

HPLC Retention Time as a Diagnostic Tool for Hemoglobin Variants and Hemoglobinopathies: A Study of 60 000 Samples in a Clinical Diagnostic Laboratory

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Background: Previous evaluations of HPLC as a tool for detection of hemoglobin variants have done so within newborn-screening programs and/or by use of stored samples. We describe a 32-month prospective study in a clinical diagnostic laboratory in which we evaluated the imprecision of HPLC retention times and determined the retention times for hemoglobin variants seen in a multiethnic setting.

Methods: We analyzed all samples on the Bio-Rad Variant II HPLC system. For normal hemoglobin fractions and hemoglobin variants, we recorded and analyzed their retention times, their proportion of the total hemoglobin (%), and the peak characteristics. We compared the imprecision of retention time with the imprecision of retention time normalized to the retention time of hemoglobin A₀ (Hb A₀) and to the retention time of Hb A₂. Alkaline and acid hemoglobin electrophoresis, and in certain cases globin chain electrophoresis, isoelectric focusing, and DNA analysis, were performed to document the identities of the hemoglobin variants.

Results: The mean (SD) imprecision (CV) of the retention time was 1.0 (0.7)% with no statistical difference compared with the imprecision for normalized retention times. Among 60 293 samples tested, we encountered 34 unique hemoglobin variants and 2 tetramers. Eighteen variants and 2 tetramers could be identified solely by retention time and 3 variants by retention time and proportion of total hemoglobin. Four variants could be identified by retention time and peak characteristics and eight variants by retention time and electrophoretic

mobility. One variant (Hb New York) was missed on HPLC. Retention time on HPLC was superior to electrophoresis for the differentiation and identification of six members of the Hb J family, four members of the Hb D family, and three variants with electrophoretic mobilities identical or similar to that of Hb C. Six variants with electrophoretic mobilities identical or similar to that of Hb S could be differentiated and identified by retention time and proportion of total hemoglobin. HPLC detected two variants (Hb Ty Gard and Hb Twin Peaks) missed on electrophoresis.

Conclusions: The retention time on HPLC is reliable, reproducible, and in many cases superior to conventional hemoglobin electrophoresis for the detection and identification of hemoglobin variants. Confirmatory testing by electrophoresis can be eliminated in the majority of cases by use of retention time, proportion of total hemoglobin, and peak characteristics of HPLC.

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The laboratory diagnosis of hemoglobinopathies and thalassemias is of growing importance, particularly because of an increasing requirement for antenatal diagnosis of significant disorders of globin chain synthesis. It has been recommended that all individuals of all ethnic groups except Northern European Caucasians be screened for variant hemoglobins, all ethnic groups for β -thalassemia trait, and selected ethnic groups for α -thalassemia trait (1). The identity of a hemoglobin variant is generally inferred from its electrophoretic mobility, its quantity, and the patient's ethnic background. Family studies can be of considerable importance in elucidating the nature of disorders of hemoglobin synthesis, but definite identification can be achieved only by DNA analysis or amino acid sequencing (1–3).

Alkaline and acid hemoglobin electrophoresis are the most widely used methods for investigating hemoglobin

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variants and hemoglobinopathy. Alkaline electrophoresis is rapid, reproducible, and capable of separating common hemoglobin variants, such as hemoglobin A (Hb A), Hb F, Hb S, and Hb C, but Hb S, Hb D, Hb G, and Hb Lepore are unresolved from each other, as are Hb C, Hb A₂, Hb O-Arab, and Hb E. In addition, there are other variants with electrophoretic mobilities identical or similar to those of Hb S and Hb C. Consequently, acid electrophoresis is needed for the identification of the aforementioned variants. Nevertheless, these electrophoretic methods are still not able, in most cases, to separate Hb D from Hb G and Hb Lepore and, in some cases, Hb E from Hb O-Arab (2).

Hemoglobin fraction analysis by cation-exchange HPLC has the advantage of quantifying Hb F and Hb A₂ along with hemoglobin variant screening in a single, highly reproducible system, making it an excellent technology to screen for hemoglobin variants and hemoglobinopathies along with the thalassemias (1, 4–7). The simplicity of the automated system with internal sample preparation, superior resolution, rapid assay time, and accurate quantification of hemoglobin fractions makes this an ideal methodology for the routine clinical laboratory (5, 6). Numerous automated HPLC systems are now commercially available, and evaluations have been published (8–11). The use of HPLC technology in the clinical laboratory setting has increased ~12.5-fold in the past 10 years (12).

Much of the published literature on the use of HPLC for the investigation of hemoglobinopathies and thalassemias has evaluated its effectiveness in newborn-screening programs (13–16). Other publications have evaluated its performance, in comparison with various other technologies (6, 17, 18), in the analysis of complicated α -thalassemia and β -thalassemia syndromes in Southeast Asia (19) and in the analysis of a small patient population and a reference collection of rare hemoglobin variants (9). We report here the results for HPLC performance in a large prospective study of 60 293 samples over a 32-month period in a multiethnic population.

Material and Methods

Specimens were drawn into tubes containing dipotassium EDTA (Becton Dickinson Vacutainer Systems). All specimens were analyzed on the Bio-Rad Variant II HPLC system with use of the Variant II β -Thalassemia Short Program Reorder Pack (Bio-Rad Laboratories) as described in the instruction manual for the assay. Briefly, in this system the samples are mixed by the Variant II sampling station, diluted with the specific hemolyzing/wash buffer, and injected into an assay-specific analytic cartridge. The Variant II dual pumps deliver a programmed buffer gradient of increasing ionic strength to the cartridge, where the hemoglobin fractions are separated based on their ionic interaction with the cartridge material. The separated hemoglobin fractions pass through a flow cell, where absorbance is measured at 415

nm; background noise is reduced with the use of a secondary wavelength at 690 nm. The raw data are integrated by the Clinical Data Management software (Bio-Rad Laboratories), and a chromatogram/sample report is generated. The integrated peaks are assigned to manufacturer-defined windows derived from the retention time, i.e., the time in minutes from sample injection to the maximum point of the elution peak, of normal hemoglobin fractions and common variants (Table 1). If a peak elutes at a retention time not predefined, it is labeled as an unknown.

Over a 32-month period, 60 293 samples were analyzed in the Special Hematology Laboratory at Bellevue Hospital Center for quantification of hemoglobin fractions and screening for hemoglobin variants. For specimens that showed chromatogram patterns consistent with sickle trait, the presence of Hb S was confirmed by use of the sodium metabisulfite reduction test (20). All non-A non-S variants were confirmed by alkaline and acid electrophoresis on the Helena Hemoglobin Electrophoresis System or the Helena SPIFE 3000 (Helena Laboratories) according to the manufacturer's recommendations. The presence of Hb H was confirmed by use of the brilliant cresyl blue test for inclusion bodies (21). Certain specimens were forwarded to the Mayo Medical Laboratories (Rochester, MN) for confirmation, where additional testing was performed by isoelectric focusing, globin chain electrophoresis, and unstable hemoglobin screen. Amino acid sequencing was performed on selected specimens.

