Hematopathology / HbA2 Quantification in 2 Analytic Systems

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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Key Words: HbA2 quantification; Hemoglobin variants; High-performance liquid chromatography; HPLC; Capillary electrophoresis

DOI: 10.1309/AJCP28QKSOPHYOBC

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 Capillaries 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 Capillaries 2.

Between-day imprecision on the VARIANT II is less than that for the Capillaries 2 system. The Capillaries 2 is superior to the VARIANT II for quantifying HbA2 in the presence of HbE and HbD Punjab traits. The Capillaries 2 offers only slight advantages over the VARIANT II for quantifying HbA2 in the presence of heterozygous and homozygous HbS. The Capillaries 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.1 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.2 Studies performed in the 1970s3-6 showed poor precision for HbA2 quantification methods based on electrophoresis. Steinberg and Adams1 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 Bain7 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 increased HbA2 levels in patients with the HbD Punjab trait due to a rising baseline,8,9 falsely increased HbA2 levels in patients with HbS (both homozygous and heterozygous),10 and coelution of various hemoglobins, including HbE, Hb Osu Christianborg, HbG Coushatta, and Hb Lepore with HbA2.9 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{S} 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 HbS (homozygous and heterozygous), and with heterozygous HbD Punjab, HbE, or HbC.

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 Capillars 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 Capillars 2 system. For this reason, samples were first analyzed on the VARIANT II and then analyzed by the Capillars 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 Capillars 2 analyzers for the 4-month study period. The results are given in Table 1. 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 1.

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

Deming analysis (x = VARIANT II; y = Capillars 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>
<thead>
<tr>
<th>Table 1</th>
<th>Precision Data for the Bio-Rad Lyphocheck HbA\textsubscript{2} Control Samples for the Bio-Rad VARIANT II and Sebia Capillars 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>HbA\textsubscript{2}</td>
<td>No. of Samples</td>
</tr>
<tr>
<td>Level 1</td>
<td>87</td>
</tr>
<tr>
<td>Level 2</td>
<td>85</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 2</th>
<th>HbA\textsubscript{2} Means and Ranges for Patients With and Without β-Thalassemia Trait, HbS, HbD Punjab, HbE, and HbC on the Bio-Rad and Sebia Capillars 2 Systems</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bio-Rad VARIANT II</td>
</tr>
<tr>
<td></td>
<td>Mean (%)</td>
</tr>
<tr>
<td>Normal (n = 207)</td>
<td>2.95</td>
</tr>
<tr>
<td>β-thalassemia (n = 91)</td>
<td>5.50</td>
</tr>
<tr>
<td>HbS (n = 107)</td>
<td>3.81</td>
</tr>
<tr>
<td>HbD Punjab (n = 27)</td>
<td>1.48</td>
</tr>
<tr>
<td>HbE (n = 26)</td>
<td>NA</td>
</tr>
<tr>
<td>HbC (n = 19)</td>
<td>3.25</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; 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  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 \((x = \text{VARIANT II}; y = \text{Capillarys 2})\) between HbA\(_2\) values on the \text{VARIANT II} and \text{Capillarys 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 \text{Capillarys 2} \(\text{HbA}_2\) result to be consistently lower than the \text{VARIANT II} result in patients with heterozygous or homozygous HbS.

**HbA\(_2\) in the Presence of HbD Punjab**

Deming analysis \((x = \text{HPLC}; y = \text{Capillarys 2})\) 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 .0029. The Pearson correlation coefficient was 0.36.

The difference plot shows the \text{HbA}_2\) results on the \text{Capillarys 2} to be higher than the \text{VARIANT II} results in patients with the HbD Punjab trait with a substantial degree of scatter.

**HbA\(_2\) in the Presence of HbE**

No comparison data were available because no \text{HbA}_2\) values were available from the \text{VARIANT II} owing to coelution of \text{HbA}_2 and \text{HbE}. The \text{HbA}_2 concentrations in 2 patients homozygous for \text{HbE} were 5.8% and 5.1%.

**HbA\(_2\) in the Presence of HbC**

Deming analysis \((x = \text{HPLC}; y = \text{Capillarys 2})\) 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 \text{HbA}_2 results on the \text{VARIANT II} were 3.3% and 3.6% and on the \text{Capillarys 2} were 2.2% and 4.0%. The \text{HbA}_2 results for a patient with homozygous HbC were 6.4% and 6.0% on the \text{VARIANT II} and \text{Capillarys 2}, respectively.

The difference plot shows a substantial amount of scatter between \text{HbA}_2 results for patients with HbC trait on the \text{Capillarys 2} and the \text{VARIANT II}.

**Discussion**

The between-day imprecision (expressed as the coefficient of variation) for the \text{Capillarys 2} quantification of \text{HbA}_2 is not as good as that for the \text{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\(^{14}\) stated that the capillary electrophoresis method they developed was satisfactory for precise quantifications of high \text{HbA}_2 but provided no data. Based on 10 data points obtained on 10 days for each \text{HbA}_2 value, Cotton et al\(^{13}\) obtained a coefficient of variation of 6% at \text{HbA}_2 concentrations of 2.0%, 3.1%, and 5.6% and 3% at an \text{HbA}_2 concentration of 2.4%. The precision for \text{HbA}_2 measurements on the Sebia \text{Capillarys 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 \text{HbA}_2 values on the \text{Capillarys 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 \text{HbA}_2 reference range (mean ± 2 SD) for the \text{VARIANT II} method was less than 3.6%, close to that previously established in this laboratory (<3.5%), and for the \text{Capillarys 2}, the upper limit of the \text{HbA}_2 reference range was established as less than 3.1%. This difference in the upper end of the reference range between the \text{VARIANT II} and \text{Capillarys 2} methods is a reflection of the lower \text{HbA}_2 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 \text{HbA}_2 values by capillary electrophoresis were higher than those from HPLC.

Although the International Federation of Clinical Chemistry working group on the standardization of \text{HbA}_2 has made substantial progress in the preparation of calibration material for \text{HbA}_2, there is to date, unlike \text{HbA}_1c, no reference \text{HbA}_2 calibrator. Also, there is no reference method for \text{HbA}_2 quantification. A review of \text{HbA}_2 values on the 2008 CAP hemoglobinopathy survey program Hg-B (the first hemoglobinopathy survey to include Sebia \text{Capillarys 2} results) showed a spread of between 0.31% and 0.65% in \text{HbA}_2 values in samples without a hemoglobin variant between the Bio-Rad \text{VARIANT II} and the Sebia \text{Capillarys 2} methods, and the difference in \text{HbA}_2 values observed in this study falls within that found in the survey. \text{HbA}_2 values found in the CAP survey showed a spread between 0.31% and 0.93% between methods, indicating that there is no standardization of \text{HbA}_2 results between methods. Laboratories using the \text{Capillarys 2} method would need to perform a reference range study before implementing the method and note that \text{HbA}_2 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 (\text{VARIANT II}, 1.5%; \text{Capillarys 2}, 2.8%) and range (\text{VARIANT II}, 1.1-%1.9%; \text{Capillarys 2}, 2.0%-3.6%) of \text{HbA}_2 values between the \text{VARIANT II} and \text{Capillarys 2} is significant \((P < .0001)\), with the \text{Capillarys 2} having higher values. In every other group we studied, the \text{HbA}_2 values on the \text{VARIANT II} were higher than on the \text{Capillarys 2}. The observation by Dash\(^9\) 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.

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

### References


