Quantification of HbA₂ in Patients With and Without β-Thalassemia and in the Presence of HbS, HbC, HbE, and HbD Punjab Hemoglobin Variants

Comparison of Two Systems

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Abstract

We studied whether problems quantifying hemoglobin A₂ (HbA₂) could be resolved by using capillary electrophoresis. HbA₂ was quantified on whole blood samples from patients with and without β-thalassemia trait and patients heterozygous for HbE, HbS, HbC, and HbD Punjab using the VARIANT II β-thalassemia (Bio-Rad, Hercules, CA) and Capillarys 2 (Sebia, Norcross, GA).

HbA₂ results in patients with and without β-thalassemia trait were lower with the Capillarys 2 system. Reasonable HbA₂ results were obtained for patients with HbD Punjab and HbE traits on the Capillarys 2. HbA₂ results for patients with HbS, heterozygous and homozygous, were similar by both methods. Interference due to coelution for HbA₂ results for patients with HbC trait was noted on the Capillarys 2.

Between-day imprecision on the VARIANT II is less than that for the Capillarys 2 system. The Capillarys 2 is superior to the VARIANT II for quantifying HbA₂ in the presence of HbE and HbD Punjab traits. The Capillarys 2 offers only slight advantages over the VARIANT II for quantifying HbA₂ in the presence of heterozygous and homozygous HbS. The Capillarys 2 gives inferior HbA₂ results for patients with HbC trait.

In the presence of thalassemic parameters in the CBC, the accurate and precise quantification of hemoglobin (Hb)A₂ (α₂δ₂) is essential for the diagnosis of β-thalassemia trait. It is necessary to have good precision in quantitative HbA₂ methods because the difference in HbA₂ concentrations between people with and without β-thalassemia trait is narrow. (In our laboratory, the upper limit of the reference range for HbA₂ is 3.5%; HbA₂ in β-thalassemia trait is usually >4.0%.)

Analytic methods to quantify HbA₂ include electrophoresis at an alkaline pH, high-performance liquid chromatography (HPLC), and tandem mass spectrometry. Studies performed in the 1970s showed poor precision for HbA₂ quantification methods based on electrophoresis. Steinberg and Adams concluded that although electrophoresis at an alkaline pH with densitometric tracings of electrophoretograms for quantification of HbA₂ was an ideal clinical laboratory method from an ease-of-use perspective, it was inaccurate. Wild and Bain claim that the diagnosis of β-thalassemia trait requires approximately 10 times greater precision for HbA₂ quantitation than densitometry can provide. The College of American Pathologists (CAP) has strongly recommended that electrophoretic methods not be used for quantification for HbA₂ because of poor precision, and it no longer includes data from these methods in hemoglobinopathy proficiency surveys.

HPLC methods, although precise, have some limitations, including falsely decreased HbA₂ levels in patients with the HbD Punjab trait due to a rising baseline, falsely decreased HbA₂ levels in patients with HbE, Hb Osu Christianborg, HbG Coushatta, and Hb Lepore with HbA₂. The increase in HbA₂ levels in patients with heterozygous HbS was originally thought to be
due to the coelution of glycated HbS\textsuperscript{11} with HbA\textsubscript{2} but was later shown to be due to the coelution of several HbS adducts, including carbamylated α and the β\textsuperscript{5} chains with HbA\textsubscript{2}.\textsuperscript{12}

Cotton et al\textsuperscript{13} described a capillary electrophoresis method for the routine determination of HbA\textsubscript{2} and HbF and concluded that the method gave excellent precision for both. Cotton et al\textsuperscript{13} also described the measurement of HbF and HbA\textsubscript{2} in the presence of HbS. Sebia (Norcross, GA) recently introduced a commercial capillary electrophoresis method that may resolve some of the problems in HbA\textsubscript{2} quantification associated with HPLC while providing the excellent precision described by Cotton et al.\textsuperscript{13}

The purpose of this study was to evaluate the between-day imprecision of the capillary electrophoresis method and compare the HbA\textsubscript{2} values obtained by this method with those from a widely used HPLC method in patients with and without β-thalassemia trait, with HbD Punjab trait, and with homozygous or heterozygous HbE, Hbc, or Hbs.

