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Automation and Analytical Techniques

Capillary isoelectric focusing and high-performance cation-exchange chromatography compared for qualitative and quantitative analysis of hemoglobin variants

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We have developed two assays for complete analysis of hemoglobins (Hbs) in the field of hemoglobinopathies: a high-performance cation-exchange liquid chromatography (HPLC) assay on the weak cation-exchanger Poly Cat A and a two-step capillary isoelectric focusing (CIEF) assay on the neutral-coated capillary from Beckman in a narrow pH gradient. The resolution was satisfactory for both HPLC and CIEF and allowed separation of normal and common abnormal Hbs, i.e., Hb A, Hb F, Hb A_2, Hb S, Hb C, and Hb E; slight differences were shown for the resolution of unusual variants such as Hb C-Harlem and Hb D-Punjab. The reproducibility of retention times was satisfactory as well for HPLC (CV 3.3%) and CIEF (CV 4.9%). The imprecision of quantification of Hb A_2, evaluated at two concentrations, and of Hb F and Hb S was <5%, except for low concentrations of Hb A_2 quantified by CIEF. Quantitative data obtained for these three Hb forms were highly correlated between the two assays. These results suggest that the new CIEF assay can be competitive with HPLC for complete routine analysis of Hb variants.

Hemoglobins (Hbs)[3] are polypeptide tetramers consisting of two pairs of unlike globin chains (α, β, δ, γ), to each of which is bound a heme group. Hb in normal adult human blood is >96% Hb A ($\alpha_2\beta_2$), ~2–3% Hb A_2 ($\alpha_2\delta_2$), and <1% Hb F ($\alpha_2\gamma_2$). Normal newborn blood contains Hb F as the major constituent (60–80%) and the rest is Hb A *[1]*. Analysis of the composition of human blood is of major clinical interest in several congenital defects associated with abnormal Hb content. These hemoglobinopathies are grouped into defective variants of Hbs (such as Hb S and >600 other variants) and thalassemias, which are characterized by abnormal proportions of normal globin chains *[2–4]*.

Various electrophoretic approaches, including cellulose acetate electrophoresis at alkaline pH, citrate agar electrophoresis at acidic pH, and isoelectric focusing (IEF) in polyacrylamide or agarose gels, as well as HPLC, immunological assays, structure analysis, and genotypic methods are used to investigate hemoglobinopathies *[5, 6]*. The electrophoretic methods, still widely used, are declining, given their difficulty of automation and inaccurate quantification of minor Hb constituents. HPLC is now considered to be a sensitive, specific, and reproducible alternative to electrophoresis and its use has been significantly expanded, especially with the development of rapid and well-resolving methods, some of them being applied to fully automated analyzers *[7–9]*. Moreover, newborn screening programs currently developed in various areas of Europe and the US need a universal method to detect and quantify Hb variants in small amounts of samples. In this field IEF emerges as a remarkable resolving method that allows unequivocal identification of a large number of Hb variants; HPLC with its automation and its quantitative power is starting to be used for such purposes *[10–12]*.

Capillary electrophoresis is a new versatile analytical electrophoresis technique that uses numerous separation principles, including capillary isoelectric focusing (CIEF) *[13–18]*. Capillary zone electrophoresis of intact Hbs and of globin chains, as well as the characterization of tryptic digests, has been reported by different groups *[19–23]*, and several have recently shown that Hb separation can also be achieved by CIEF with various capillaries *[21, 24–26]*. The aim of the present work was to develop and

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[3] Nonstandard abbreviations: Hb, hemoglobin; CIEF, capillary isoelectric focusing; IEF, isoelectric focusing; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol.

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compare, for both resolution and quantification capabilities, two methods suitable for complete routine analysis of Hbs—a CIEF assay and a HPLC assay.

