

# Comparison of Sebia Capillary Electrophoresis With the Primus High-Pressure Liquid Chromatography in the Evaluation of Hemoglobinopathies

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## Abstract

*The Sebia Capillarys (capillary zone electrophoresis [CE]) and the Primus Resolution high-pressure liquid chromatography (HPLC) were used to prospectively evaluate 297 samples for hemoglobinopathies. Hemoglobin (Hb) A levels were similar on both techniques (mean, 96.2% and SD, 5.7% by CE; mean, 96.8% and SD, 5.5% by HPLC), but HbA<sub>2</sub> levels were higher by CE (mean, 2.8%; SD, 0.8%) than by HPLC (mean, 2.3%; SD, 0.8%). HbS had higher values by CE (mean, 40.6%; SD, 18.9%) than by HPLC (mean, 38.4%; SD, 18.9%). In cases with Hg S, HbA<sub>2</sub> levels were greater by HPLC (mean, 4.0%; SD, 1.0%) than by CE (mean, 3.1%; SD, 0.8%). HbA<sub>2</sub> was occasionally not separated sufficiently from HbC for measurement by CE, but did separate from HbE by CE. Both methods identified HbS, HbC, HbE, HbS, and HbC together, HbA<sub>2</sub>', HbD-Los Angeles, HbF variant, HbG-Philadelphia, HbS-G Philadelphia, and Hb Lepore.*

Detection of structural hemoglobin (Hb) variants and thalassemias has become increasingly important in clinical laboratories in the past few years. Early detection of variants such as sickle disease or  $\beta$ -thalassemia major has important therapeutic consequences early in life.<sup>1-3</sup> The appropriate application of transfusion, augmentation of HbF production, and chelation therapy can dramatically improve the course of disease in the patients. The necessity for precise estimates of HbA<sub>2</sub> and HbF to aid in the identification of patients with thalassemias, as well as detection of an ever-expanding number of variants that may have clinical significance, has resulted in the development of more sensitive methods than alkaline and acid gel techniques for use in screening.<sup>4-8</sup> In the past decade, high-pressure liquid chromatography (HPLC) has provided a robust automated method for rapid identification of variants and improved precision of HbA<sub>2</sub> and HbF measurement.<sup>9-12</sup>

Nevertheless, HPLC techniques require special instrumentation and training and result in patterns that are relatively complex. Consequently, many clinical laboratories still use alkaline and acid gel electrophoresis to screen for hemoglobinopathies. A relatively recent entry into the field is automated capillary zone electrophoresis (CE).<sup>7,13-17</sup> The Sebia Capillarys system (Sebia, Norcross, GA) was approved in 2007 by the US Food and Drug Administration (FDA) for the evaluation of hemoglobinopathies. CE is an automated method that has been shown to be an effective technique for detection of monoclonal gammopathies and other common protein abnormalities in serum.<sup>18-21</sup>

In this prospective study, we compared the ability of CE with that of the Primus Resolution HPLC method (Primus, Kansas City, MO) to detect most common variants and evaluated the effect of the most common hemoglobin variants

(HbS, HbC, and HbE) on the measurement of HbA<sub>2</sub> by these systems. Both methods were able to detect the most common variants but differed with regard to measurement of HbA<sub>2</sub>. The CE was able to measure HbA<sub>2</sub> in the presence of HbE, whereas the HPLC, like other screening methods, could not. The CE had difficulty measuring HbA<sub>2</sub> in the presence of HbC, whereas the HPLC method could measure HbA<sub>2</sub> but included some breakdown product, as it does with HbS.

## Materials and Methods

### Specimens

This was a prospective study of 297 consecutive samples of whole blood collected in EDTA that were submitted for routine evaluation of hemoglobinopathies. Each sample was examined by HPLC and CE. Our study was conducted in accordance with a protocol (R-06-703) approved by the institutional review board of St Joseph Mercy Hospital, Ann Arbor, MI.

### High-Pressure Liquid Chromatography

HPLC was performed using the Primus Resolution HPLC method, a cation exchange column. This method relates the retention time of unknown hemoglobins to that of a calibrating standard containing 4 hemoglobins: HbF, HbA, HbS, and HbC. Whole blood specimens collected in EDTA were lysed with the hemolyzing reagent (provided by the manufacturer) for injection into the HPLC column. Because a bar code reader was not present on our instrument, sample placement was used to identify patients. However, all other procedures, including aspiration of the sample, lysis, and final pattern, were performed without a requirement for technical work beyond placing the sample tube in the instrument rack. Elution of adsorbed hemoglobins used a gradient formed by 2 mobile phases of Bis-tris(hydroxymethyl)aminomethane and 1 mmol of potassium cyanide with different pH and ionic strength values as previously described.<sup>9,11</sup> Our laboratory determined normal ranges for adults are as follows: HbA, 97.0% to 99.0%; HbF, less than 2.0%; and HbA<sub>2</sub>, 1.7% to 3.1%.