For hemoglobin elution peaks with retention times <0.63 min, the proportion of total hemoglobin (%Hb) was determined by densitometry of the alkaline electrophoresis gel using the QuickScan 2000 (Helena Laboratories) and/or manual calculation. Manual calculation was accomplished by determining the peak area of each elution peak, i.e., the product of the height and the width at half-height as measured with a Vernier caliper. The %Hb was calculated by determining the area of a peak as a fraction of the total area of all hemoglobin peaks seen on the HPLC chromatogram.

All data analyses were performed with Minitab Statistical Software (Minitab Inc.). Student's *t*-test was used to

Table 1. Manufacturer-assigned windows for Bio-Rad Variant II HPLC system.

Peak name	Retention time, min
P1 window	0.63–0.85
F window	0.98–1.20
P2 window	1.24–1.40
P3 window	1.40–1.90
A ₀ window	1.90–3.10
A ₂ window	3.30–3.90
D window	3.90–4.30
S window	4.30–4.70
C window	4.90–5.30

calculate statistical significance where n was ≥ 3 for each group being analyzed.

Results

RETENTION TIMES AND PROPORTIONS OF HEMOGLOBIN VARIANTS

Shown in Table 2 are the number of observations, retention times, %Hb, interval period in months between the first and last observation for each hemoglobin variant, and the type of variant with the specific amino acid substitution for the 34 observed variants, the 2 tetramers, Hb F, Hb A₀, and Hb A₂. The mean retention times and %Hb for Hb F, Hb A₀, and Hb A₂ were determined by use of the first five normal specimens seen in each month of the study period.

EVALUATION OF THE RETENTION TIMES

The CVs of the retention times for Hb F, Hb A₀, Hb A₂, and each of the 15 variants seen on three or more occasions were calculated. Hb New York was not included in these calculations because it coelutes with Hb A₀. It was postulated that normalization of the retention time of the hemoglobin variant to that of either Hb A₀ or Hb A₂ at the time of assay might minimize the imprecision of the assay attributable to changes in lots of reagents and columns. The CVs of the retention time normalized to the retention time for Hb A₀ or the retention time for Hb A₂ at each occurrence for each of the 15 variants were calculated. For each incident of the six variants with an retention time in the Hb A₂ window, the mean retention time for Hb A₂ in the preceding five samples and the following five samples was determined and used in the calculation. The mean (SD) of the CVs for the retention time, the retention time normalized to the retention time for Hb A₀, and the retention time normalized to the retention time for Hb A₂ were calculated. There was no statistical difference in imprecision between the mean (SD) CV for the retention time [1.0 (0.7%)] and the mean CV for the retention time normalized to the retention time for Hb A₀ [1.3 (0.5%); $P = 0.10$] or normalized to the retention time for Hb A₂ [1.0 (0.4%); $P = 0.51$]. For this reason, subsequent analysis of the data was done with only the retention time.

The SD for the retention times of Hb F, Hb A₀, Hb A₂, and the 15 variants seen on three or more occasions did not correlate with either the retention time ($P = 0.889$) or the %Hb ($P = 0.288$), demonstrating that the SD is independent of these two variables. The SD, the measure of the variation around the mean for the retention time, was therefore used to predict the statistical difference of the retention time of a variant seen fewer than three times from that of another variant. The mean (SD) for the individual SDs of the retention times observed in these different hemoglobins was 0.026 (0.016) min. A difference (d) in the retention time of two hemoglobins greater than the mean of the individual SDs + 2 SD [0.026 + (2 × 0.016) = 0.058 min] was considered significant.

HEMOGLOBIN VARIANTS WITH RETENTION TIMES

<0.63 MIN

The Clinical Data Management software does not integrate elution peaks that occur at <0.63 min. The tetramers Hb Barts ($\gamma 4$) and Hb H ($\beta 4$), and Hb F₁, the acetylated form of Hb F, all elute before chromatogram integration; they therefore are not indicated on the chromatogram report. The elution peaks are detected only by visual analysis of the chromatogram. Hb Barts was seen in newborns at risk for at least two gene deletions of the α -globin. Of the 12 cases of Hb H disease seen, 3 did not show a discernible fast-moving band on electrophoresis. Hb F₁ was seen mostly in newborns and was ~10–15% of the total Hb F present.

HEMOGLOBIN VARIANTS WITH RETENTION TIMES IN THE P1 WINDOW (0.63–0.85 MIN)

No hemoglobin variants were detected in this window.

HEMOGLOBIN VARIANTS WITH RETENTION TIMES IN THE F WINDOW (0.98–1.20 MIN)

A survey of the Globin Gene Server (22) revealed that at least seven hemoglobin variants (four β - and three α -variants) are expected to elute in this window, all in quantities >10%. To eliminate the possibility of incorrectly designating an elution peak as Hb F, 16 months into the study the laboratory began performing electrophoresis on all samples with a Hb F fraction >7%, which was not age appropriate. Of the 288 specimens analyzed by electrophoresis, all were confirmed to be Hb F.

HEMOGLOBIN VARIANTS WITH RETENTION TIMES IN THE P2 WINDOW (1.24–1.40 MIN)

Hb A_{1C} eluted in the P2 window. When the elution peak was >7% of the total hemoglobin, the patient records were checked for indication of diabetes and Hb A_{1C} quantification. If no quantification was available, the Hb A_{1C} was quantified in the hospital's chemistry laboratory by phenol-borate affinity HPLC (Primus Corporation). If the %Hb in the P2 window and the Hb A_{1C} values were concordant, i.e., within 15% of each other, no further studies were performed. The only hemoglobin variant found to elute in this window was Hb Hope, which had a mean (SD) %Hb [45.9 (2.2)%] much greater than would be expected for Hb A_{1C}.

HEMOGLOBIN VARIANTS WITH RETENTION TIMES IN THE P3 WINDOW (1.40–1.90 MIN)

Nine hemoglobin variants (four α - and five β -variants) had elution peaks in the P3 window. It is predicted that Hb Camden ($d = 0.10$ min from Hb Hope) and Hb J-Oxford ($d = 0.11$ min from Hb Camden) can be differentiated and identified based solely on their retention times. Hb Austin, Hb N-Baltimore, and Hb Fukuyama could not be differentiated from each other by their respective retention times; hemoglobin electrophoresis was required (Fig. 1, lanes 3, 6, and 4, respectively). The %Hb was

Table 2. Hemoglobins seen.