Materials and Methods

CHEMICALS

All chemicals used were of analytical-reagent grade. Methylcellulose with a viscosity of 1500 cP (20 g/L aqueous solution at 25 °C), 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol (Bis-Tris), potassium cyanide, and sodium citrate were obtained from Sigma Chemical Co. Carrier ampholytes, Pharmalyte pH 6–8 and pH 7–9, were obtained from Pharmacia.

ORIGIN AND PREPARATION OF SAMPLES

Samples from adults and newborns were collected at the maternity ward of Hôpital Saint-Antoine and sent to the laboratory for routine Hb analysis. Blood was drawn by venipuncture into EDTA-containing tubes. The samples used for this study were also analyzed by conventional methods for Hb analysis, i.e., alkaline and acidic electrophoreses, microcolumn ion-exchange chromatography for Hb A_2, and HPLC for Hb F. Blood sampling was in accordance with the ethical standards of our hospital's committee. Whole-blood samples (30 μL) were lysed by addition of 1 mL of hemolyzing solution (KCN, 5 mmol/L), after which the hemolysate was centrifuged for 3 min at 12 000*g*. The clear supernatant was used directly for CIEF or diluted twofold for HPLC.

Normal and abnormal controls (Lyphochek Hemoglobin A_2 Bi-Level; Bio-Rad) were stored and used according to the manufacturer's instructions. The pathological control containing Hb A_2, S, F, and A (pI 7.42, 7.21, 7.15, and 7.10, respectively) was included in each series of analyses.

CIEF

Instrumentation. We used a P/ACE 5000 System with a UV/visible detector set at 415 nm and System Gold software (vers. 8.1) from Beckman Instruments. The separations were performed on 50 μm (i.d.) × 37 cm (total length) [30 cm (length to detector)] neutral (polyacrylamide)-coated capillaries from Beckman.

Separation program. The instrument was set up with the anode at the inlet end of the capillary (anolyte: H_3PO_4, 20 mmol/L) and the cathode at the outlet (catholyte: NaOH, 20 mmol/L). The capillary was thermostated at 25 °C. The ampholyte solution consisted of a mixture of Pharmalyte pH 6–8 and Pharmalyte pH 7–9 (3:1, by vol), at a final concentration of 20 mL/L in a 4 g/L methylcellulose solution. Before each run, the capillary was pressure-rinsed and filled with the ampholyte solution (138 kPa, 3 min), then backflushed with the catholyte (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 3 min at 30 kV, and then mobilized by low pressure while maintaining a high voltage (25 kV). Between each analysis the capillary was rinsed with deionized water (138 kPa, 2 min). The mobility of each Hb fraction in CIEF was expressed as a retention time, as for HPLC.

HPLC

Instrumentation. We used the integrated HPLC System Gold from Beckman, consisting of a Model 126 pump gradient, a Model 166 UV/visible detector set at 418 nm, a Model 507E autosampler, and System Gold software (vers. 8.1). The system was equipped with a 100 × 4.0 mm column packed with a weak cation-exchanger, porous (100-nm pore size) 5-μm microparticulate polyaspartic acid–silica (Poly Cat A) purchased from Touzart & Matignon (Les Ulis, France).

Gradient program. Separation of Hbs was accomplished by a salt gradient obtained by mixing buffers A (Bis-Tris 20 mmol/L, KCN 2 mmol/L, pH 5.8) and B (Bis-Tris 20 mmol/L, KCN 2 mmol/L, sodium citrate 75 mmol/L, pH 5.8). The flow rate was 1.5 mL/min. The column was equilibrated with B:A (33:67 by vol). After injection of the sample, the proportion of B was increased linearly to B:A (45:55 by vol) at 5 min and to 100:0 at 9 min; finally, the mobile phase was returned to 33:67 at 11 min, for reequilibration.

DATA ANALYSIS

The System Gold software quantifies the data on the basis of peak areas; values are expressed as a percentage of total Hb. Because of the selective absorption of heme at 415–418 nm, all major and minor peaks detected were attributed to Hb.

The differences between groups of data were analyzed by Student's paired *t*-test with a two-tail probability distribution. The correlations between variables were calculated by linear regression analysis.