### Capillary Zone Electrophoresis (CE)

CE was performed using the Sebia Capillarys system. Manufacturer guidelines were followed in performing the analysis. Initial processing of the sample required centrifugation of the whole blood at 5,000 rpm for 5 minutes. Thereafter, the overlying plasma was removed and the erythrocyte pellet was vortexed for 5 seconds. Because the instrument was able to read the bar code, the identification, sampling, electrophoresis, and production of the electrophoretogram pattern were all performed by the instrument without further

requirement for technical intervention. Electrophoresis was performed in alkaline buffer, pH 9.4, provided by the manufacturer (Sebia), with separation primary by the pH of the solution and endosmosis. The hemoglobins were measured at 415-nm wavelength. Electrophoretograms were recorded with the location of specific hemoglobins in specific zones. The presence of HbA was required for the appearance of the zone demarcations that help guide interpretation of structural variants. If HbA was not present, the test of the sample was repeated, being premixed with a 1:1 mixture of a normal control. Quantitative results for such a case were reported from the original sample alone, and the 1:1 mixture was used for qualitative identification only. Manufacturer's recommended normal ranges for healthy adults were as follows: HbA, 96.8% or more; HbF, less than 0.5%; and HbA<sub>2</sub>, 2.2% to 3.2%.

## Results

### CE Reproducibility

The between-run consistency of the CE was evaluated on 17 consecutive runs of the standard preparation containing HbA, HbF, HbS, and HbC. The mean, SD, and coefficient of variation (CV) were as follows: HbA, 28.1% (SD, 0.54%; CV, 1.91%); HbF, 31.0% (SD, 0.43%; CV, 1.39%); HbS, 30.8% (SD, 0.49%; CV, 1.60%); and HbC, 10.2% (SD, 0.28%; CV, 2.76%).

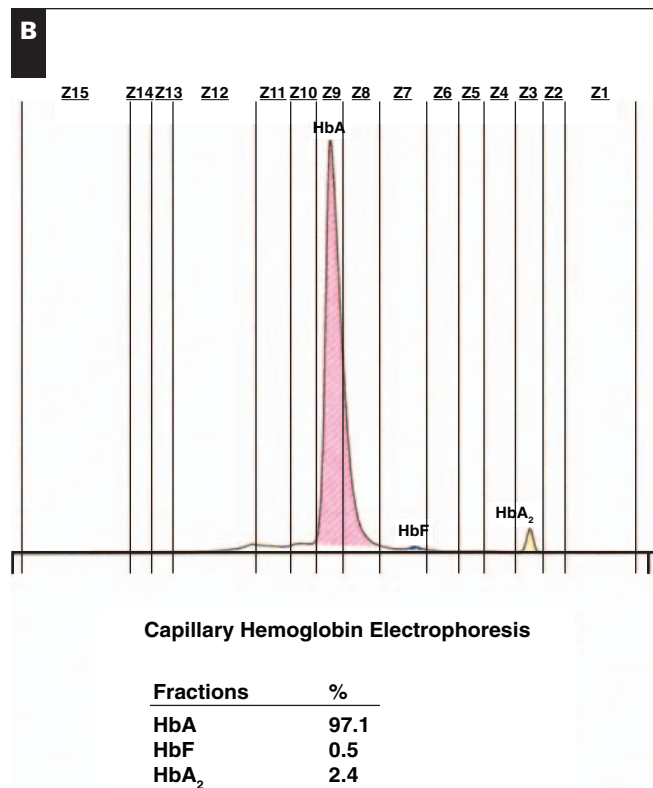
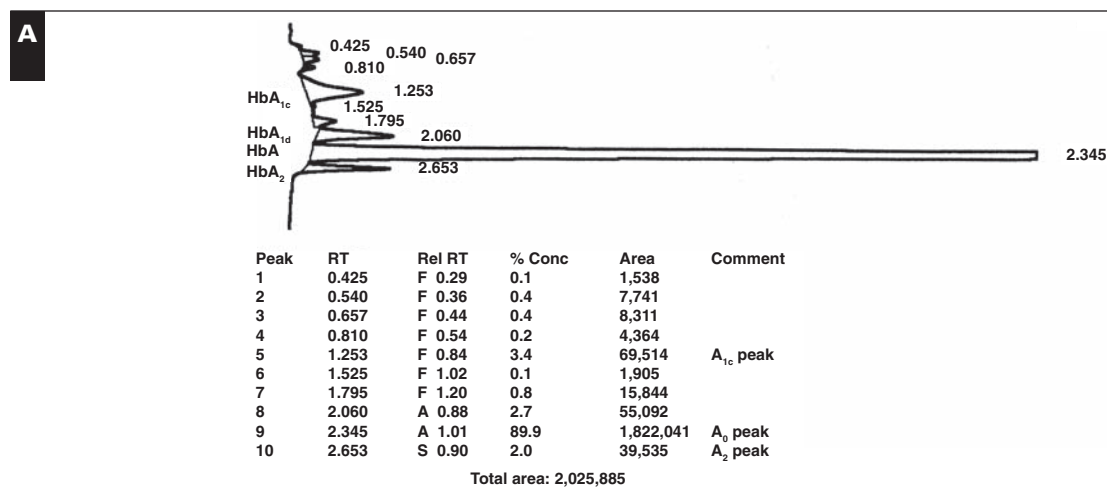
### Comparison of Patterns