Variant name	n ^a	Retention time, ^b min	%Hb ^c	ΔTime, ^d months	Variant ^e
Hb Barts	ND ^f	0.2	ND	32	γ4
Hb H	12	0.2	12.5 (4.0)	32	β4
Hb F ₁	ND	0.5	ND	32	—
Hb F	160 ^g	1.10 (0.017)	1.0 (0.5)	32	—
Hb Hope	11	1.39 (0.007)	45.9 (2.2)	32	β 136Gly→Asp
Hb Camden	2	1.50; 1.48	52.4; 49.3	5	β 131Gln→Glu
Hb J-Oxford	1	1.60	24.7	—	α 15Gly→Asp
Hb Austin	3	1.68 (0.017)	47.1 (0.4)	12	β 40Arg→Ser
Hb N-Baltimore	6	1.70 (0.031)	47.8 (0.9)	21	β 95Lys→Glu
Hb Fukuyama	2	1.72; 1.73	— ^h	0	β 77His→Tyr
Hb Fannin-Lubbock	7	1.75 (0.024)	35.0 (3.0)	21	β 119Gly→Asp
Hb J-Anatolia	2	1.75; 1.75	19.9; 21.2	0.5	α 61Lys→Thr
Hb J-Mexico	2	1.74; 1.78	22.7; 22.3	10	α 54Gln→Glu
Hb J-Meerut	2	1.88; 1.88	25.4; 25.2	11	α 120Ala→Glu
Hb J-Toronto	1	1.94	— ⁱ	—	α 5Ala→Asp
Hb J-Bangkok	1	2.02	43.6	—	β 56Gly→Asp
Hb Ty Gard	1	2.20	34.1	—	β 124Pro→Gln
Hb Köln ^j	2	2.26; 2.26 (4.93; 4.87) ^j	26.8; 23.5 (7.0; 7.3) ^j	24	β 98Val→Met
Hb A ₀	160 ^g	2.43 (0.041)	86.3 (1.5)	32	—
Hb New York	4 ^k	2.43 (0.010)	Does not separate	12	β 113Val→Glu
Hb Twin Peaks	3	Appears as hump	Does not separate	11	α 113Leu→His
Hb Lepore	3	3.37 (0.019)	12.1 (1.5)	24	δβ-hybrid
Hb D-Iran	1	3.49	47.7	—	β 22Glu→Gln
Hb A ₂	160 ^g	3.63 (0.035)	2.7 (0.4)	32	—
Hb E	83 ^l	3.69 (0.069)	30.3 (4.0) ^m	32	β 26Glu→Lys
Hb Osu-Christiansborg	1	3.77	44.0	—	β 52Asp→Asn
Hb G-Honolulu	1	3.86	27.4	—	α 30Glu→Gln
Hb Korle-Bu	8	3.92 (0.050)	46.5 (3.7)	16	β 73Asp→Asn
Hb D-Punjab	7	4.18 (0.007)	33.1 (1.8)	24	β 121Glu→Gln
Hb G-Philadelphia	8	4.22 (0.037)	26.4 (6.6)	29	α 68Asn→Lys
Hb E-Saskatoon	2	4.34; 4.32	39.3; 40.4	2	β 22Glu→Lys
Hb S	3587 ⁿ	4.51 (0.030)	34.9 (4.1) ^m	32	β 6Glu→Val
Hb Manitoba	1	4.58	16.5	—	α 102Ser→Arg
Hb Montgomery	7	4.58 (0.020)	15.7 (2.2)	32	α 48Leu→Arg
Hb A ₂ '	81 ^o	4.59 (0.030)	1.2 (0.1)	2	δ 16Gly→Arg
Hb Q-Thailand	1	4.67	29.3	—	α 74Asp→His
Hb Hasharon	7	4.83 (0.016)	17.9 (1.5)	32	α 47Asp→His
Hb O-Arab	6	4.91 (0.008)	35.9 (3.0)	24	β 121Glu→Lys
Hb G-Siriraj	1	5.08	24.2	—	β 7Glu→Lys
Hb C	962 ^p	5.18 (0.013)	35.6 (4.0) ^m	32	β 6Glu→Lys

^a Number of observations.

^b Mean (SD) relative retention time except when n was <3, for which the individual retention times are given.

^c Mean (SD) percentage of the hemoglobin variant as a fraction of the total hemoglobin.

^d Time period between the first and last observations.

^e Globin chain on which the mutation exists along with the amino acid substitution.

^f ND, not determined.

^g The mean retention times and %Hb values for Hb F, Hb A₀, and Hb A₂ were calculated based on the first five normal specimens assayed in each month of the study period.

^h Variant seen in conjunction with Hb S (mother) and with β-thalassemia trait (child). The expected %Hb in a heterozygote is ~45% (22).

ⁱ Variant seen in conjunction with Hb S. The expected %Hb in a heterozygote is ~30% (22).

^j Peaks for intact (denatured) Hb Köln.

^k Observations in a 1-year prospective study of 632 Asian patients.

^l Hb AE, Hb EE, Hb SE, Hb E/β-thal, and Hb ED-Punjab included.

^m %Hb determined in heterozygous state only.

ⁿ Hb AS, Hb SS, Hb SC, Hb S/β-thal, Hb SE, Hb SG-Philadelphia, Hb SO-Arab, Hb SJ-Toronto, and Hb SFukuyama included.

^o Observations in a 6-month prospective survey.

^p Hb AC, Hb SC, Hb CC, Hb CHope, and Hb C/β-thal included.

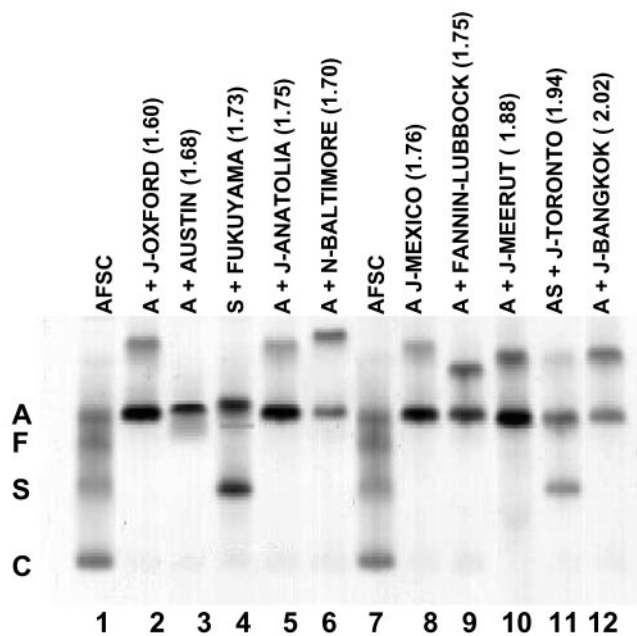


Fig. 1. Alkaline hemoglobin electrophoresis performed on Helena SPIFE 3000.

The reference (AFSC) is seen in lanes 1 and 7. The other lanes are labeled with the hemoglobin patterns, with the mean retention times of the hemoglobin variants (in minutes) in parentheses. All electrophoresis patterns show the variant in the heterozygous state with Hb A except for Hb Fukuyama and Hb J-Toronto. Hb Fukuyama is shown in the heterozygous state with Hb S. Hb J-Toronto, an α -variant, was found in a patient with Hb AS. The fast, anodal band is Hb J-Toronto, the band in the A position is a combination of Hb A and the Hb SJ-Toronto hybrid, and the band in the S position is Hb S. Hb Austin (lane 2) migrates cathodal to Hb A and appeared as an unresolved band in all three cases seen in this laboratory.

sufficient, however, to distinguish these variants from Hb Fannin-Lubbock, Hb J-Anatolia, and Hb J-Mexico. To distinguish these latter three variants, however, retention times and electrophoresis were required (Fig. 1, lanes 9, 5, and 8, respectively). It is predicted that Hb J-Meerut can be differentiated and identified based solely on its retention time ($d = 0.12$ min from Hb J-Mexico).

