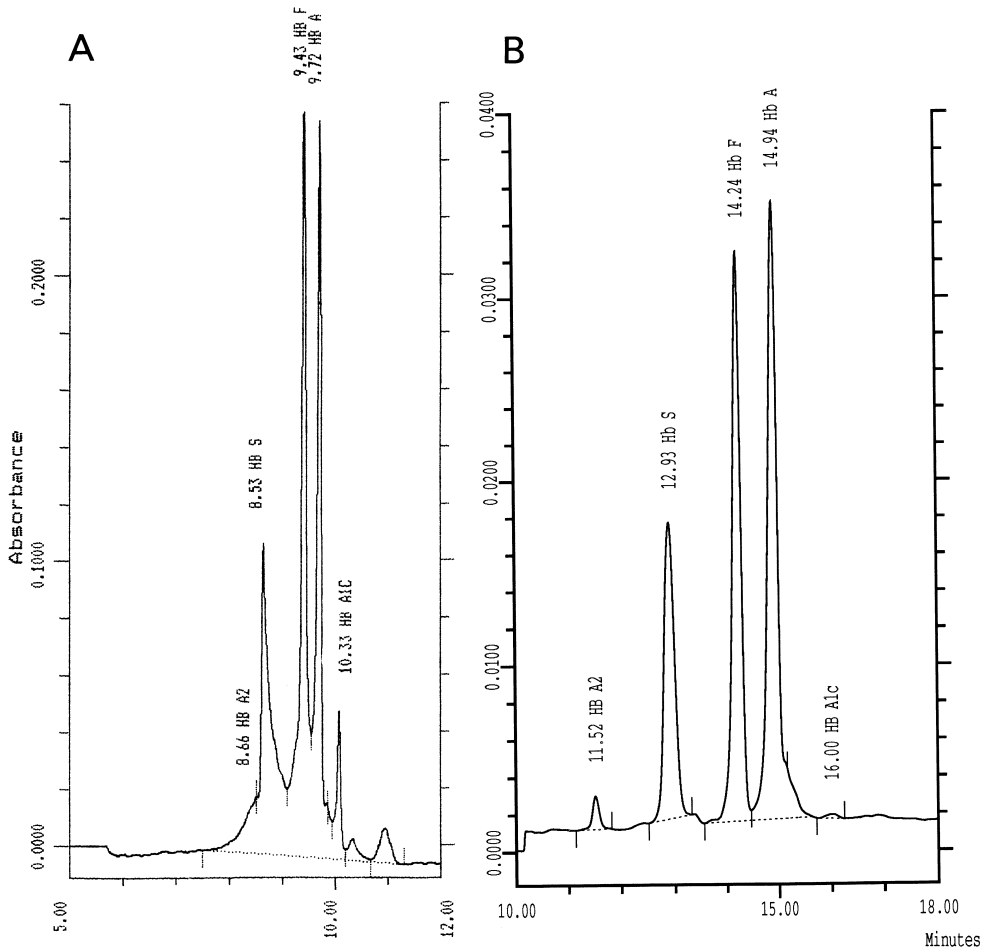

Fig. 3. Separation of hemoglobins A_2, S, F, A and A_{1c} by CIEF under the finally adopted conditions; (A) one-step assay, (B) two-step assay.

acrylamide-coated capillaries [5,6]. We obtained a baseline resolution between Hb F and Hb A which was not the case when a polyaminoethoxyethanol-coated capillary was used, even in the presence of separators added to flatten the pH gradient in this portion [10]. The 0.02 pH unit R_s of the two-step assay allowed separation of some Hb variants that comigrate with Hb A_2 in alkaline electrophoresis: e.g., Hb A_2, Hb E and Hb C-Harlem were partially resolved from Hb C, but Hb E still comigrated with Hb A_2 (Fig. 4). Hb D-Korle-Bu, which comigrates with Hb S in alkaline electrophoresis, could be differentiated from Hb S as shown by the superimposition of the two-step CIEF profiles obtained from a newborn with double heterozygous S/C disease and another newborn with D-Korle-Bu trait (Fig. 5).

3.2. Quantitation of hemoglobins

With the one-step assay, Hb quantitation was not evaluated because Hb A_2 was not totally resolved from Hb S. For the two-step assay, the precision was evaluated with various samples: one with low levels of Hb A2, a second with increased levels of both Hb A_2 and Hb F, and a third containing Hb S. Quantitation of Hbs A_2, F and S was previously realized by high-performance cation-exchange chromatography on Poly CatA column with a method derived from that of Ou and Rognerud [11]. Intra-assay variability