Patterns for a normal control sample by HPLC and CE respectively are shown in **Figure 1**. In the HPLC pattern, the elution time is expressed in minutes. The HPLC demonstrates the HbA molecule along with several posttranslational modifications including glycosylated forms such as HbA<sub>1c</sub> and others (Figure 1A). The data in the CE are relative migration times of the hemoglobin past the detector. It is divided into zones Z1 through Z15 based on standardizing on the location of HbA. Unlike HPLC, the CE pattern does not separate the various posttranslational fractions of HbA (Figure 1B). Both techniques provide a single, discrete peak for measuring HbA<sub>2</sub>.

### HbA, HbA<sub>2</sub>, and HbF in Samples Without or With HbS

In 228 samples lacking a variant hemoglobin, there was good agreement between the techniques for HbA and HbF. HbA gave a mean value of 96.2% (SD, 5.7%) by CE and 96.8% (SD, 5.5%) by HPLC. HbF gave an identical mean of 0.9% by both techniques with an SD of 5.6% by CE and 5.4% by HPLC.

However, these samples consistently had higher HbA<sub>2</sub> percentages by CE (mean, 2.8%; SD, 0.8%) than by HPLC (mean, 2.3%; SD, 0.8%) **Figure 2**. With the exception of



**Figure 1** **A**, Primus high-pressure liquid chromatography on a normal sample. Times are given in minutes. **B**, Sebia capillary zone electrophoresis on the same sample as in Figure 1A. The data are relative migration times of the hemoglobin past the detector in zones Z1 through Z15 based on standardizing on the location of HbA. Conc, concentration; Hb, hemoglobin; RT, retention time; Rel RT, relative RT. The reference percentages are reported in the "Materials and Methods" section.

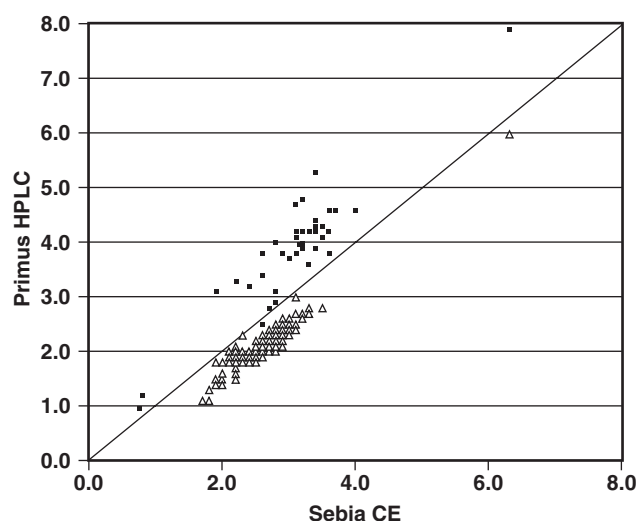
a couple of outliers, this was true at all levels of HbA<sub>2</sub>. The slope was 0.931 (0.908-0.953) with an intercept of -0.32 (95% confidence interval, -0.38 to -0.25). The correlation coefficient was 0.9832 with a bias of -0.51.

In contrast, in 39 samples containing HbS, the percentage of HbA<sub>2</sub> was significantly greater when measured by HPLC (mean, 4.0%; SD, 1.0%) vs CE (mean, 3.1%; SD, 0.8%) due to a major fraction of glycated products of HbS comigrating with the HbA<sub>2</sub> peak in the HPLC method (Figure 2). This difference is even more striking because HbA<sub>2</sub> gave a higher mean value in the CE method than in the HPLC method in samples from people lacking a variant hemoglobin. Because an increase of only 1% or 2% can be

significant for HbA<sub>2</sub>, we routinely place a caveat noting this carryover in our interpretive reports when the HPLC technique is used.