HEMOGLOBIN VARIANTS WITH RETENTION TIMES IN THE A₀ WINDOW (1.90–3.10 MIN)

Six hemoglobin variants (two α - and four β -variants) had elution peaks in the A₀ window. Hb J-Toronto ($d = 0.06$ min from Hb J-Meerut), Hb J-Bangkok ($d = 0.08$ min from Hb J-Toronto), and Hb Ty Gard ($d = 0.18$ min from Hb J-Bangkok) could all be differentiated and identified solely by their retention times. Hb Köln ($d = 0.06$ min from Hb Ty Gard), an unstable hemoglobin, could be identified by its retention time along with its characteristic chromatogram. In addition to the elution peak for the intact hemoglobin at retention time 2.26 min, there was a secondary peak at retention time ~ 4.90 min representing the denatured Hb Köln (Fig. 2B). Hb Ty Gard (data not shown) and Hb Twin Peaks (Fig. 3, lane 4) did not separate from Hb A on hemoglobin electrophoresis and were detected only by HPLC. Hb Ty Gard appeared to

have a unique retention time, whereas Hb Twin Peaks had a characteristic chromatogram in which there was a hump on the downward slope of the Hb A₀ elution peak (Fig. 2C). Hb New York appeared to have a retention time identical to that of Hb A₀. Alkaline electrophoresis was required to detect this β -variant; it moved more anodal than Hb A under these conditions (Fig. 3, lane 6).

HEMOGLOBIN VARIANTS WITH RETENTION TIMES IN THE A₂ WINDOW (3.30–3.90 MIN)

Five hemoglobin variants (one $\delta\beta$ -hybrid, one α -, and three β -variants) had elution peaks in the A₂ window. Hb Lepore could be differentiated and identified based solely on its retention time. The %Hb and the characteristic hump on the downward slope of the elution peak (Fig. 2D) were additional distinguishing features of Hb Lepore. The retention time for Hb D-Iran appeared to be significantly different from those of both Hb Lepore ($d = 0.12$ min) and Hb A₂ ($d = 0.14$ min). In addition, the %Hb of Hb D-Iran (47.7%) was significantly greater than either of these variants [Hb Lepore, 12.1 (1.5)%; Hb A₂, 3.63 (0.04)%]. The retention times and %Hb for Hb A₂ and Hb E were significantly different ($P = 0.001$ for both). The retention time for Hb Osu-Christiansborg appeared to be significantly different from that for Hb E ($d = 0.08$ min) in addition to the %Hb [44.0% vs 30.3 (4.0)%, respectively]. Hb G-Honolulu appeared to have a retention time ($d = 0.09$ min) and %Hb significantly different from those of Hb Osu-Christiansborg (27.4% vs 44.0%, respectively). Although Hb G-Honolulu and Hb Korle-Bu appeared to have significantly different retention times ($d = 0.06$ min), the lower %Hb [27.4% vs 46.5 (3.7)%, respectively] and characteristic chromatogram of Hb G-Honolulu allowed further differentiation of the two variants. Hb G-Honolulu, an α -variant, showed the presence of the characteristic minor Hb A₂ variant peak ($\alpha_2^{G-Honolulu}\delta_2$) immediately after the variant peak, which was missing in Hb Osu-Christiansborg, a β -variant.

HEMOGLOBIN VARIANTS WITH RETENTION TIMES IN THE D WINDOW (3.90–4.30 MIN)

Three hemoglobin variants had elution peaks in the D window, all of which were β -variants. The retention times, along with the %Hb, were statistically different for Hb Korle-Bu vs Hb D-Punjab ($P < 0.001$, respectively) and Hb Korle-Bu vs Hb G-Philadelphia ($P < 0.001$, respectively). Although the retention times for Hb D-Punjab and Hb G-Philadelphia were statistically different ($P = 0.015$), there was no statistical difference in %Hb ($P = 0.21$). The mean Hb A₂ values for Hb D-Punjab trait [1.4 (0.4)%] and Hb G-Philadelphia trait [1.3 (0.4)%] were significantly lower ($P < 0.001$, respectively) than the range for Hb A₂ in the normal specimens. The chromatogram for Hb G-Philadelphia (Fig. 2F), an α -variant, showed the presence of the characteristic minor Hb A₂ variant peak ($\alpha_2^{G-Philadelphia}\delta_2$) in all heterozygous cases. Because the range of retention times for Hb Korle-Bu straddled both

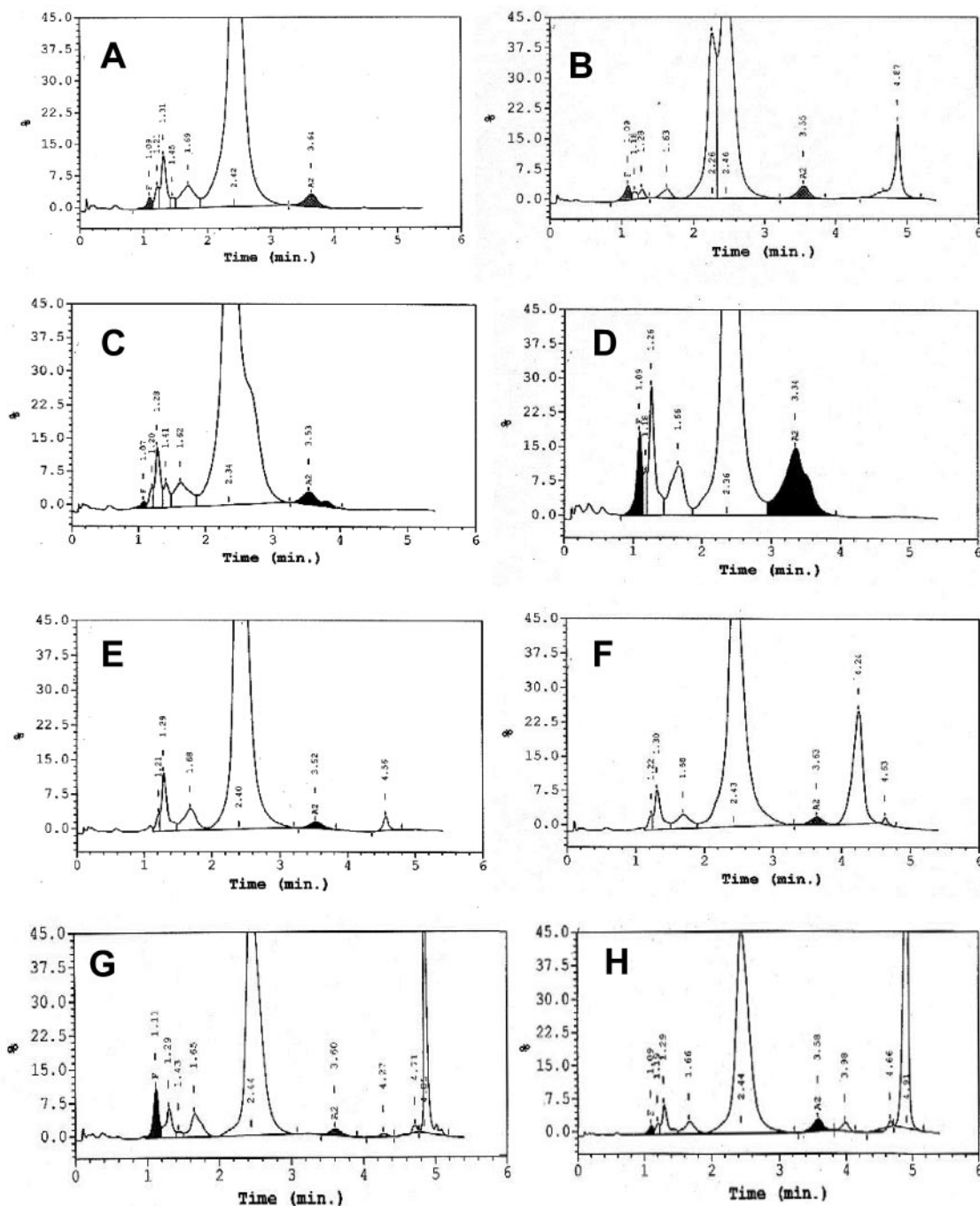


Fig. 2. Elution chromatograms of patient specimens on Bio-Rad Variant II HPLC System.