Results

IDENTIFICATION OF HB VARIANTS AND RESOLUTION

The separation of Hbs A, A_2, F, and Hb S (this last being the most common variant in our laboratory) was achieved by both CIEF and HPLC in <20 min and 15 min, respectively (Fig. 1). The resolution in CIEF (Rs) can be defined as the difference in isoelectric point of adjacent peaks and, in a linear pH gradient, can be calculated with the formula: $Rs = (t_2 - t_1)/\Delta t$, where t_2 and t_1 are the migration times of two adjacent peaks, and Δt is the peak width *[27]*. The gradient was linear between pH 7.0 and 7.5; based on the separation of Hb F from Hb A, therefore, the resolution was 0.02 pH unit.

The analysis by HPLC and CIEF of some β-variants that comigrate with Hb A_2 in alkaline electrophoresis are presented in Figs. 2 and 3. With HPLC, Hb A_2 and Hb E were completely separated from Hb C, but Hb C-Harlem coeluted with Hb C (not shown) and Hb E with Hb A_2. With CIEF, Hb A_2, Hb E, and Hb C-Harlem were partially

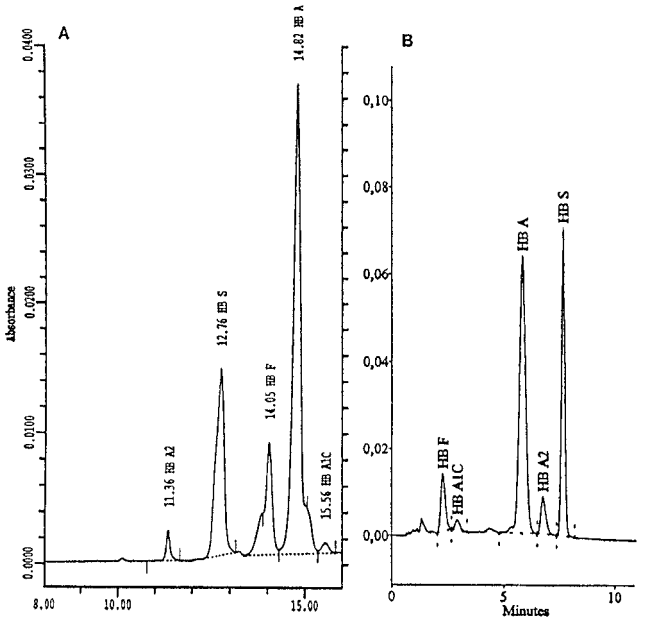

Fig. 1. Separation of hemoglobins A_2, S, F, A and A_{1c} (*A*) by two-step CIEF on a neutral capillary and (*B*) by HPLC with a Poly Cat A column.

resolved from Hb C, but Hb E always comigrated with Hb A_2. Fig. 4 compares the separations by CIEF of Hb S and Hb D-Punjab, which comigrate in alkaline electrophoresis; as shown by the superimposition of the CIEF profiles, these hemoglobins could be differentiated. However, there was no difference between the hemoglobins of group D, such as Hb D-Punjab and Hb D-Korle Bu (not shown).

REPRODUCIBILITY OF RETENTION TIMES

The reproducibility of retention times was evaluated for both methods with a normal adult sample stored at −20 °C. Intraassay reproducibility was calculated from the retention times of Hb A obtained in 20 successive runs made in 1 day and interassay reproducibility from the retention times obtained in 10 different days with use of different reagent preparations. The within-day CV (n = 20) was 1.2% for HPLC and 1.0% for CIEF. The between-day CV (n = 8) was 3.3% for HPLC and 4.9% for CIEF.