#### Analysis of Variant Hemoglobins in the Presence of HbA

In **Figure 3A**, the HPLC pattern for a case of HbC trait is shown. As with HbA, the HPLC measures posttranslational forms of HbC. For example, the HbC<sub>1c</sub> glycated fraction can be seen at peak 13 with an absolute retention time of 5.823 minutes and a relative retention time (REL RT) relative to the closest standard, in this case HbS, of S 1.02 minutes. To obtain the total percentage of HbC, we add the values of peaks 12, 14, 15, 16 (breakdown products), and 13 (mainly HbC<sub>1c</sub>) to the percentage of the main HbC peak 17 (REL RT C, 1.04 minutes). Some of the breakdown products will migrate in the HbA<sub>2</sub> peak and cannot be accounted for with this technique. We include a note in our interpretive report that HbA<sub>2</sub> may be slightly elevated owing to this issue. The same issue applies to a larger extent with HbS, as previously reported.<sup>19,22-24</sup>



**Figure 2** Percentage of hemoglobin (Hb) A<sub>2</sub> in 228 samples with no structural abnormality (triangles) and in 39 samples containing HbS (squares). CE, capillary zone electrophoresis; HPLC, high-pressure liquid chromatography.

In **Figure 3B**, the CE pattern for the same case is shown. With CE, the system standardizes the position of HbA in zone 9, the middle of the 15 zones provided on the manufacturer's system. The CE instrument has indicated the presence and percentage of HbF, HbA<sub>2</sub>, and HbC based on their migration relative to the position of HbA. Note that HbA<sub>2</sub> overlaps somewhat with HbC. In some cases, this overlap causes the instrument to combine the HbC and the HbA<sub>2</sub> values. When this occurs, in the interpretive report for this method we note that the percentage reflects HbC and HbA<sub>2</sub>. In the present case, the instrument determined there was sufficient separation to provide the HbA<sub>2</sub> level as 3.0%, compared with the 2.9% reported by HPLC. Unlike the HPLC system, glycosylated fractions are not delineated.

#### Analysis of Variant Hemoglobins in the Absence of HbA

In **Figure 4A**, the HPLC pattern is shown for a patient with HbSC disease. Here, the HPLC uses the REL RT to identify HbS (peak 6 with a REL RT of S 1.02 minutes) and HbC (peak 7 with a REL RT of C 1.03 minutes). Some of the breakdown products and glycosylated HbC will be present in the HbS peak 6, and a little will also be in the HbA<sub>2</sub> peak 5 (REL RT, S 0.89 minutes). That peak also contains some HbS glycosylated and breakdown hemoglobin that we note in our interpretive report to account for the apparent elevation of HbA<sub>2</sub> in the sample.

In **Figure 4B**, the CE pattern for the same case is shown. Because with CE the system standardizes the position of HbA as zone 9, the lack of this hemoglobin precludes the zones from being displayed with the initial run (peaks were labeled

here after they were identified by the procedure to produce the data in **Figure 4C**). To identify the peaks, the patient's sample was mixed 1:1 with a normal control sample, and the CE procedure was repeated (as described in the "Materials and Methods" section). This allowed identification of HbS and HbC. The percentages derived from the areas under the peaks in **Figure 4B** are used in the interpretive report. In this sample, HbC and HbA<sub>2</sub> did not separate sufficiently for the instrument to record a separate measurement for these hemoglobins.

#### Measurement of HbS

There was good agreement between HPLC and CE in measuring the percentage of HbS in the 39 samples that contained this variant—a small, but consistently higher value was found in samples measured by CE (mean, 40.6%; SD, 18.9%) than in samples evaluated by HPLC (mean, 38.4%; SD, 18.9%). This small increase in HbS in samples evaluated by HPLC may have reflected different handling of glycosylated fractions of HbS by these methods.