Time (min.) represents the retention time in minutes for each fraction to elute; % represents the percentage of hemoglobin in the elution peak. The retention time for each fraction is shown with the peak. (A), a normal patient. (B), Hb Köln elutes at a retention time of 2.26 min and the characteristic denatured Hb Köln at retention time 4.87 min. (C), Hb Twin Peak elution demonstrating the characteristic hump on the downward slope of the Hb A₀ elution peak at 2.34 min. (D), Hb Lepore elution demonstrating the characteristic increased proportion of Hb A₂ (11.6%) and hump on the downward slope of the Hb A₂ peak at 3.34 min. (E), Hb A₂' (4.56 min) elution chromatogram. (F), Hb G-Philadelphia elution demonstrating the characteristic lower proportion of Hb A₂ (0.8%) at 3.63 min, the Hb G-Philadelphia elution peak at 4.24 min, and the presence of the characteristic minor peak ($\alpha_2^{\text{G-Philadelphia}}\delta_2$) at 4.63 min. (G), Hb Hasharon elution demonstrating the characteristic decreased proportion of Hb A₂ (1.5%) at 3.60 min, a characteristic minor peak at 4.27 min, and the characteristic minor peaks preceding and succeeding ($\alpha_2^{\text{Hasharon}}\delta_2$) the Hb Hasharon elution peak at 4.85 min. (H), Hb O-Arab elution demonstrating the characteristic minor peak at 3.98 min, which is absent in Hb C samples.

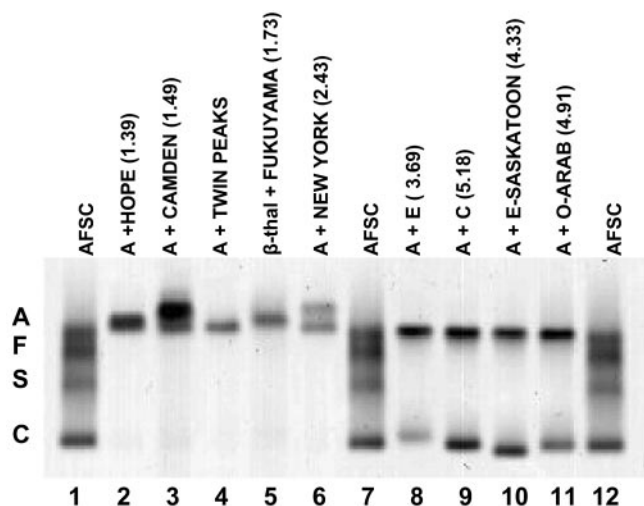


Fig. 3. Alkaline hemoglobin electrophoresis performed on Helena SPIFE 3000.

The reference (AFSC) is in lanes 1, 7, and 12. The other lanes are labeled with the hemoglobin patterns, with the mean retention times of the hemoglobin variants (in minutes) in parentheses. All electrophoresis patterns show the variant in the heterozygous state with Hb A except for Hb Fukuyama, which is shown in combination with β^0 -thalassemia trait, explaining the absence of the Hb A band.

the A_2 and D windows, one-half of the specimens seen in this series fell in the A_2 window although the mean retention time was in the D window.

HEMOGLOBIN VARIANTS WITH RETENTION TIMES IN THE S WINDOW (4.30–4.70 MIN)

Six hemoglobin variants (three α -, two β -, and one δ -variant) had elution peaks in the S window. Hb E-Saskatoon and Hb S appeared to have significantly different retention times ($d = 0.18$ min). Although Hb Manitoba, Hb Montgomery, and Hb A_2' all appeared to have identical retention times, their retention times and %Hb were statistically different from those of Hb S ($P < 0.001$ for all). The %Hb values for Hb Manitoba and Hb Montgomery did not appear to be statistically different [16.5% vs 15.7 (2.2)%, respectively]; however, the %Hb for Hb A_2' was statistically different from these two variants [1.2 (0.1)%; $P < 0.001$]. The retention time for Hb Q-Thailand appeared to be different from the retention times of Hb Manitoba, Hb Montgomery, and Hb A_2' ($d = 0.09$, 0.09, and 0.08 min, respectively). At least in this single case, the %Hb for Hb Q-Thailand appeared to be greater than those for the other three variants (Table 2). Hb Manitoba and Hb Montgomery showed different mobilities on electrophoresis, which was necessary for identification (data not shown).

The incidence for Hb A_2' (0.76%) was determined in a 6-month prospective study in samples submitted to this laboratory for hemoglobin analysis. In patients heterozygous for Hb A_2' , the %Hb for Hb A_2 [1.64 (0.17)%] was significantly lower ($P < 0.001$) than the value for the normal specimens.

HEMOGLOBIN VARIANTS WITH RETENTION TIMES IN THE UNKNOWN WINDOW (4.70–4.90 MIN)

The elution peak for Hb Hasharon, an α -variant, fell in the time interval for unknowns. Although its retention time appeared to be significantly different from that of Hb Q-Thailand ($d = 0.16$ min), the retention time and %Hb were statistically different ($P < 0.001$ for both) from those for Hb O-Arab. This variant also had a characteristic chromatogram. In addition to the expected Hb A_2 variant peak ($\alpha_2^{\text{Hasharon}}\delta_2$), immediately after the variant peak there were two small peaks in all examples of Hb Hasharon seen in this laboratory (Fig. 2G). One minor peak appeared at a retention time of ~ 4.27 min, presumably glycosylated or degraded Hb, and another appeared immediately preceding the elution peak.

HEMOGLOBIN VARIANTS WITH RETENTION TIMES IN THE C WINDOW (4.90–5.30 MIN)

Three hemoglobin variants (three β -variants) had elution peaks in the C window. As reported previously (23), Hb O-Arab and Hb C had statistically different retention times ($P < 0.001$), whereas the %Hb values were not statistically different ($P = 0.84$). In addition, all examples of Hb O-Arab seen in this laboratory had a minor peak in the D window (Fig. 2H), which was not seen in Hb C trait. The retention time for Hb G-Siriraj appeared to be statistically different from that of Hb O-Arab ($d = 0.17$ min) and that of Hb C ($d = 0.10$ min). Hb G-Siriraj, a β -variant, is reported to be 33–40% of the total hemoglobin in heterozygotes (22). This patient appeared to have a concomitant α -thalassemia, which would account for the lower %Hb for the variant.