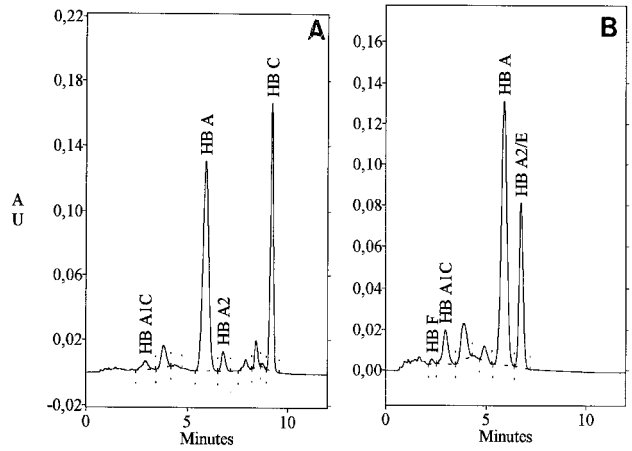

Fig. 2. HPLC profiles of hemoglobin variants that comigrate with Hb A_2 in alkaline electrophoresis: (*A*) patient with Hb C trait, (*B*) patient with Hb E trait (*AU*, absorbance unit).

QUANTIFICATION OF HB VARIANTS

For each method, imprecision was evaluated by assaying various samples: one with normal Hb A_2, a second with increased values of Hb A_2 and Hb F, and a third containing Hb S. Intraassay variability was determined by analyzing each specimen 20 times in a series and interassay by analyzing each one, stored at −20 °C, on 10 different days. The results yielded CVs between 1% and 12%, as reported in Table 1. Intraassay CVs always were less than corresponding interassay CVs, more particularly for HPLC. All but low proportions of Hb A_2 by CIEF were analytically suitable.

Table 2 summarizes results of correlation between CIEF and HPLC for quantification of Hb A_2, Hb F, and Hb S. The values of Hb A_2 measured in 43 samples (35 from apparently normal individuals and 8 with minor β-thalassemia) were significantly ($P < 0.001$) lower by CIEF than by HPLC but with a strong correlation. In the 35 apparently normal individuals, the mean Hb A_2 value was 2.0% (range 1.6–2.7%) by HPLC and 1.8% (range 1.3–2.4%) by CIEF. In the 8 patients with minor β-thalassemia, Hb A_2 was 4.9% (range 3.7–6.6%) of total Hb by HPLC and 4.1% (range 3.4–5.8%) by CIEF. The values of Hb F measured in 24 samples (16 adults and 8 newborns) did not statistically differ between both methods and were highly correlated between them. The values of Hb S measured in 20 samples (15 adults, 3 newborns with Hb S trait, and 2 adults with Hb S disease) were significantly ($P < 0.001$) higher by CIEF than by HPLC but still correlated strongly.

Discussion

Classical IEF carried out in slab gels with narrow pH gradients, usually between 6 and 8, is well recognized for its resolving power in analysis of Hb variants *[28]*. However, quantification by densitometry does not allow accurate measurement of Hb fractions present in low amounts, such as Hb A_2 and Hb F in adults. Conversely, in the past decade ion-exchange HPLC has appeared to be an appropriate candidate for direct identification and sensitive quantification of major and minor, normal and abnormal, Hb fractions *[9, 29]*. CIEF now appears to be an alternative to HPLC by combining the advantages of the previous methods: (*a*) It offers the same high resolution and specificity of identification based on isoelectric point as classical IEF, and (*b*) it shares with HPLC the benefits of direct on-line detection and automation with full computerization. The purposes of our study were to develop an IEF assay on capillary electrophoresis for complete Hb analysis; to adapt to this procedure the HPLC assay routinely used in our laboratory for quantification of Hb A_{1c} and Hb F; and, then, to compare both assays for the diagnosis