#### Measurement of HbC and HbA<sub>2</sub> in Samples Containing HbC

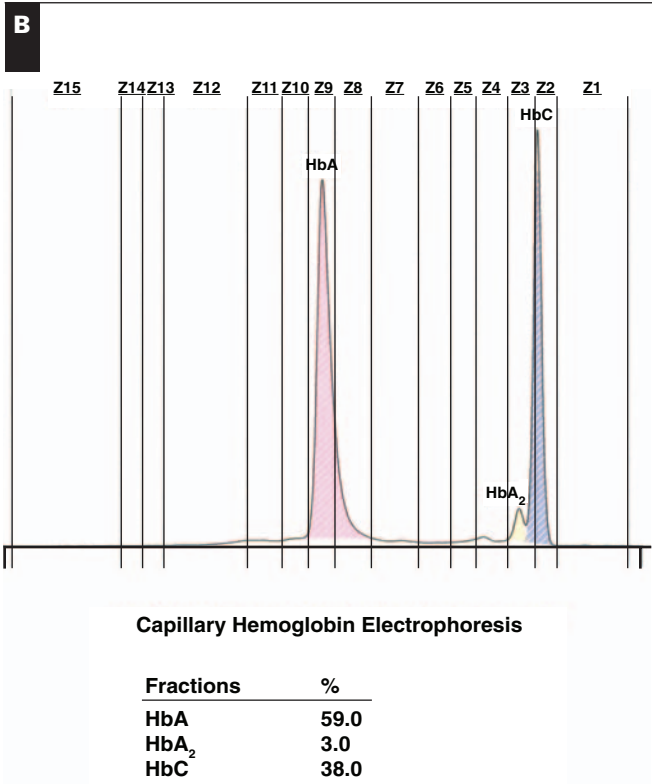
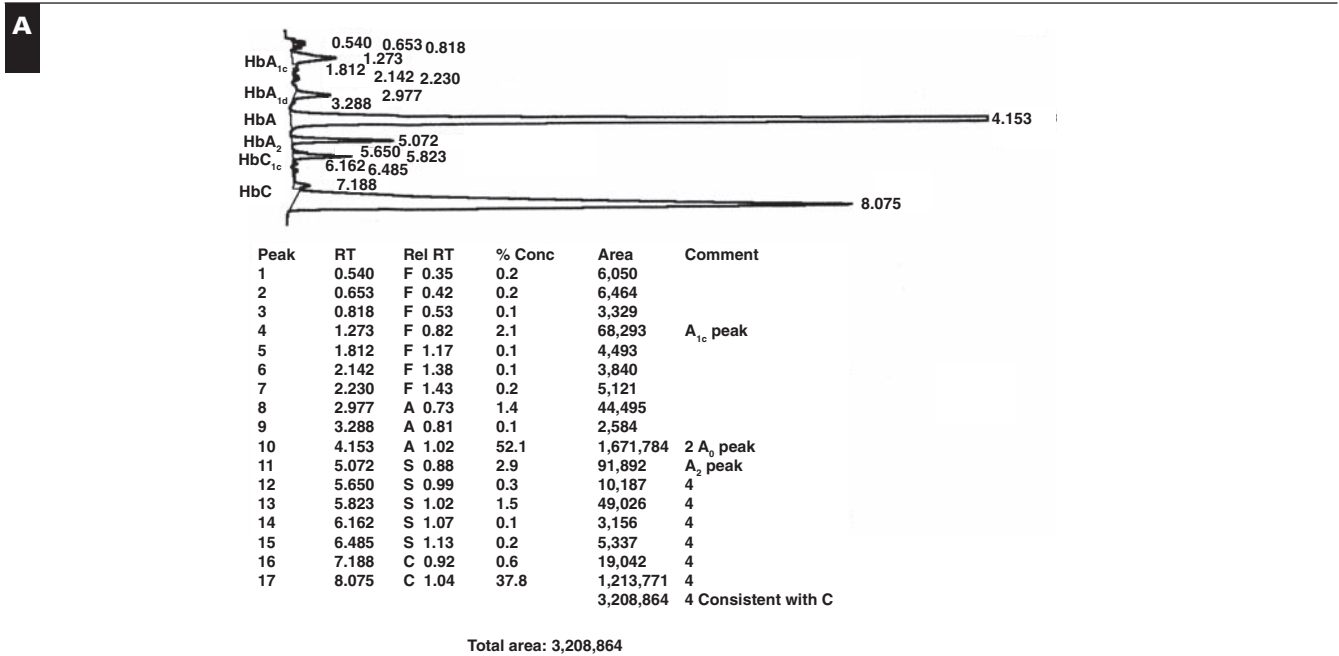
As noted in **Figure 3**, carryover of HbC breakdown products into HbA<sub>2</sub> occurs with HPLC, although to a lesser extent than with HbS. However, with the CE, the HbA<sub>2</sub> peak shows considerable overlap with HbC (**Figure 3**). This resulted in HbA<sub>2</sub> being included with the HbC percentage in 2 of 14 samples with HbC trait and 1 sample with HbS and HbC (**Figure 4B**).

#### Measurement of HbE and HbA<sub>2</sub> in Samples Containing HbE

Whereas only 3 cases containing HbE were encountered during this prospective study, the complete separation of HbA<sub>2</sub> from HbE by CE in all 3 compared with the lack of measurable HbA<sub>2</sub> by HPLC deserves note. In **Figure 5A**, the HPLC pattern from a patient with HbE trait is shown. While the HPLC attempts to distinguish HbA<sub>2</sub> (peak 13), the overlap with HbE (peak 12) is too great for a reliable estimate. Our interpretive report for the HPLC notes that the HbE value includes HbA<sub>2</sub>. However, the CE pattern in this same case **Figure 5B** demonstrates a clean separation of HbA<sub>2</sub> from HbE. The amount of HbA<sub>2</sub> in the 3 cases is slightly elevated for HbA<sub>2</sub> **Table 1**. This is consistent with the fact that HbE is a structural form of  $\beta$ -thalassemia. Consequently, at least a relative increase in the amount of HbA<sub>2</sub> in such cases would be expected. This pattern has been consistent in several cases of HbE trait evaluated after the present study (data not shown).

