Comparison of Capillary Zone Electrophoresis with High-pressure Liquid Chromatography in the Evaluation of Hemoglobinopathies

Çakır Madenci Ö. et al: Evaluation of Hemoglobinopathies

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Introduction
Genetic mutations in the amino acid sequence of Alpha (α-) and Beta-(β-) chains that alter their structure or cause a reduction in the amount synthesized, or both, lead to hemoglobinopathies (1,2). Many of these mutations are silent and do not cause clinical manifestations (3,4).

Cellulose acetate electrophoresis, isoelectric focusing, high-pressure liquid chromatography (HPLC), and capillary zone electrophoresis (CZE) are presumptive methods that can be readily applied in laboratories (1,5-7). The final identification of hemoglobin (Hb) variants can still only be obtained by DNA sequencing of the affected globin gene or amino acid sequencing of the abnormal protein, generally by mass spectrometry (MS) (8-10).

HbA2 levels may differ only slightly between people with and without β-thalassemia carriers and the performance of HbA2 analyses must be both precise and accurate (12). Accuracy of HbA2 measurement is particularly important in the critical range between 3.0% and 4.0%, and imprecision should be such that an SD of 0.05 or the coefficient of variation (CV) of 2% can be achieved (12).

For more than thirty years, HPLC has been used in clinical laboratories for the evaluation of hemoglobinopathies, and the experience with retention times of the different variants has been satisfactory (13,14). However, the HbA molecule is quantified along with several posttranslational modifications, including glycated forms such as HbA1c and others (13). Recently, the CZE method has been one of the most commonly used techniques with adequate precision for the determination of HbA2 and HbF (15-17). In comparison studies, CZE and MS/MS showed acceptable analytical and diagnostic performance and were accepted as an accurate and appropriate method choice for HPLC (18). By CZE, accurate measurements of HbA2 can be observed without separating the glycated fraction and other Hb adducts in the presence of HbS (19, 20). However, the presumptive identification of variants is related to their migration zones on the electropherogram and these zones are less specific than retention time (19).

In this prospective study, we aimed to compare the Sebia Capillaries 3 OCTO (CZE) system with Premier Resolution (HPLC) which was the routine method in our laboratory for premarital screening testing. Besides the identification of 5 common (Hb D-Punjab, Hb E, Hb C, HbS/A, Hb S/S) and 8 rare (Hb S/D, Hb O-Arap, Hb Lepore, Hb G-Coashata, Hb Setif, Hb Hamadan, Hb Q-Iran, and Hb H) Hb variants were compared.

Materials and Methods
Study Design and Subjects
A total of 455 whole blood samples selected from patients who were screened for hemoglobinopathy at ............ between November 2022 and February 2023 were included.

The mean age of the patients was 29.6±14.5 years (range: 1-74 years), with 239 females (52.5%) and 216 males (47.5%). Pregnants and children <1 year of age were excluded. There were no patients with high HbA1c values in this group as observed in the chromatograms of the HPLC system. This study was approved by our institution’s Ethical Committee (.................Ethical Committee), 11.01.2023 2022/514/241/1.
Blood Collection
Blood was collected after overnight fasting between 8:00 and 10:00 into Becton Dickinson (BD) 3-ml Vacutainer® tubes containing 5.4 mg K2EDTA (lot 1023106) (Becton, Dickinson and Company, BD Plymouth PL6 7BP, UK).

Methods
Complete Blood Count Analyses (CBC)
CBC analyses were performed using the Sysmex XN 9000 (Sysmex America, Mundelein, IL). The Mentzer index was calculated by dividing the mean corpuscular value (MCV) by the red blood cells (RBC) (21).

High-performance Liquid Chromatography (HPLC)
HPLC analysis was performed in the Premier Resolution (Kansas City, Kansas, USA) system using cation exchange chromatography and spectrophotometric detection. The Hb fractions were identified according to their retention times taking Hb-containing calibrators as reference: HbF, HbA, Hb S, and HbC (13). The screening was performed using the Quick Scan assay (4 minutes), which measures HbF, HbA2, and HbA0 and flags abnormal peaks. In the presence of abnormal peaks, analysis was repeated with the high-resolution assay (8 minutes), the system indicating abnormal hemoglobins for identification. Selected residual samples were re-evaluated within 24 hours using the CZE system. The adult reference range used in our laboratory was: HbA;97.0%-99.0%, HbF;<2.0%, and HbA2; 1.5 -3.5% as recommended by the manufacturer.