### Materials and Methods

#### HPLC Method

The Bio-Rad VARIANT II β-thalassemia method (Bio-Rad, Hercules, CA) was used as directed by the manufacturer.

#### Capillary Electrophoresis Method

The Sebia Capillarys 2 analyzer, software version 6.10, using the HEMOGLOBIN (E) kit was used as directed by the manufacturer.

### Samples

The study was performed during a 4-month period. Samples submitted to the laboratory for hemoglobinopathy/thalassemia investigation, with EDTA as the anticoagulant, were analyzed by both methods. The VARIANT II system uses a whole blood sample, but an RBC sample is required for the Capillarys 2 system. For this reason, samples were first analyzed on the VARIANT II and then analyzed by the Capillarys 2 system within 12 to 72 hours of each other. Data analysis was performed using Analyse-It for Microsoft Excel, version 2.07 (Analyse-It Software, Leeds, England).

### Results

#### Precision

Bio-Rad Lyphocheck HbA\textsubscript{2} control samples (levels 1 and 2), which are whole blood control samples, were included in each of the analytic runs on the VARIANT II and Capillarys 2 analyzers for the 4-month study period. The results are given in Table I. The excellent correlation of patient results between methods shows that good precision exists in both methods.

#### Equivalence of Results

The number of samples analyzed and the mean and range for HbA\textsubscript{2} with and without β-thalassemia trait, with HbS (homozygous and heterozygous), and with heterozygous HbD Punjab, HbE, or Hbc are shown in Table 2. Difference plots for these groups are shown in Figure II.

### HbA\textsubscript{2} in Patients With and Without β-Thalassemia Trait

Deming analysis (x = VARIANT II; y = Capillarys 2) on the 207 samples with no evidence of β-thalassemia trait in the CBC with a HbA\textsubscript{2} value within the laboratory-established reference range (up to 3.5%) or presence of hemoglobin variant showed a constant bias of 0.24 with a proportional bias of 0.93 (t statistic, 37.5; 2-tailed P < .0001) with a mean difference of 0.46. The Pearson correlation coefficient was 0.83.

#### Table I

<table>
<thead>
<tr>
<th>HbA\textsubscript{2}</th>
<th>No. of Samples</th>
<th>Mean (%)</th>
<th>1 SD</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level 1</td>
<td>87</td>
<td>2.52</td>
<td>0.12</td>
<td>4.83</td>
</tr>
<tr>
<td>Level 2</td>
<td>85</td>
<td>3.34</td>
<td>0.14</td>
<td>2.62</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HbA\textsubscript{2}</th>
<th>No. of Samples</th>
<th>Mean (%)</th>
<th>1 SD</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (n = 207)</td>
<td>2.95</td>
<td>2.3-3.6</td>
<td>2.49</td>
<td>1.9-3.1</td>
</tr>
<tr>
<td>β-thalassemia (n = 91)</td>
<td>5.50</td>
<td>3.9-7.1</td>
<td>5.01</td>
<td>3.5-6.6</td>
</tr>
<tr>
<td>HbS (n = 107)</td>
<td>3.81</td>
<td>2.8-4.8</td>
<td>3.06</td>
<td>2.2-3.9</td>
</tr>
<tr>
<td>HbD Punjab (n = 27)</td>
<td>1.48</td>
<td>1.1-1.9</td>
<td>2.76</td>
<td>2.0-3.6</td>
</tr>
<tr>
<td>HbC (n = 28)</td>
<td>NA</td>
<td>NA</td>
<td>3.65</td>
<td>2.8-4.5</td>
</tr>
<tr>
<td>HbC (n = 19)</td>
<td>3.25</td>
<td>2.6-3.9</td>
<td>2.91</td>
<td>1.6-4.1</td>
</tr>
</tbody>
</table>