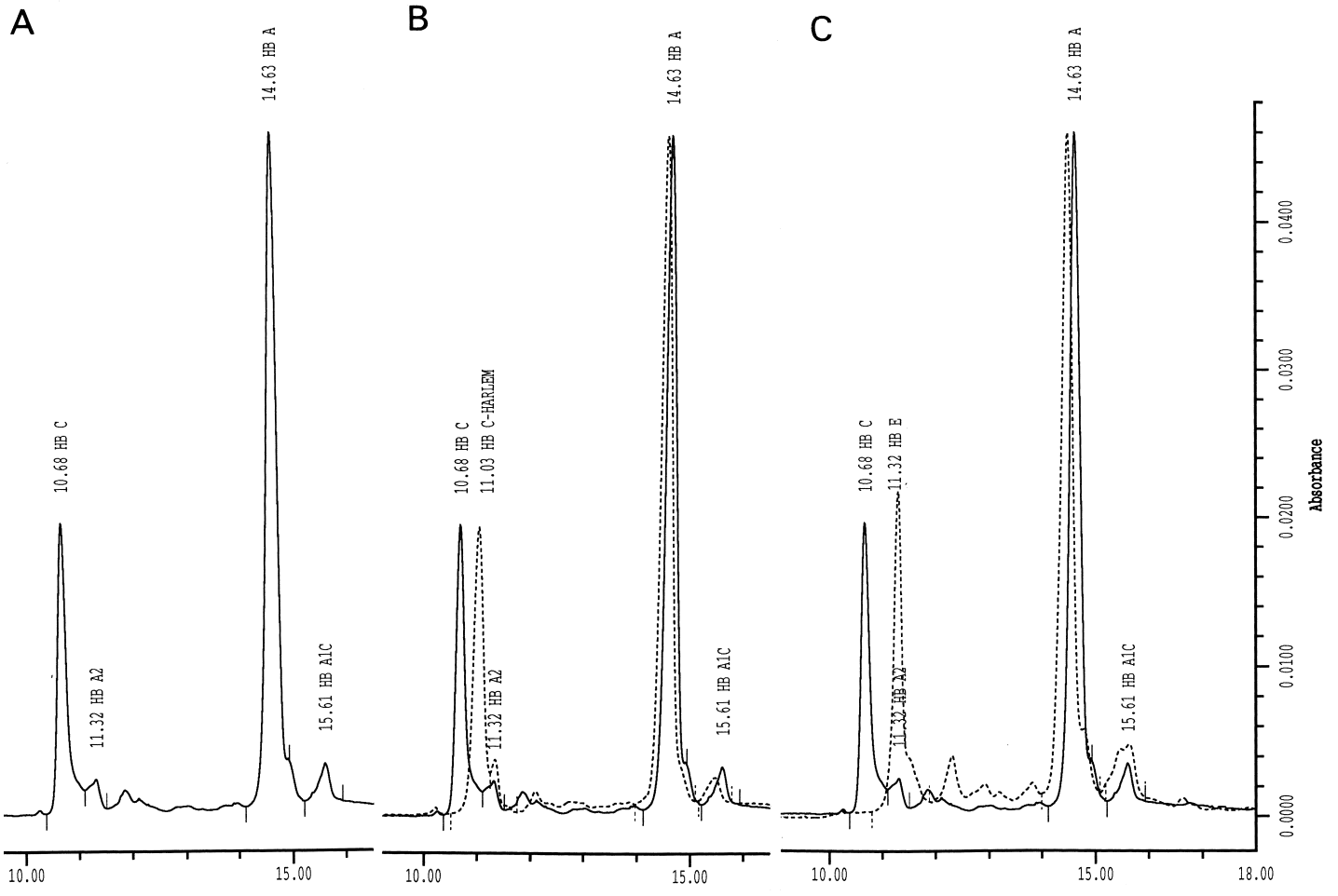

Fig. 4. Two-step CIEF profiles of hemoglobin variants that comigrate with Hb A_2 in alkaline electrophoresis; (A) patient with Hb C trait, (B) patient with Hb C-Harlem trait (- - -) superimposed on patient with Hb C trait (———), (C) patient with Hb E trait (– – –) superimposed on patient with Hb C trait (———).

was determined by analyzing each specimen 20 times in a series and inter-assay by analyzing each one, stored at −20°C, on 10 different days. The results yielded coefficients of variation (C.V.s) between 2.9 and 12%, the intra-assay C.V.s being always lower than corresponding inter-assay C.V.s (Table 1). The relatively high C.V. for Hb A_2 at low level is in agreement with the unique precision study reported by Hempe and Craver [8] and is without clinical consequence. Indeed, the pathological values observed in β-thalassemias are related to an increase in Hb A_2. We also performed a linearity study for Hb A_2 demonstrating that the measure is analytically suitable from 0.8% (data not shown).