Capillary Zone Electrophoresis (CZE)
The CZE was performed using the Sebia Capillaries 3 OCTA system (Sebia, Lisses, France). The Hb fractions are separated according to their electrical charges in an alkaline buffer, and the instrument records Hb migration on the x-axis from 0 to 300 (19). The migration position for each Hb is normalized relative to the standardized position of HbA (position 150) and HbA2 (position 243). In the presence of HbA and/or HbA2, the electrophoretic profile is divided into 15 migration regions, and the possible identification of an abnormal fraction is based on the migration site. In the absence of HbA, the sample is mixed 1:1 with the normal patient sample, and the migration positions were normalized by introducing Hb A and Hb A2 into the test sample (19).

The manufacturer's suggested reference range was HbA> 96.8%, HbF< 0.5%, and HbA2 2.2-3.2%.

Imprecision
For imprecision studies, 2 levels of commercial control materials for both systems were used. (Trinity Biotech Premier Resolution A2+F Control for HPLC (Lot number:13420) and Sebia Capillaries HbA2 Control for CZE (Lot number:05111/XX). Within-run CVs were determined by analyzing two levels of HbA and HbA2 in 10 consecutive runs on the same day. Ten replicates of two control materials were analyzed in 8 different capillaries for CZE. Between-run CVs were determined by measuring two commercially available control materials on 10 consecutive days. The mean, standard deviation (SD), and CV were calculated.

Reference Range
A reference range study was performed for HbA2 using CZE. A total of 321 samples with HbA, HbA2, and HbF values within the reference range of the HPLC system were evaluated. The reference range was derived by a non-parametric method and reported as the 2.5 and 97.5 percentiles according to the recommendations of the IFCC (CLSI C28-A3) (22).

Statistical Analysis
Statistical analysis was carried out using MedCalc Statistical Software (version 12, Med-CalcSoftware, Mariakerke, Belgium). The distribution of data was assessed by the Shapiro-Wilk test, and the results were expressed as mean ± SD or median (2.5-97.5 percentile) according to the distribution. Continuous variables were compared with the Wilcoxon rank sum test. Deming regression analysis, Spearman correlation analysis, and Bland-Altman analysis were used in the method comparison study.

Agreement of the methods in Hb A2 interpretation was expressed with the Kappa (κ) coefficient. Statistical significance was set at P<0.05.

Results
Imprecision Study
The mean ± SD values for HbA (%) and HbA2 (%) and within-run and between-run CVs are given in Table 1.

The Reference Range for HbA2 for CZE
The reference range showed a modest shift: with a normal HbA2 level of 1.6-3.1%.