#### Other Structural Variants Identified

In addition to the 39 cases containing HbS, 14 cases containing HbC and 3 cases containing HbE variants correctly



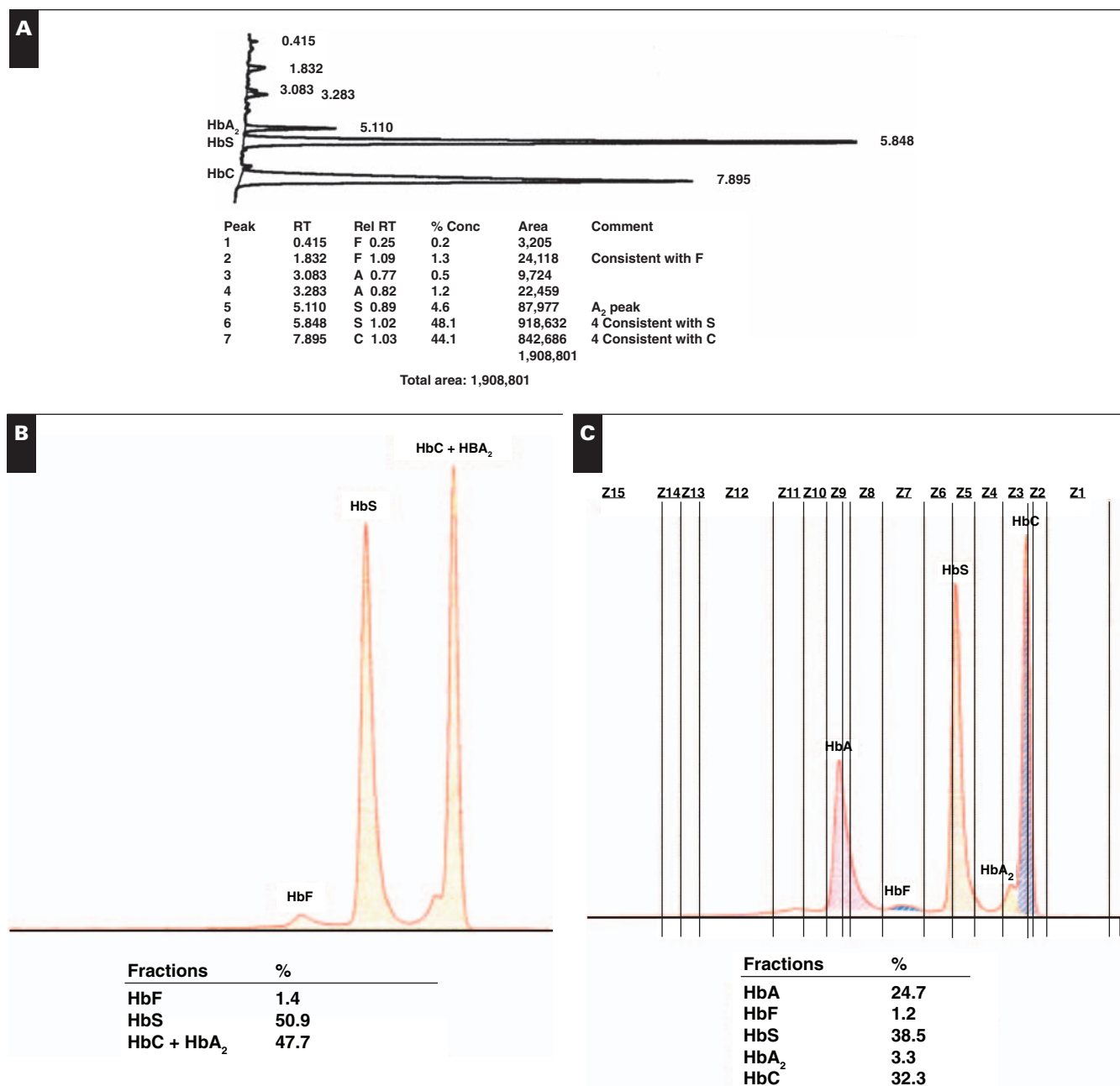
**Figure 3** **A**, High-pressure liquid chromatography pattern on a sample with hemoglobin (Hb) C trait. Times are given in minutes. **B**, Capillary zone electrophoresis pattern for the same case as in Figure 3A. Conc, concentration; Hb, hemoglobin; RT, retention time; Rel RT, relative RT; Z, zone. The reference percentages are reported in the “Materials and Methods” section.

**Discussion**

The comparison between 2 automated, FDA-approved methods demonstrated the usefulness of both methods in the routine evaluation of patients for the presence of hemoglobinopathies. As with other studies, there was good correlation of measurements of HbA and HbF between the methods.<sup>19</sup> Although the correlation between HbA<sub>2</sub> results was good, there was a consistent bias, with higher values determined by the CE method in patients who did not have a structural variant. Similar results were found by Cotton et al<sup>19</sup> when comparing the Beckman P/ACE 5500 CE system (Beckman Coulter, Fullerton, CA) with the Bio-Rad Variant HPLC (Bio-Rad, Hercules, CA).