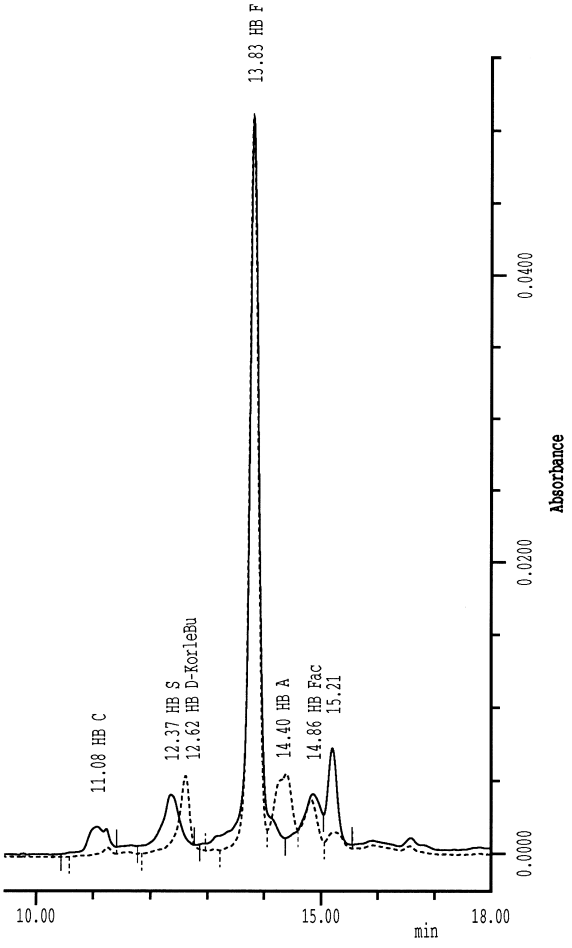

Fig. 5. Superimposition of two-step CIEF profiles of two newborn samples, one from a newborn with Hb S/C disease (———) and another from a newborn with Hb D-Korle-Bu trait (- - -).

Table 1
Precision of hemoglobin analysis by two-step CIEF assay

	Hb A_2		Hb S	Hb F
	Normal	High		
Intra-assay (n=20)				
Mean[a]	1.7	4.9	22.3	7.7
C.V. (%)	7.5	2.9	5.4	1.4
Inter-assay (n=10)				
Mean[a]	1.7	5.2	21.7	7.8
C.V. (%)	12	4.6	6.1	2.1

[a] Mean values in percent of total hemoglobin.

The linearity of the two-step CIEF assay for Hb F was evaluated on lysates, assayed as duplicates and prepared with various volume ratios of bloods from two individuals: a normal adult without measurable Hb F and a normal newborn with predominant Hb F; the results are depicted in Fig. 6. As determined by linear regression, the linearity was excellent (r^2= 0.999) for values above 0.5%, showing a sensitivity adapted to Hb F measurement in both adult and newborn.

Hb A_{1c}, a glycated form of Hb A, was also separated but its quantitation was not evaluated because it is out of the hemoglobinopathies field.

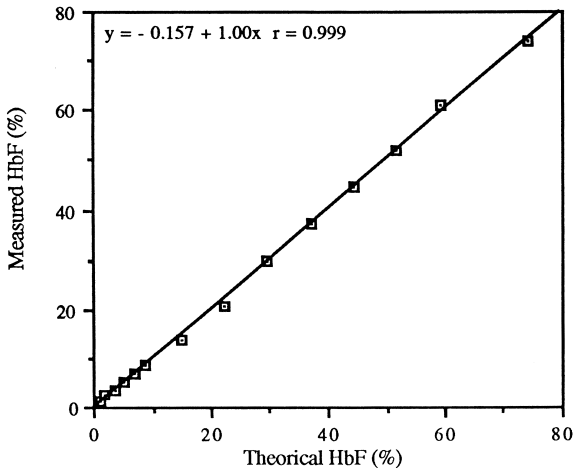

Fig. 6. Linearity of two-step CIEF assay for Hb F. Samples were prepared by mixing in various volume ratios (adult:infant) blood from a normal adult and blood from a normal newborn. Theoretical Hb F % in the newborn sample was determined by high-performance cation-exchange chromatography on Poly CatA column, as routinely realized in our laboratory.

3.3. Reproducibility of retention times

The reproducibility of retention times was evaluated for the two-step assay on a normal adult sample stored at −20°C. Intra-assay reproducibility was calculated from the real retention times of Hb A obtained in 20 successive runs in one day and inter-assay reproducibility on 10 different days, with different reagent preparations. Some authors standardized the retention times on the one of Hb A to enhance the reproducibility [8], but this approach cannot be used when Hb A is not the predominant Hb fraction, as in newborn samples. Within-day C.V. was 1.0% and between-day C.V. 4.9%. The satisfactory reproducibility of retention times combined with a good resolving power allowed unequivocal identification of common Hb variants. Nevertheless, we observed a progressive decrease in the retention times leading to a loss of resolution after about 200 runs; the instability of the coating or of the inner treatment of the capillary certainly is responsible for this trouble as it has been reported for other capillaries using narrow pH gradients [12]. Variation in retention times is still a problem in CIEF, whatever the capillary and the assay [7,13].

4. Conclusions

In conclusion, CIEF on a neutral capillary is suitable for routine investigation of hemoglobinopathies. The one-step CIEF assay does not allow quantitation of all the Hb fractions of clinical interest but is suitable for the screening of Hb variants. The two-step CIEF assay can be performed for both identification and direct quantitation of major and minor Hb fractions; it allows the complete Hb analysis since it is able to characterize the most common Hb variants and to diagnose β-thalassemias as defined by elevated Hbs A_2 and F. The development of such a configuration offers an alternative to high-performance ion-exchange chromatography currently used in clinical laboratories [11,14]. The two methods use small sample volumes; then they can be applied to the diagnosis of hemoglobinopathies in newborns, for example in screening programs.

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