HbA2 Levels in Patients without Hemoglobin Variant
A total of 321 patients with HbA, HbA2, and HbF values within the manufacturer's recommended reference ranges according to HPLC were evaluated. The study group was matched in terms of gender (p=0.911). The median (2.5-97.5 percentile) values for HbA (%) were 97.4 (97.0-98.0) and 97.5 (96.6-98.4) for HPLC and CZE methods, respectively (p=0.060). The median (2.5-97.5 percentile) values for HbA2 (%) were 2.4(1.6-3.0) and 2.5(1.6-3.1) for the HPLC and CZE methods, respectively (P<0.001) (Table 2). In method comparison studies for HbA2; higher
results were obtained with the CZE with a bias of 0.10% limit of agreement (LOA);(0.33--0.53) and a constant error
of 0.255 (CI; 0.062-0.448) even with a strong correlation of 0.782 (CI: 0.623-0.802) between the methods (Figure
1A).
HbA2 in Patients with β-Thalassemia Trait
A total of 113 patients with HbA2 values that were above the manufacturer's recommended reference range (>3.5%)
according to HPLC were evaluated. The median (2.5-97.5 percentile) for HbA2 (%) was 5 (3.6-6.3) and 5.1 (3.7-
5.9) for the HPLC and CZE methods, respectively (p=0.1831) (Table 2). In method comparison studies for HbA2;
higher results were obtained with the CZE with a bias of 0.07% (LOA:0.76--0.91) and a constant error of 0.795
(0.083-1.506) even with a strong correlation of 0.725 (CI: 0.623-0.802) between the methods (Figure 1B).
CBC values in patients without the hemoglobin variant and with β-thalassemia trait were given in Table 3.
Agreement of Methods in the Interpretation of Hb A2 with the Kappa (κ)-Coefficient
Using the newly determined reference ranges for HbA2 by CZE, both methods similarly identified 113 β-
thalassemia trait patients. The methods showed almost perfect interrater agreement (κ=0.911).
Analysis of Common β-Chain Hb Variants (Hb D-Punjab, Hb E, Hb C, Hbs/A, Hb S/S)
HbA2 and Hb variant values of the patients with Hb D-Punjab, Hb E, Hb C, Hbs/A, and Hb S/S are given in Table
4. Both methods detected and similarly identified the common variants. HbA2 values interfered differently in either
HPLC or CZE.
HbA2 in the presence of HbD Punjab: In four patients with HbD Punjab lower HbA2 values were observed using
the HPLC system. The mean HbA2 values were 2.4% (range; 2.1%-3.0%) and 3.0% (range; 2.8%-3.2%) for HPLC
and CZE, respectively.
HbA2 in the presence of HbE: In one patient with HbE, the HPLC system could not clearly distinguish HbE and
HbA2, while it was adequately separated by CZE. The HbA2 values were 3% and 3.2% with the HPLC and CZE
systems, respectively.
HbA2 in the presence of HbC: In one patient with HbC, the CZE system could not clearly distinguish HbC and
HbA2, while it was adequately separated by HPLC. The HbA2 results were 3.3% and 3.7% with the HPLC and CZE
systems, respectively.
HbA2 in the presence of Heterozygous HbS: In five patients with heterozygous HbS, higher A2 values were
obtained with the HPLC method. The mean HbA2 values were 3.4% and 3.1% with HPLC and CZE, respectively.
HbA2 in the presence of Homozygous S: In a patient with homozygous S, HbS and HbF values were higher with
CZE. (HbF; 9 % vs. 3.8 % and HbS;87.3% vs. 78.6 %, with CZE and HPLC respectively).
Analysis of Rare Variants
All rare variants, except HbS/D with CZE and HbH with HPLC, were detected as separate peaks with both methods.
In one patient, a 77.2% Hb variant was detected in the D zone, with overlapping migration zones of the HbS and
HbD variants with CZE. In this sample, HPLC distinguished 39.6% Hb D and 33.3% HbS variants. In two patients
with Hb O-Arap HbA2 could not be quantified with CZE because it co-eluted with the variant. Hb Lepore, Hb G-
Coushata, Hb Setif, Hb Hamadani, and Hb Q-Iran were detected by both methods and all except Hb Lepore were
confirmed by DNA analysis because of limitations of libraries of either system (Table 4). A comparison of results
with HPLC and CZE for HbH, HbS/D, and Hb O-Arap is given in Figure 2. HPLC, CZE, and DNA analyses for the
rare α-chain Hb variants; HbSetif and Hb Q-Iran are given in Figure 3.
Discussion
This study aimed to compare CZE with HPLC in the evaluation of hemoglobinopathies. In patients without
hemoglobinopathy, HbA values were in agreement with the two methods but HbA2 values were higher with CZE
(Bias 0.10%) which is consistent with the previous studies (16,17). For the β-thalassemia trait, a negative bias of
0.07% was observed. It seems a better agreement is achieved with methods, especially in the clinical cut-off levels
used to diagnose B-Thalassemias. A good correlation was obtained between the methods in patients with and
without Thalassemias. For HbA2, different correlation coefficients between 0.55 and 0.999 have been reported in
the literature. This difference could be related to the study population (with or without Hb variants) or the
performance characteristics of the system used (15-16,23).
In this study, the within-run and between-run CVs for HbA2 were higher than the recommended maximum
imprecision of 2% with both methods (12). This target could not be established in any of the studies; in the quality
control program organized by the Italian Society of Haemoglobinopathy, normal, borderline, and pathological HbA2
results were distinguished with HPLC in all participating laboratories, but the overall interlaboratory CV was
between 4 % and 8.2% (24). In the survey results from CAP 2008, HbA2 values showed a Bias between 0.31% and
0.93% between different methods, suggesting that there is no standardization of HbA2 measurements (15).
Thus, it is crucial to standardize and improve the analytical quality of HbA2 measurements.
Forty-eight individuals within the normal range of the HPLC method were below the CZE's lower reference range suggesting that HbA2 reference ranges should be determined in this study. The lower cut-off value for HbA2 determined in this study (1.6%) was more consistent with HPLC results.