Whereas the HPLC method has the advantage of a broad literature and great precision in detection of a large number of

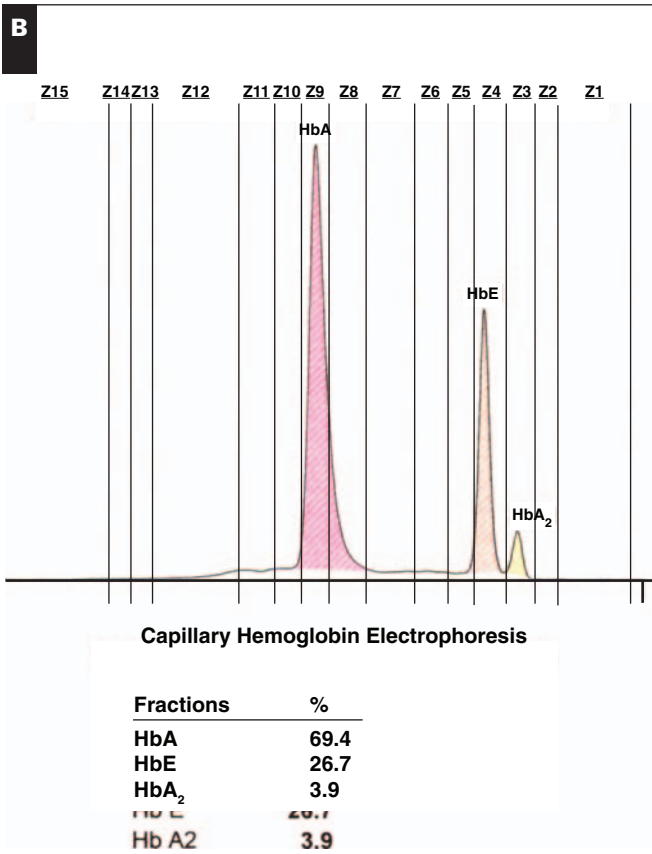
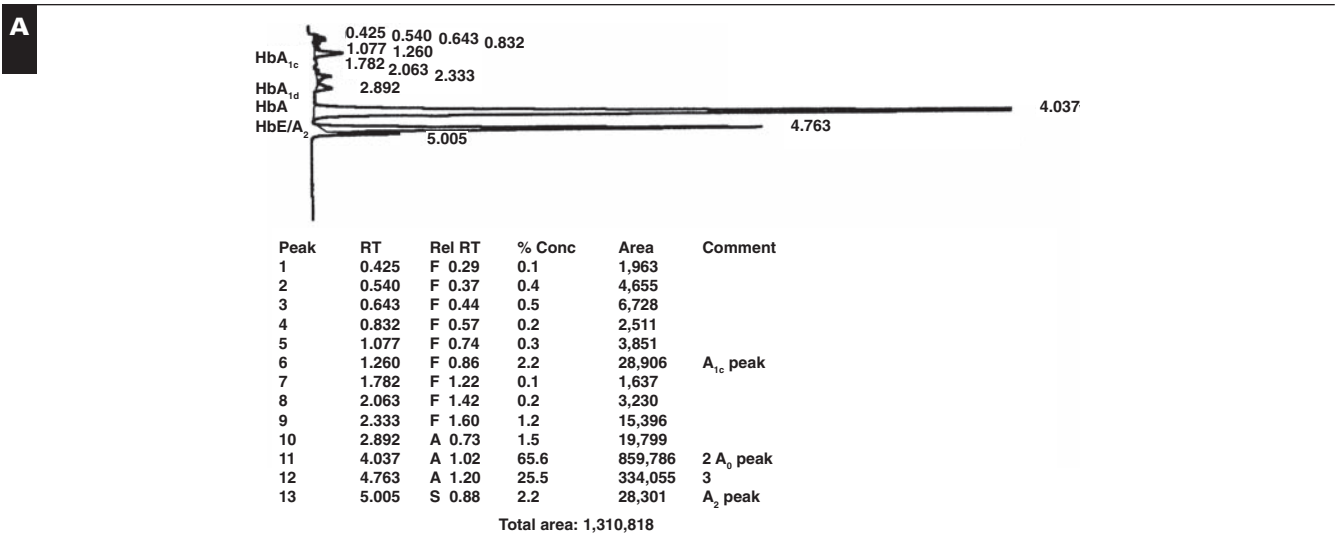