Hb, hemoglobin; NA, not available.
Deming analysis (x = VARIANT II; y = Capillarys 2) on 91 samples with laboratory evidence of β-thalassemia trait in the CBC (mean corpuscular volume <72 µm³ [72 fl]; increased RBC count) from patients who were iron-replete (ferritin >15 µg/L [34 pmol/L]) with an HbA₂ concentration higher than the in-laboratory–established VARIANT II HbA₂ reference interval (upper limit, 3.5%) showed a constant bias of –0.44 with a proportional bias of 0.99 (t statistic, 16.65; 2-tailed P < .0001) with a mean difference of 0.49. The Pearson correlation coefficient was 0.94. The difference plots shown in Figure 1 for patients with and without β-thalassemia trait show a bias with the Capillarys 2 results consistently lower than the VARIANT II results.

**HbA₂ in the Presence of HbS**

The mean (range) HbA₂ concentrations and range of 10 samples (from patients who had not received transfusion) with HbS concentrations less than 30% (suggestive of the presence of coinherited α-thalassemia) were 3.4% (2.4%-4.4%) on the VARIANT II and 2.8% (1.7%-3.9%) on the Capillarys 2. In 13 samples from patients with homozygous HbS (had not received transfusion), the mean (range) HbA₂ on the VARIANT II was 3.7% (3.2%-4.2%) and was 3.2% (2.7%-3.7%) on the Capillarys 2. In 84 samples from patients with heterozygous HbS (HbS trait), the mean (range) HbA₂ on the VARIANT II was 3.9% (3.0%-4.8%) and was 3.1% (2.4%-3.8%) on the Capillarys 2.

![Figure 1](https://academic.oup.com/ajcp/article-abstract/131/3/357/1760489)

**Figure 1** Difference plots for hemoglobin (Hb)A₂ in patients without β-thalassemia (A), with β-thalassemia (B), with HbS (C), with HbD (D), and with HbC (E): CE, capillary electrophoresis; CI, confidence interval; HPLC, high-performance liquid chromatography.
For the 107 samples with homozygous or heterozygous HbS, the Deming comparison (x = VARIANT II; y = Capillaries 2) between HbA₂ values on the VARIANT II and Capillaries 2 showed a constant bias of −0.02 and a proportional bias of 0.81 with a Pearson correlation coefficient of 0.79, a \( t \) statistic of 13.24, and a 2-tailed \( P \) value of less than .0001.

The difference plot shows the Capillaries 2 HbA₂ result to be consistently lower than the VARIANT II result in patients with heterozygous or homozygous HbS.

### HbA₂ in the Presence of HbD Punjab

Deming analysis (x = HPLC; y = Capillaries 2) of the results for 27 patients with heterozygous HbD Punjab showed a constant bias of −3.82 and a proportional bias of 4.45 with a mean difference of −1.29, a \( t \) statistic of −1.98, and a 2-tailed \( P \) value of less than .001. The Pearson correlation coefficient was 0.36.

The difference plot shows the HbA₂ results on the Capillaries 2 to be higher than the VARIANT II results in patients with the HbD Punjab trait with a substantial degree of scatter.

### HbA₂ in the Presence of HbE

No comparison data were available because no HbA₂ values were available from the VARIANT II owing to coelution of HbA₁ and HbE. The HbA₂ concentrations in 2 patients homozygous for HbE were 5.8% and 5.1%.

### HbA₂ in the Presence of HbC

Deming analysis (x = HPLC; y = Capillaries 2) of the results for 19 patients with heterozygous HbC showed a constant bias of −15.81 and a proportional bias of 5.76, a \( t \) statistic of 2.37, a 2-tailed \( P \) value of less than .0294, and a mean difference of 0.34. The Pearson correlation coefficient was 0.27. For 2 patients with SC disease, the HbA₂ results on the VARIANT II were 3.3% and 3.6% and on the Capillaries 2 were 2.2% and 4.0%. The HbA₂ results for a patient with homozygous HbC were 6.4% and 6.0% on the VARIANT II and Capillaries 2, respectively.