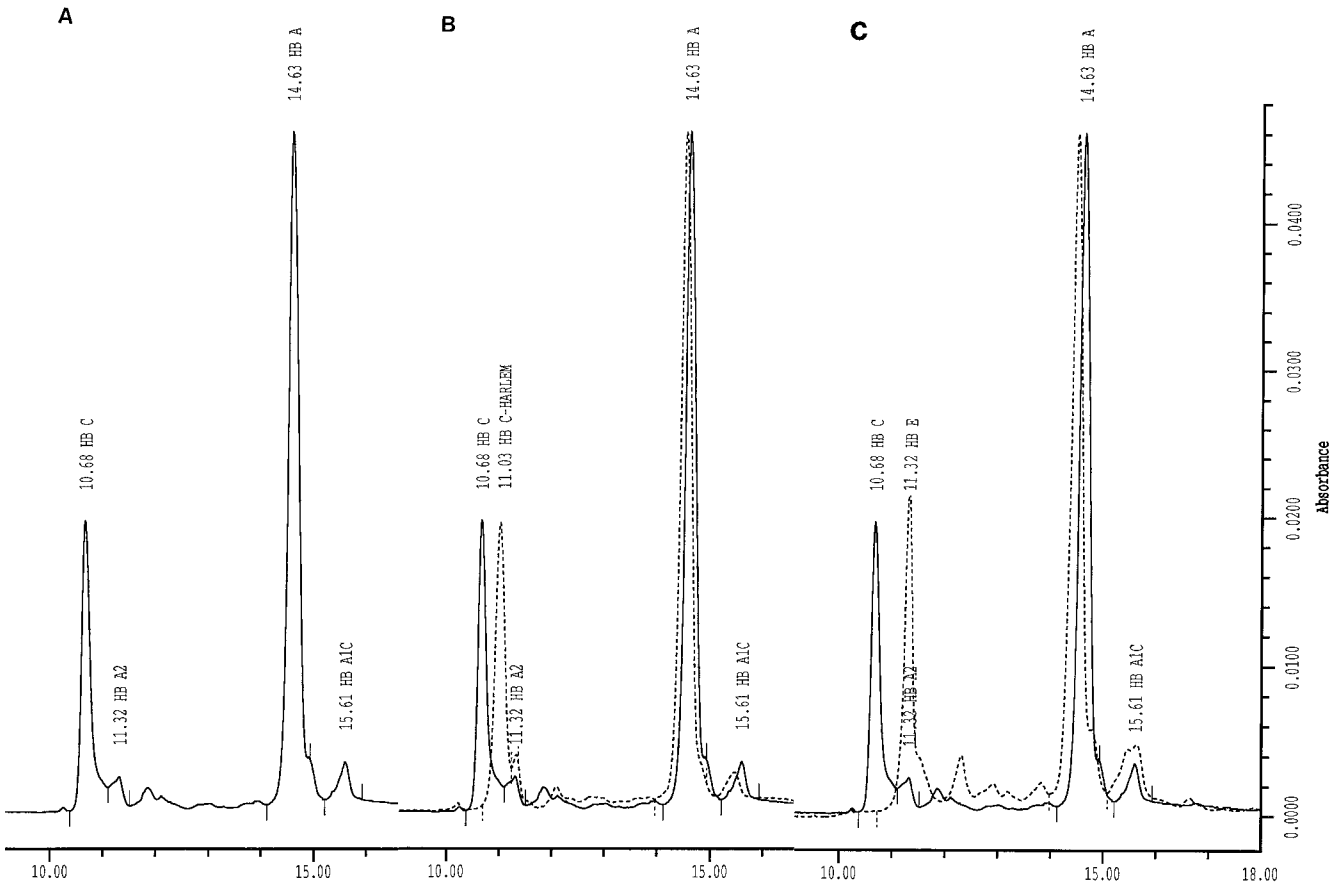

Fig. 3. CIEF profiles of hemoglobin variants that comigrate with Hb A_2 in alkaline electrophoresis: (*A*) patient with Hb C trait, (*B*) patient with Hb C-Harlem trait (—) superimposed with patient with Hb C trait (—), (*C*) patient with Hb E trait (—) superimposed with patient with Hb C trait (—).

of hemoglobinopathies both qualitatively and quantitatively.