**Figure 4** **A**, High-pressure liquid chromatography pattern of a sample with hemoglobin (Hb) S and HbC. Times are given in minutes. **B**, Initial capillary zone electrophoresis (CE) on the same sample as in Figure 4A (no zones are shown because no HbA is present). **C**, CE on the same sample as in 4A, but the sample has been mixed 1:1 with a normal control sample to provide HbA. The presence of HbA from the control sample allows identification of the variant hemoglobins. However, because of the dilution of the current sample by the control sample, the percentages from the original CE run (Figure 4B) are used in our report. Conc, concentration; Hb, hemoglobin; RT, retention time; Rel RT, relative RT; Z, zone.

variants, it has the disadvantage of a complex elution pattern that may be difficult for many routine chemistry laboratories to apply to their daily workload. Indeed, the difference in values of HbS between the methods related to the need to account for some fractions of HbS that elute in the same location as HbA. Fractions that coelute with HbA by HPLC are unmeasurable,

except in people with sickle disease (lacking transfused HbA). Furthermore, the inclusion by the HPLC technique of HbS and, in some reports HbC, posttranslational and/or breakdown products in the region of HbA<sub>2</sub> interfere with the accurate measurement of HbA<sub>2</sub> for detecting  $\beta$ -thalassemia.<sup>19,23,25,26</sup> In general, variants are distinct on HPLC; however, in a sample



**Figure 5** **A**, High-pressure liquid chromatography pattern for a patient with hemoglobin (Hb) E trait. HbA<sub>2</sub> is not completely separated from HbE, and the 2 hemoglobins must be measured together. Times are given in minutes. **B**, Capillary zone electrophoresis pattern on the same sample as in Figure 5A. HbA<sub>2</sub> does completely separate from HbE. Conc, concentration; Hb, hemoglobin; RT, retention time; Rel RT, relative RT; Z, zone. The reference percentages are reported in the “Materials and Methods” section.

**Table 1**  
Comparison of HbA<sub>2</sub> and HbE by CE and HPLC in 3 Cases Containing HbE\*

Case No.	CE HbA <sub>2</sub>	HPLC HbA <sub>2</sub>	CE HbE	HPLC HbE
1	3.9	ND	26.7	27.7 <sup>†</sup>
2	3.6	ND	27.2	27.2 <sup>†</sup>
3	3.9	ND	17.8	17.4 <sup>†</sup>

CE, capillary zone electrophoresis; Hb, hemoglobin; HPLC, high-pressure liquid chromatography; ND, not determined.  
\* Values are given as percentages.  
<sup>†</sup> Includes HbA<sub>2</sub>.

containing Hb Athens/Waco, which was not detected by the screening HPLC in this study but was detected by CE, this is not always the case.

Although CE has been evaluated in research laboratories for several years, the recent FDA approval of the present automated CE facilitates use of this new technique for evaluating hemoglobinopathies in routine clinical laboratories. We found that the CE patterns are easier to read than HPLC patterns as long as HbA is present in the sample, and, since the study

was completed, we have noted that HbH and Hb Bart’s are more readily detected and measured by CE than by the HPLC method (data not shown).

However, when HbA is absent, the laboratory must perform a second analysis to obtain the appropriate zones for verifying the identity of the hemoglobin. While not difficult, it is an additional step that is unnecessary with HPLC. Separation of HbC from HbA<sub>2</sub> by CE usually is sufficient to allow measurement of HbA<sub>2</sub>, but in 2 of the 14 cases that

contained HbC trait in our study, a separate HbA<sub>2</sub> measurement was not obtained.

One outstanding feature of the CE method is the ability to obtain a clean measurement of HbA<sub>2</sub> in patients with HbE. Because HbE results from a mutation that introduces a splice site in exon 1, it produces fewer HbE  $\beta$  chains and a picture of  $\beta$ -thalassemia.<sup>1</sup> However, routine screening methods—alkaline and acid gels—and currently approved HPLC methods have not been able to separate HbA<sub>2</sub> sufficiently to allow for this measurement in routine chemistry laboratories.<sup>27</sup> Tandem mass spectrometry has been able to measure HbA<sub>2</sub> in the presence of HbE.<sup>8</sup> However, the present CE method provides this information in a simplified format.

We have found HPLC and CE to be complementary techniques and use both routinely. Our current triage uses HPLC as the screening method and CE to confirm relatively standard variants, such as HbS, HbC, HbE, HbD, HbG, and Hb Lepore. We occasionally use routine alkaline and acid gels because of the more extensively documented characterization of unusual variants by gel techniques in the literature. As the use of CE expands for characterizing variant hemoglobins, we predict that it will become the first line of screening in many clinical laboratories.

Both methods provide efficient, automated detection of hemoglobins but differ in the logistics of identifying variant hemoglobins, especially in the absence of HbA and with regard to measurement of HbA<sub>2</sub> when HbS, HbC, and HbE are present.

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Reagents for the capillary electrophoresis studies were provided by Sebia.

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