The difference plot shows a substantial amount of scatter between HbA₂ results for patients with HbC trait on the Capillaries 2 and the VARIANT II.

### Discussion

The between-day imprecision (expressed as the coefficient of variation) for the Capillaries 2 quantification of HbA₂ is not as good as that for the VARIANT II (6.4% vs 4.83% for the low-value control sample and 6.0% vs 2.62% for the high-value control sample). Mario et al.\(^4\) stated that the capillary electrophoresis method they developed was satisfactory for precise quantifications of high HbA₂ but provided no data. Based on 10 data points obtained on 10 days for each HbA₂ value, Cotton et al.\(^13\) obtained a coefficient of variation of 6% at HbA₂ concentrations of 2.0%, 3.1%, and 5.6% and 3% at an HbA₂ concentration of 2.4%. The precision for HbA₂ measurements on the Sebia Capillaries 2 method is as good as that previously described by Cotton et al.\(^13\) for a capillary electrophoresis method and, according to Cotton et al.\(^13\), is acceptable for establishing the diagnosis of \( \beta \)-thalassemia.

The HbA₂ values on the Capillaries 2 for patients with and without \( \beta \)-thalassemia trait showed an average bias (as expressed in mean values) of approximately 0.5%. The upper limit of the HbA₂ reference range (mean ± 2 SD) for the VARIANT II method was less than 3.6%, close to that previously established in this laboratory (<3.5%), and for the Capillaries 2, the upper limit of the HbA₂ reference range was established as less than 3.1%. This difference in the upper end of the reference range between the VARIANT II and Capillaries 2 methods is a reflection of the lower HbA₂ values obtained by capillary electrophoresis. There is good correlation between the methods, in agreement with the work by Mario et al.\(^14\) In contrast, Cotton et al.\(^9\) found that HbA₂ values by capillary electrophoresis were higher than those from HPLC.

Although the International Federation of Clinical Chemistry working group on the standardization of HbA₂ has made substantial progress in the preparation of calibration material for HbA₂, there is to date, unlike HbA₁c, no reference HbA₂ calibrator. Also, there is no reference method for HbA₂ quantification. A review of HbA₂ values on the 2008 CAP hemoglobinopathy survey program Hg-B (the first hemoglobinopathy survey to include Sebia Capillaries 2 results) showed a spread of between 0.31% and 0.65% in HbA₂ values in samples without a hemoglobin variant between the Bio-Rad VARIANT II and the Sebia Capillaries 2 methods, and the difference in HbA₂ values observed in this study falls within that found in the survey. HbA₂ values found in the CAP survey showed a spread between 0.31% and 0.93% between methods, indicating that there is no standardization of HbA₂ results between methods. Laboratories using the Capillaries 2 method would need to perform a reference range study before implementing the method and note that HbA₂ values generated by their laboratory may be lower than quoted in the literature for other methods.

In patients with the HbD Punjab trait, the increase in the mean (VARIANT II, 1.5%; Capillaries 2, 2.8%) and range (VARIANT II, 1.1%-1.9%; Capillaries 2, 2.0%-3.6%) of HbA₂ values between the VARIANT II and Capillaries 2 is significant (\( P < .0001 \)), with the Capillaries 2 having higher values. In every other group we studied, the HbA₂ values on the VARIANT II were higher than on the Capillaries 2. The observation by Dash\(^5\) and Cotton et al.\(^9\)
that HbA₂ is decreased owing to integration error in the baseline by HPLC methods rather than a real decrease in value in patients with the HbD Punjab trait is confirmed by this study. On the Capillaries 2, the upper limit of the reference interval for HbA₂ in patients with the HbD Punjab trait was not different from that found in patients without the β-thalassemia trait or a hemoglobin variant.