In this study, HbD-Punjab, HbE, HbC, HbS/A, and HbS/S were similarly identified using both methods with different interferences on HbA2 levels. Lower HbA2 levels were found in HbD-Punjab with the HPLC system and this was attributed to baseline noise errors rather than a real decrease. In individuals with coinheritance of Hb D Punjab and β-thalassemia quantification of Hb A2 with HPLC presents a challenge (15,16). A clear separation and reliable estimation of HbA2 could not be achieved since HbE coeluted with HbA2 in the HPLC system which was also reported in the previous studies (15).

On the other hand, the HbA2 result with heterozygous HbC was higher with the CZE system because the peaks of the HbC and HbA2 curves slightly overlapped, interfering with the quantification of both HbC and HbA2 (18,25). In cases with heterozygous HbS, higher HbA2 values were found with the HPLC method because of the co-elution of glycated HbS and various HbS adducts, such as carbamylated α- and βS-chains (15,26).

In a patient with homozygous S, higher HbF levels were found with CZE compared to HPLC because the peaks of the HbC and HbA2 curves slightly overlapped, interfering with the quantification of both HbC and HbA2 (18). In cases with heterozygous HbS, higher HbA2 values were found with the HPLC method because of the co-elution of glycated HbS and various HbS adducts, such as carbamylated α- and βS-chains (15,26).

The discrepancy in identifying HbA2 values in the presence of HbS, HbC, HbD, and HbE may be attributed to different methodological techniques (14). For HPLC the Hb fractions were identified according to their retention times taking specific variant Hb-containing calibrators as reference. On CZE identification of variants is related to their migration positions on the electropherogram, taking HbA and HbA2 as the reference (19).

In the study period, we didn’t have an adequate number of results with comparable HbF values which may be considered as a limitation of the study.

In this large-scale study, we compared the hemoglobin screening results of HPLC and CZE on 455 samples. A total of 13 different Hb variants were identified in the course of the study and migration patterns of 4 of them- Hb G-Coushata, Setif, Hamadan, and Q-Iran- were identified using CZE for the first time and confirmed genetically.

**Conclusion**

Both methods were in agreement in the preliminary identification of β-thalassemia patients. Different Hb variants were detected by both methods but with a possible methodologic interference on HbA2 measurements. CZE is a reliable and simple alternative for the evaluation of hemoglobinopathies. Standardization of HbA2 measurement should be prioritized as more techniques be available in routine laboratory practice.

**Financial Disclosure Statement:** Reagents for capillarys 3 OCTA was provided by Sebia (Medisis Medical Systems).

**Conflict of Interest:** The authors declare no conflict of interest.

**References**


### Table 1. Precision study for HPLC and CZE methods

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HPLC</th>
<th>CZE</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Within-run CV (%)</td>
</tr>
<tr>
<td>HbA</td>
<td>Level 1</td>
<td>81.8±0.2</td>
</tr>
<tr>
<td></td>
<td>Level 2</td>
<td>46.0±0.3</td>
</tr>
<tr>
<td>HbA2</td>
<td>Level 1</td>
<td>3.0±0.5</td>
</tr>
<tr>
<td></td>
<td>Level 2</td>
<td>6.3±1.3</td>
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### Table 2. Characteristics of the study population

<table>
<thead>
<tr>
<th>Study group</th>
<th>Demographics</th>
<th>HPLC (%) Median (2.5-97.5 percentile)</th>
<th>CZE (%) Median (2.5-97.5 percentile)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F/M</td>
<td>Age (years) (mean ± SD)</td>
<td>HbA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>97.4 (97.0-98.0)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>97.5 (96.6-98.4)</td>
</tr>
<tr>
<td>Without variant (n=321)</td>
<td>162/159</td>
<td>30.9±13.6</td>
<td>94.5 (89.3-96.2)</td>
</tr>
<tr>
<td>β-Thalassemia trait (n=113)</td>
<td>66/47</td>
<td>24.8±16.8</td>
<td>94.3 (88.0-96.8)</td>
</tr>
</tbody>
</table>