Several CIEF configurations for Hb analysis utilizing various capillaries and pH gradients have been described; among these, two-step methods have emerged, first on fused-silica capillaries *[21]* and then on coated capillaries, for better control of electroosmotic flow and improved resolution *[25, 26]*. Many of the electropherograms presented in those studies showed poor resolution and contained several unidentified minor peaks. An enhanced resolution was obtained in narrow pH gradients but depended on the capillary used. The best resolution was observed by Hempe and Craver *[25]*, using a dimethylpolysiloxane-coated capillary (DB-1); in our experience, however, this capillary gave extremely high variability in retention times. A neutral capillary was developed in a one-step CIEF configuration and in a wide range of pH values, initially to separate monoclonal antibodies *[30]*.

To improve Hb analysis by CIEF, we developed a new CIEF assay, keeping the physico-chemical conditions of the two-step CIEF assay described for use with DB-1 but instead using the neutral capillary applied to CIEF of monoclonal antibodies. In our configuration, the resolution did not reach that obtained with DB-1 CIEF. However, it was better than that obtained with a linear acrylamino-coated capillary, which did not allow baseline resolution between Hb F and Hb A, as reported by Conti et al. *[26]*. The satisfactory reproducibility on retention times at this resolution allowed unequivocal identification of common Hb variants. However, we observed a progressive decrease in the retention times, leading to a loss of resolution after ~200 runs; the instability of the coating or of the inner treatment of the capillary was presumably responsible, as has been reported for other capillaries with use of narrow pH gradients *[31]*. Thus, the capillary must be changed when baseline resolution between Hb F and A is not obtained.

The variability on retention times was less in our HPLC assay with the Poly Cat A column than in our CIEF assay, perhaps related to greater stability of the former. The resolving powers of the CIEF and HPLC assays differ slightly, sometimes in complementary ways, because they involve different modes of separation. For example, (*a*) CIEF allowed separation of Hb C and Hb C-Harlem, whereas HPLC did not—although this separation was effected in another HPLC assay, which seemed to allow more resolution but with longer run times *[8]*; (*b*) HPLC could separate Hb C from Hb A_2, whereas CIEF did not allow baseline resolution between these Hbs, so HPLC is unique in distinguishing homozygous Hb C disease from

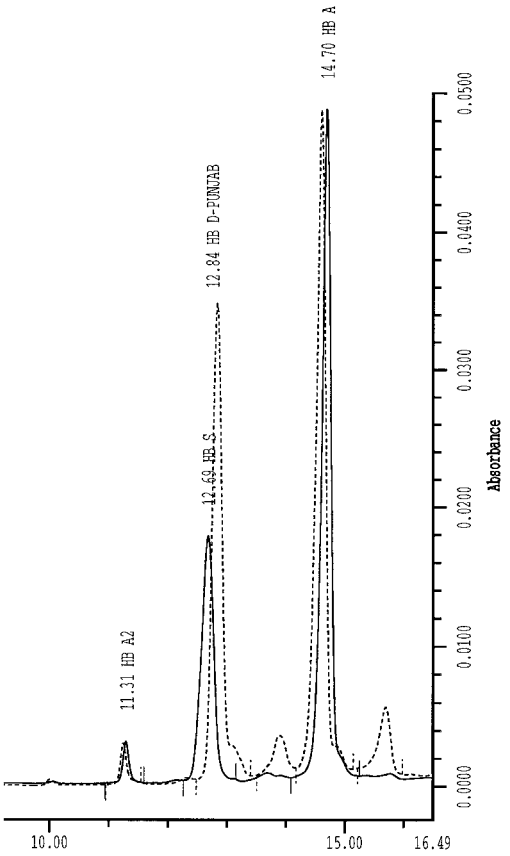

Fig. 4. Superimposition of CIEF profiles of two adult samples, one with D-Punjab trait (—) and another with Hb S trait (– – –).

double heterozygous inheritance of Hb C/β_o-thalassemia, in which Hb A_2 is increased.