The increase in HbA₂ means (VARIANT II, 2.95 vs 3.81, Δ 0.86; Capillaries 2, 2.49 vs 3.06, Δ 0.57; P < .0001) without and with HbS is different, with the greater difference noted with the HPLC method. This finding would suggest that any interference from HbS adducts that affects the HbA₂ value in the Bio-Rad VARIANT II method and, by extension, all HPLC methods is also present, but to a lesser extent, in the Sebia Capillaries 2 capillary electrophoresis method. In contrast with the findings of Cotton et al., who found that there was no interference in HbA₂ values generated by capillary electrophoresis in patients with HbS, we found that the HPLC HbA₂ results are the same as those generated by the capillary electrophoresis method for 2 of 3 distinct groups of people with HbS and that any interference present in the Bio-Rad VARIANT II HPLC method is present in the Sebia Capillaries 2 method to a lesser extent. It is noteworthy that the HbA₂ concentrations in patients with homozygous HbS, in whom any interference due to HbS adducts would be maximized, are identical. Also, the difference in HbA₂ in patients with less than 30% HbS is smaller than that when the HbS is between 30% and 45% of the total hemoglobin.

The mean of HbA₂ values by the Capillaries 2 in patients with the HbE trait was 3.65% (range, 2.85%-4.45%). The mean and range are higher than found in patients without the β-thalassemia trait. Bain commented that HbA₂ in people with HbE is increased. This may be due to the decreased synthesis of the abnormal β-globin chain allowing for increased binding between the excess α and δ globin chains. Investigation of the CBC in these patients did not show thalassemia-associated parameters such as an increased RBC count. The mean corpuscular volume is decreased in patients with the HbE trait, and, therefore, it cannot be used to evaluate coinherited α-thalassemia. The mean HbE concentration on the Capillaries 2 was 23.91% (range, 20.41%-27.33%), which is significantly different from the mean HbE concentration of 30% (range, 27%-33%) found on the Bio-Rad VARIANT II system in previous reference range studies in our laboratory and stated by Bain to be normal. Patients with HbE concentrations less than 25% indicate the presence of coexisting α-thalassemia using current methods, and this value must be established for the Sebia Capillaries 2 system. One explanation for the lower HbE concentrations on the Capillaries 2 may be that the value of HbE on the VARIANT II represents the combined HbE and HbA₂ concentrations owing to the coelution of HbE and HbA₂, whereas on the Capillaries 2 system, HbA₂ and HbE are resolved from each other, and, therefore, the value of approximately 24% probably represents the true concentration of HbE.

On the Sebia Capillaries 2, HbA₂ and HbC are poorly separated. This is probably why there was poor agreement between results for HbA₂ in the presence of HbC, with the Bio-Rad VARIANT II providing the better result because HbA₂ is well resolved from the HbC peak.

Conclusion

The Sebia Capillaries 2 method shows similar analytic performance for HbA₂ quantification to previously published work using capillary electrophoresis, but performance is not as good as the Bio-Rad VARIANT II method. However, the precision is acceptable for the diagnosis of β-thalassemia trait. A hematopathologist who reviewed the data expressed the opinion that if an appropriate reference range for HbA₂ was quoted with all HbA₂ results produced by the Capillaries 2, there was no difference in establishing the correct diagnosis of β-thalassemia trait or coinheritance of β-thalassemia with a hemoglobin variant.

The Sebia Capillaries 2 is superior to the Bio-Rad VARIANT II for the quantification of HbA₂ in the presence of HbE and HbD Punjab. HbE concentrations in patients with the HbE trait obtained in the Capillaries 2 system are lower than from the VARIANT II and probably represent the true concentration of HbE. The Sebia Capillaries 2 offers minimal advantages over the Bio-Rad VARIANT II for the quantification of HbA₂ in the presence of HbS and is not as good for the quantification of HbA₂ in the presence of HbC.

From DynaLIFE Dx, Edmonton, Canada.

Sebia and Somagen provided the Sebia Capillaries 2 and reagents for the study without charge.

Address reprint requests to Mr Higgins: DynaLIFE Dx #200, 10150 102 St, Edmonton, Alberta, Canada T5J 5E2.

This study was performed as part of the requirements for a degree in medical laboratory technology by Ms Mack.

References