<sup>a</sup>, <sup>b</sup>: Statistically different results (p<0.001)

### Table 3. Complete Blood Count Analyses for Healthy and Beta Thalassemia Patients

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Healthy subjects (Median (2.5-97.5 percentile))</th>
<th>Beta thalassemia patients (Median (2.5-97.5 percentile))</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red blood cell (10&lt;sup&gt;6&lt;/sup&gt;/uL)</td>
<td>4.9 (3.8-6.0)</td>
<td>5.5 (4.3-7.6)</td>
<td>*0.001</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>14 (9.6-17.4)</td>
<td>11.5 (9-15.4)</td>
<td>*0.001</td>
</tr>
<tr>
<td>MCV (um&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>88 (66-97)</td>
<td>66 (55-87)</td>
<td>*0.001</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>29.1 (19.7-32.6)</td>
<td>20.2 (16.8-30.3)</td>
<td>*0.001</td>
</tr>
<tr>
<td>Mentzer Index</td>
<td>17.7 (12.3-23.1)</td>
<td>11.6 (8.4-18.3)</td>
<td>*0.001</td>
</tr>
</tbody>
</table>

MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, Mentzer index: MCV/RBC, *Statistically different results (p<0.005)

### Table 4. Patients with common and rare Hb variants

<table>
<thead>
<tr>
<th>Hb variant (n=21)</th>
<th>HPLC</th>
<th>CZE</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>HbA2 (%)</td>
<td>Variant (%)</td>
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<tr>
<td>HbD (n=4)</td>
<td>3</td>
<td>39.2</td>
</tr>
<tr>
<td></td>
<td>2.3</td>
<td>39.7</td>
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<tr>
<td></td>
<td>2</td>
<td>39.3</td>
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<tr>
<td></td>
<td>2.1</td>
<td>34.7</td>
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<tr>
<td>Hb E (n=1)</td>
<td>3</td>
<td>29.5</td>
</tr>
<tr>
<td>Hb C (n=1)</td>
<td>3.3</td>
<td>28</td>
</tr>
<tr>
<td>HbS Heterozygote (n=5)</td>
<td>3.5</td>
<td>38.2</td>
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<tr>
<td></td>
<td>3.3</td>
<td>37.5</td>
</tr>
<tr>
<td></td>
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<td>27.9</td>
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<tr>
<td></td>
<td>4</td>
<td>26.1</td>
</tr>
<tr>
<td></td>
<td>2.9</td>
<td>37.5</td>
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<tr>
<td>HbS Homozygote (n=1)</td>
<td>3.7</td>
<td>76.6</td>
</tr>
<tr>
<td>#Hb H (n=1)</td>
<td>1</td>
<td>#</td>
</tr>
<tr>
<td>*HbS/D Heterozygote (n=1)</td>
<td>1.9</td>
<td>39.6</td>
</tr>
<tr>
<td>**Hb Q-Arab (n=2)</td>
<td>2.5</td>
<td>33.7</td>
</tr>
<tr>
<td></td>
<td>2.9</td>
<td>37.4</td>
</tr>
<tr>
<td>Hb Lepore (n=1)</td>
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<td>12.2</td>
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<tr>
<td>Hb G-Coushata (n=1)</td>
<td>3.0</td>
<td>38.4</td>
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<td>Hb Setif (n=1)</td>
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<tr>
<td>Hb Hamadan (n=1)</td>
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<td>46.2</td>
</tr>
<tr>
<td>Q-Iran (n=1)</td>
<td>3.2</td>
<td>19.4</td>
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# Not Detected with HPLC, *Hb S/D weren’t seperated with CZE, ** HbA2 was eluted with variant

**Figure 1.** Deming Regression and Bland Altman Analyses of HbA2 209x297 mm (300x300 DPI)
Figure 2. Comparison of HPLC and CZE reports for the rare Hb variants 2A Hb H; migrated at zone 15 with CZE, but wasn’t detected by HPLC. in the D zone with CZE. 209x297 mm (300 x 300 DPI)
Figure 3. HPLC, CZE and DNA analyses for the rare α-chain Hb variants respectively. 209x297 mm (300 x 300 DPI)