Both assays were able to separate and quantify in a single run normal Hb forms and common pathological variants, i.e., Hb A, Hb F, Hb A_2, Hb S, Hb C, Hb E, and even Hb A_{1c}. Both also appeared to be more efficient than classical gel electrophoresis and more convenient and accurate than gel IEF. The imprecision of quantification estimated from peak area was satisfactory for both methods, although CV reached 12% for low amounts of Hb A_2 on CIEF. The greater between-day variabilities may be linked to a progressive degradation with time of the column or, more specifically, of the capillary.

Table 1. Precision of hemoglobin analysis by CIEF and HPLC.[a]

		Hb A_2			
		Normal	High	Hb S	Hb F
HPLC					
Intraassay (n = 20)	Mean[b]	2.2	5.2	31.1	6.8
	CV, %	0.7	0.8	1.0	0.2
Interassay (n = 10)	Mean	2.0	5.1	32.0	7.2
	CV, %	4.0	2.9	1.7	3.9
CIEF					
Intraassay (n = 20)	Mean	1.7	4.9	22.3	7.7
	CV, %	7.5	2.9	5.4	1.4
Interassay (n = 10)	Mean	1.7	5.2	21.7	7.8
	CV, %	12	4.6	6.1	2.1

[a] The samples used for the 2 methods were different.
[b] Mean percent of total Hb.

Table 2. Correlation of Hb A_2, F, and S values determined by CIEF and HPLC.

	Hb A_2	Hb F	Hb S
n	43	24	20
HPLC			
Mean %	2.54	25.7	36.8
Range %	1.61–6.62	0.2–77.2	5.5–89.1
CIEF			
Mean %	2.26[a]	25.7	41.4[a]
Range %	1.32–5.76	0.2–79.1	6.1–92.6
Slope	0.96	1.00	0.99
Intercept %	−0.15	−0.07	4.9
r	0.980	0.998	0.986

[a] Statistically significant difference between CIEF and HPLC means ($P < 0.001$).

Quantitative values for Hb A_2, Hb F, and Hb S were highly and linearly correlated between both methods. The lower Hb A_2 values obtained with CIEF may be related to the separation of glycated Hb A_2 from the nonglycated Hb A_2, as previously described *[25]*. Both CIEF and HPLC allowed the diagnosis of β-thalassemia by an accurate quantification in a single run of both Hb F and Hb A_2, without overlap between Hb A_2 values for normal individuals and for β-thalassemia patients. For this differentiation, both assays were more convenient than classical methods, which need two experiments, i.e., alkali denaturation for Hb F and microcolumn ion-exchange for Hb A_2. Nevertheless, the reference range for Hb A_2 by CIEF needs to be established, particularly the lower limit for normal values, because the diagnosis of minor α-thalassemia is characterized by a low Hb A_2 value. The mean values of Hb F given by both methods were identical, and both allowed accurate quantification of Hb F at low values (±1%), as observed in adults (normal or with minor β-thalassemia), and at high values, as found in newborns (≤90% in premature infants).

Both CIEF and HPLC require a relatively high investment in materials, but this can be widely compensated for by savings in technical time, as realized by the complete automation, and by the fact that either method can be used as a unique assay for complete Hb analysis. The HPLC columns are more expensive than the CIEF capillaries but have a longer lifespan. Unlike HPLC, CIEF uses very low volumes of buffer, which reduces both reagent and waste-disposal costs. The duration of analysis, ~15 min for HPLC and 20 min for CIEF, allows a relatively high daily throughput, adapted to primary use in medium-size laboratories.

In conclusion, HPLC on Poly Cat A columns as well as our new CIEF assay on a neutral capillary are suitable for

routine investigation on hemoglobinopathies. Each or both can be performed as a unique assay for identification and quantification of major and minor Hb fractions in small sample volumes and can also be used for the diagnosis of hemoglobinopathies in newborns, e.g., in screening programs. These two methods, which offer common qualities with different separation principles, could become complementary but also competitive for Hb analysis.

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