Hb J FAMILY

Six members of the Hb J family (five α - and one β -variants) were identified in this series of hemoglobin variants. Three α -variants (Hb J-Oxford, Hb J-Anatolia, and Hb J-Mexico) had identical electrophoretic mobilities (Fig. 1, lanes 2, 5, and 8, respectively). Although the retention times for Hb J-Anatolia and Hb J-Mexico did not appear to be significantly different ($d = 0.01$ min), the retention time for Hb J-Oxford appeared to be significantly different ($d = 0.15$ min and 0.16 min, respectively). Two other α -variants (Hb J-Meerut and Hb J-Toronto) with identical electrophoretic mobilities (Fig. 1, lanes 10 and 11, respectively) had significantly different retention times ($d = 0.06$ min). The β -variant (J-Bangkok), with yet a different electrophoretic mobility (Fig. 1, lane 12), appeared to have a unique retention time and a significantly higher %Hb [43.6% vs 22.6 (2.4)% for the α -variants].

Hb D FAMILY

Three members of the Hb D family were identified in this series, all of which were β -variants. Whereas Hb D-Punjab and Hb D-Iran had identical electrophoretic mobility (Fig. 4, lanes 6 and 7, respectively), the retention

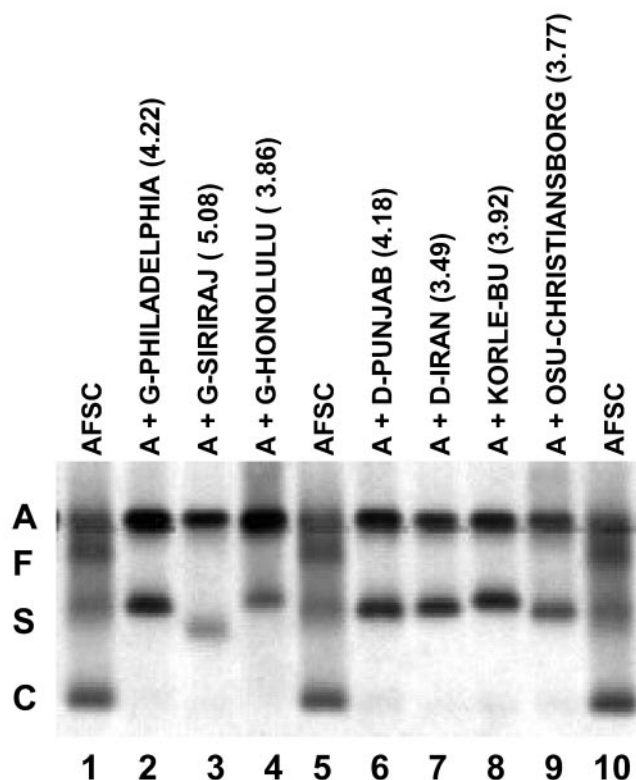


Fig. 4. Alkaline hemoglobin electrophoresis performed on Helena SPIFE 3000.

The reference (AFSC) is in lanes 1, 5, and 10. The other lanes are labeled with the hemoglobin patterns, with the mean retention times of the hemoglobin variants (in minutes) in parentheses. All electrophoresis patterns show the variant in the heterozygous state with Hb A.

times were quite different ($d = 0.69$ min). Hb Osu-Christiansborg, which had alkaline electrophoretic mobility identical on the Helena Hemoglobin Electrophoresis System (data not shown) and similar on SPIFE (Fig. 4, lane 9), and an acid electrophoresis mobility identical to these two hemoglobins, had a retention time apparently significantly different from both of them ($d = 0.41$ and 0.28 min, respectively).

Hb G FAMILY

Three members of the Hb G family were identified in this series (two α - and one β -variant). Hb G-Honolulu and Hb G-Philadelphia had mobilities that were similar, but not identical, on electrophoresis (Fig. 4, lanes 4 and 2, respectively). Their retention times, however, appeared to be significantly different ($d = 0.36$ min); they eluted in different windows. Hb G-Siriraj had a significantly different retention time from the other two members ($d = 1.22$ and 0.86 min, respectively) and exhibited a unique mobility pattern on electrophoresis. It was slightly cathodal to Hb S on alkaline electrophoresis (Fig. 4, lane 3) and slightly cathodal to Hb C on acid electrophoresis (data not shown).

Hb C FAMILY

Two β -variants (Hb E and Hb E-Saskatoon) were identified, each of which had alkaline mobilities on the Helena Hemoglobin Electrophoresis System identical to that of Hb C (data not shown) and SPIFE mobilities (Fig. 3, lanes 8 and 10, respectively) similar to that of Hb C. On acid electrophoresis, the two hemoglobin variants, however, had mobilities identical to Hb A but not Hb C. The two variants had significantly different retention times ($d = 0.65$ min), and the relative amount of Hb E-Saskatoon appeared to be greater than that of Hb E [39.9% vs 30.3 (4.0)%, respectively]. An additional β -variant (Hb O-Arab) with alkaline electrophoretic mobility identical to that of Hb C (Fig. 3, lane 11) had an acid electrophoretic mobility slightly cathodal to Hb A (data not shown).

HEMOGLOBIN VARIANTS WITH ALKALINE ELECTROPHORETIC MOBILITY SLIGHTLY ANODAL FROM Hb A₀

Four β -variants in this series (Hb Hope, Hb Camden, Hb Fukuyama, and Hb New York) all had similar alkaline electrophoretic mobilities (Fig. 3, lanes 2, 3, 5, and 6, respectively), but their retention times were significantly different (1.39, 1.49, 1.73, and 2.43 min, respectively).

Discussion

The laboratory diagnosis of hemoglobinopathies and thalassemias, both of which are common, may be required (a) to confirm a provisional diagnosis, such as significant sickling disorders or β -thalassemia major; (b) to explain a hematologic abnormality such as anemia, microcytosis, or polycythemia; (c) to identify an abnormality in the pre-symptomatic phase, as in neonatal screening; (d) to predict serious disorders of globin-chain synthesis in the fetus and offer the option of termination of pregnancy; (e) to permit genetic counseling of prospective parents; and (f) to allow preoperative screening for the presence of sickle cell hemoglobin (1).

The most common investigative tools in the clinical laboratory are alkaline and acid electrophoresis for hemoglobin variants and hemoglobinopathies, Hb A₂ quantification by ion-exchange column chromatography, and Hb F quantification by alkali denaturation or radial immunodiffusion for thalassemia. Whereas the more common sickling disorders (Hb SS, Hb SC, Hb SD-Punjab, Hb SE, Hb SG-Philadelphia, Hb SHope, Hb SLepore, Hb SO-Arab, and Hb S/ β -thal) are all clinically significant, these combinations do present different manifestations and degrees of severity (24, 25), making precise identification important. None of these can be conclusively identified by a single electrophoretic technique (2). The identification of hemoglobin variants is often presumptive, based on characteristic electrophoretic mobility, quantity, and/or ethnic origin. Definite identification usually requires DNA analysis or amino acid sequencing.

HPLC has been shown to be a sensitive, specific, and reproducible alternative to electrophoresis. Its use has

been dramatically expanded, especially with the development of rapid, well-resolving, and fully automated analyzers. In the past decade HPLC, with its automation and its quantitative power, has appeared to be an appropriate candidate for direct identification and sensitive quantification of major and minor, normal and abnormal, hemoglobin fractions (6, 9, 13–19).

To date, evaluations of the performance and use of HPLC technologies in the diagnostic laboratory have been in relation to newborn screening (13–16), screening specific ethnic populations (19), evaluation of patients studied because of the presence of an abnormal hemoglobin component, and evaluation of stored library samples (9). There has been no large prospective study, however, evaluating its use in the clinical diagnostic laboratory setting.

Over a 32-month period, 60 293 samples were analyzed in the Special Hematology Laboratory at Bellevue Hospital Center for quantification of hemoglobin fractions and screening for hemoglobin variants. Twenty β -variants, 12 α -variants, 2 tetramers, 1 δ -variant, and 1 $\delta\beta$ -fusion globin were observed. The mean (SD) interval period between the first and last observations was 23.3 (6.6) months for the 15 variants seen on three or more occasions. Hb S (5.95%), Hb C (1.60%), Hb A₂' (0.76%), and Hb E (0.14%) were the most common variants encountered. Thirty unique additional variants were encountered with various incidences in 104 samples (Table 2).

Different reports have addressed the precision of the retention times obtained with stored normal samples (17); specimens containing Hb S, Hb C, and Hb E (9); and liquid controls (14). Only two reports have tabulated the retention times for various hemoglobin variants (6, 9). None of these reports, however, addressed the feasibility of using the retention time as a diagnostic tool. The mean (SD) imprecision (CV) of the retention time for the variants seen on three or more occasions over the 32-month observation period was 1.0 (0.7)%, confirming and extending the data of Eastman et al. (14). Because it has been suggested that retention times of hemoglobin peaks differ slightly, but significantly, with different columns or reagent lot numbers (9), the retention time for each variant was normalized to the retention time of Hb A₀ or to the retention time of Hb A₂ in the hope of eliminating the effects of these variables. There was no statistical difference ($P = 0.10$ and 0.51 , respectively), and all subsequent data analyses were performed with use of the retention time. During our observation period, three different lot numbers of columns and 10 different lot numbers of reagents were used. Analysis of the retention times and %Hb for Hb A₀, Hb A₂, and Hb S showed no statistical difference (data not shown) with regard to column and/or reagent changes. This was further confirmed by the CVs of the retention times for Hb F, Hb A₀, and Hb A₂ (2.5%, 1.7%, and 0.96%, respectively) determined from the retention time of the first five normal patient values for each month of observation. In addition, the CVs for those

variants seen over the 32 months (i.e., Hb Hope, Hb E, Hb S, Hb Montgomery, Hb Hasharon, and Hb C) ranged from 0.24% to 1.9%. Finally, the use of a normalized retention time did not allow the identification of any additional variants not identified by the retention time alone.

To predict whether the 17 hemoglobin variants seen on fewer than three occasions had retention times significantly different from other hemoglobins eluting in the same windows, the SD, a measure of the variation around the mean, of the retention times was used. The SD for the retention times of Hb F, Hb A₀, Hb A₂, and the 15 variants seen on three or more occasions showed no correlation with the retention time or the %Hb, demonstrating that it is independent of these two variables. The mean (SD) for the individual SDs of the retention times was 0.026 (0.016) min. A difference in retention times of two hemoglobins >0.058 min was, therefore, considered significant. This calculation is probably conservative. For example, although the retention times for Hb D-Punjab and Hb G-Philadelphia were significantly different ($P = 0.015$) according to Student's *t*-test, the difference in retention time was 0.04 min. This suggests that accumulation of further data for those hemoglobin variants seen on fewer than three occasions may give additional statistically significant different retention times, e.g., Hb Austin and Hb Fukuyama ($d = 0.045$ min).

The retention time alone ($n = 18$) or in conjunction with either the %Hb ($n = 3$) or the peak characteristics ($n = 4$) could identify 25 of the hemoglobin variants seen in this series. The %Hb can generally be the initial predictor whether the detected variant is an α - or β -variant. In this series, the α -variants all had mean %Hb values $<30\%$, whereas the β -variants all had mean %Hb values $>34\%$ except for Hb E [30.3 (4.0)%] and Hb G-Siriraj (24.2%).

The Clinical Data Management software does not integrate peaks with elution time shorter than 0.63 min, a weak point for the Bio-Rad Variant II HPLC system. Visual examination of the elution pattern for each specimen is required and is particularly important for laboratories with a patient population at high risk for α -thalassemia, Hb H disease, and Hb Barts hydrops fetalis. When Hb H or Hb Barts is noted, the %Hb must be determined by either densitometry or manual calculation. Twelve cases of Hb H disease with a %Hb range of 5–20% were observed. Interestingly, three of these specimens did not show an easily discernible band on alkaline electrophoresis when performed according to the manufacturer's recommendations. The band was noted only after a more concentrated solution was analyzed by electrophoresis. HPLC was, therefore, more sensitive to the presence of Hb H. Although Hb Barts and Hb H appear to have identical retention times, they can be differentiated based on the age of the patient. Hb Barts, the γ_4 tetramer, will be present when Hb F production is increased, i.e., generally in the newborn period, whereas Hb H, the β_4 tetramer,

will be present when β -chain production is significant, i.e., >1 year of age.

Hb A₂' is an example of a hemoglobin variant that can be identified by the retention time and %Hb. Although two variants, Hb Manitoba and Hb Montgomery, have retention times identical to that of Hb A₂', it is easily identified by its statistically significantly reduced %Hb.

The elution peak characteristic in addition to the retention time can be used to identify certain hemoglobin variants. The retention times for Hb D-Punjab and Hb G-Philadelphia were statistically significantly different. In addition, both of these variants were associated with decreased Hb A₂, confirming a previous report (26) in which a similar range (0.9–2.5%) in the presence of Hb D-Punjab was reported. It was postulated in that report that the decrease in Hb A₂ may be attributable to either coelution with the Hb A₀ or the Hb D-Punjab peaks or the mutation itself influencing the amount of δ -chain. This same rationale probably does not account for the decreased Hb A₂ seen in the presence of Hb G-Philadelphia. All cases of heterozygous Hb G-Philadelphia demonstrated the minor Hb A₂ variant peak ($\alpha_2^{\text{G-Philadelphia}}\delta_2$; Fig. 2F) falling in the S window, with no such minor peak seen in any of the cases of Hb D-Punjab. The true Hb A₂ [2.7 (0.9)%] is the sum of the normal and variant peaks and is not statistically different ($P < 0.001$) from the proportion seen for the normal specimens. Certain other variants present consistent and distinctive characteristics in their elution patterns that can be incorporated into an algorithm for hemoglobin variant identification. Such characteristics are demonstrated in Fig. 2 for Hb Köln, Hb Twin Peaks, Hb Lepore, Hb Hasharon, and Hb O-Arab.

Six hemoglobin variants with retention times of 1.68–1.78 min could be divided into two groups (group I, Hb Austin, Hb N-Baltimore, and Hb Fukuyama; group II, Hb Fannin-Lubbock, Hb J-Anatolia, and Hb J-Mexico). Although the retention time was not sufficient to distinguish the two groups, the groups could be distinguished by the %Hb. Within the groups, electrophoresis was required to distinguish the members of each group (Fig. 1). Additionally, Hb Manitoba and Hb Montgomery, both α -globin variants with statistically identical retention times, %Hb values, and peak characteristics, required electrophoresis (data not shown) to be differentiated.

Two hemoglobin variants, Hb Ty Gard (data not shown) and Hb Twin Peaks (Fig. 3, lane 4), did not separate from Hb A on electrophoresis but could be easily detected by HPLC. Hb Ty Gard appeared to have a retention time significantly different from that of Hb J-Bangkok ($d = 0.18$ min). It can be distinguished from Hb Köln by both the retention time ($d = 0.06$ min) and the characteristic secondary peak seen with this unstable variant (Fig. 2B). Hb Twin Peaks did not fully separate from Hb A₀ on HPLC, presenting a characteristic hump on the downward slope of the elution peak. Although Hb Twin Peaks is hematologically and clinically insignif-

icant, Hb Ty Gard is a high-oxygen-affinity hemoglobin that can lead to erythrocytosis, which is of potential clinical importance (22).

Hb New York, a β -globin chain variant, did not separate on HPLC and migrated slightly anodal to Hb A on alkaline electrophoresis (Fig. 3, lane 6). This slightly unstable variant is the second most common variant found among the Chinese, but it has been described infrequently in other ethnic groups. It has been found in the heterozygous state or in combination with Hb E, α -thalassemia, and β -thalassemia (22). Heterozygotes have normal hematologic indices with Hb New York representing 40–45% of the total hemoglobin. β^{NY} -chains have a higher turnover than β^{A} -chains and, in contrast to some β -chain variants, such as β^{S} , β^{C} , or β^{E} , have a higher affinity for α -chains. If there is a co-inheritance of Hb H and Hb New York, preferential synthesis of the unstable Hb New York, the small number of α chains available to synthesize Hb A, and the high excess of β^{A} - and β^{NY} -chains, which precipitate in the erythrocyte precursors and cause ineffective erythropoiesis, lead to aggravation of the anemia. Although carriers of this variant are usually not anemic, they have occasional erythrocytes with inclusion bodies and a reduced α -non- α -globin chain ratio attributable to rapid turnover of the β^{NY} -chain (27, 28). For a 12-month period, all samples from patients with an Asian-appearing name were analyzed by alkaline electrophoresis in addition to HPLC. Of 632 samples analyzed, 4 (0.63%) cases of Hb New York (3 heterozygotes and 1 Hb New York/ β -thalassemia) were detected.

Hb A₂' is a δ -globin variant commonly found in ~1–2% of the African-American population with West African heritage (29). Hb A₂' is easily detectable by HPLC, producing a minor peak [1.2 (0.1)%] in the S window [retention time, 4.59 (0.030) min; Fig. 2E], which is consistent with previously published results (30). Among laboratories participating in a recent proficiency survey by the College of American Pathology, only 32% of the laboratories using HPLC correctly identified the presence of Hb A₂' (31). Hb A₂', whether heterozygous or homozygous, is clinically and hematologically silent. Its sole importance is that it may cause an underestimation of the Hb A₂ concentrations in the work-up for thalassemia. An accurate Hb A₂ value for this purpose represents the sum of the Hb A₂ and Hb A₂' peaks (29, 30). In our own laboratory, we have seen a case of homozygous Hb A₂' (Hb A₂ = 0.0%; Hb A₂' = 2.3%) and β -thalassemia trait in conjunction with Hb A₂' (Hb A₂ = 2.5%; Hb A₂' = 2.2%; total Hb A₂ = 4.7%). This latter case might have been mistakenly diagnosed as α -thalassemia if only the Hb A₂ fraction had been reported.

The Hb J family consists of a large group of α - and β -variants that migrate faster than Hb A on alkaline electrophoresis because of an amino acid substitution, resulting most often in the gain of a negative charge and less often in the loss of a positive charge on the Hb molecule (2). The α - and β -variants can often be predicted

by the relative amounts of the variants present; however, they exhibit similar or identical alkaline electrophoretic mobilities, as shown in Fig. 1. The diagnosis of these members is often only inferred by the electrophoretic mobility, %Hb, and ethnicity of the patient. However, the retention time alone was superior to electrophoresis in the identification of members of the Hb J family encountered in this series of specimens.

Finally, HPLC was able to avoid misidentification of two hemoglobin variants having benign interaction with Hb S. Hb D-Punjab and Hb D-Iran exhibited identical electrophoretic mobilities (Fig. 4, lanes 6 and 7, respectively), but their retention times appeared to be unique and significantly different ($d = 0.069$ min). Similarly, Hb C and Hb E-Saskatoon exhibited identical mobilities on the Helena Hemoglobin Electrophoresis System (data not shown) and very similar mobilities on alkaline SPIFE (Fig. 3, lanes 9 and 10, respectively). Hb E-Saskatoon appeared to have a unique retention time ($d = 0.11$ min compared with Hb G-Philadelphia and $d = 0.18$ min compared with Hb S). These situations are clinically important because Hb SD-Punjab and Hb SC are both significant sickling disorders, whereas Hb SD-Iran and Hb SE-Saskatoon are clinically benign (24, 25). The misdiagnosis of Hb D-Iran and Hb E-Saskatoon may lead to incorrect genetic counseling in addition to undue anxiety for the family.

Electrophoresis of hemoglobin variants with similar mobilities has inherent limitations. The identification of variants is dependent on the technical performance of electrophoresis, which has many variables, e.g., hemoglobin concentration, amperage, running temperature, and length of electrophoresis run. These variables can affect the quality of separation and relative positioning of the bands. Variants that migrate identically or similarly (see Figs. 1, 3, and 4) would be very difficult, if not impossible, to evaluate without the unknown sample being electrophoresed directly adjacent to the reference hemoglobin mixture or adjacent to several known stored specimens. HPLC, on the other hand, has been shown to have a high degree of reproducibility and precision.

In this study the influence of hemoglobin stability testing and patient ethnicity were not included. Hemoglobin stability testing is an uncommon and subjective test for which controls are not readily available. The collection of ethnicity information is very difficult for the clinical laboratory, especially a reference laboratory. It requires cooperation from the requesting physician to collect from the patient information that is frequently subjective.

In conclusion, this is the first report of a large prospective study on the use of HPLC for determining the presence and identities of hemoglobin variants in a clinical laboratory setting. These data demonstrate that HPLC is an excellent, powerful diagnostic tool for the direct identification of hemoglobin variants with a high degree of precision in the quantification of major and minor, normal

and abnormal, hemoglobin fractions. HPLC is suitable for the routine investigation of hemoglobin variants, hemoglobinopathies, and thalassemia. With the integration of proper algorithms (see the Data Supplement that accompanies the online version of this article at <http://www.clinchem.org/content/vol50/issue10/>) involving retention time, %Hb, and peak characteristics, a clinical laboratory is capable of identifying ~75% of the common variants encountered without the need for confirmatory studies such as alkaline and acid electrophoresis. More importantly, identification of the common variants (i.e., Hb C, Hb D-Punjab, Hb E, Hb G-Philadelphia, Hb Hope, Hb Lepore, Hb O-Arab, and Hb S) that in combination with Hb S lead to a clinically significant sickling disorder can be quickly and accurately accomplished by use of such algorithms without the need for confirmatory testing.

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