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Effects of Hemoglobin C, D, E and S traits on Measurements of Hemoglobin A1c by Twelve Methods

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Abstract

Background—Hemoglobin C, D Punjab, E or S trait can interfere with hemoglobin A1c (HbA1c) results. We assessed whether they affect results obtained with 12 current assay methods.

Methods—Hemoglobin AA (HbAA), HbAC, HbAD Punjab, HbAE and HbAS samples were analyzed on one enzymatic, nine ion-exchange HPLC and two capillary electrophoresis methods. Trinity ultra² boronate affinity HPLC was the comparative method. An overall test of coincidence of least-squared linear regression lines was performed to determine if HbA1c results were statistically significantly different from those of HbAA samples. Clinically significant interference was defined as >7% difference from HbAA at 6 or 9% HbA1c compared to ultra² using Deming regression.

Results—All methods showed statistically significant effects for one or more variants. Clinically significant effects were observed for the Tosoh G8 variant mode and GX (all variants), GX V1.22 (all but HbAE) and G11 variant mode (HbAC). All other methods (Abbott Architect c Enzymatic, Bio-Rad D-100, Variant II NU and Variant II Turbo 2.0, Menarini HA-8180T thalassemia mode and HA-8180V variant mode, Sebia Capillarys 2 and Capillarys 3) showed no clinically significant differences.

Conclusions—Several methods showed clinically significant interference with HbA1c results from one or more variants which could adversely affect patient care.

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Conflicts of Interest Statement

Keywords
Hemoglobin A1c; Hemoglobin Variants; Diabetes; HPLC; Capillary Electrophoresis; Immunoassay

1. Introduction
Hemoglobin A$_{1c}$ (HbA$_{1c}$) is an important indicator of mean glycemia in patients with diabetes that has been shown to be strongly predictive of diabetes complications (1-2). More recently it has been recommended for use in diagnosing diabetes (3). Common hemoglobin variants have been shown to interfere with HbA1c results from some assay methods (4). Clinically significant interferences may result in either over-treatment leading to hypoglycemia or under-treatment leading to hyperglycemia and heightened risk of diabetes complications. Interferences and can also impact the diagnosis of diabetes. In the U.S. and worldwide hemoglobin S is the most common hemoglobin variant; the prevalence is estimated to be 6-8% in African Americans and as high as 40% in parts of central Africa (5-6). Hemoglobin C is second most common in the U.S. followed by E and D Punjab; worldwide hemoglobin E is second most common, followed by C and D Punjab. It is therefore important to identify which methods show interference from one or more of these variants by evaluating new methods, and periodically re-evaluating existing methods where the effects of potential interferences may have changed due to reagent lot changes and/or software revisions. Here, we evaluate 12 current HbA1c assay methods for potential interference from heterozygous hemoglobin S (HbAS), C (HbAC), E (HbAE) and D Punjab (HbAD).

2. Materials and Methods
2.1. Samples
EDTA whole blood specimens from subjects without hemoglobin variants (HbAA) and with HbAS, HbAC, HbAE and HbAD, both with and without diabetes, were analyzed by the test and comparative methods. Aliquots of each sample were shipped on dry ice to designated laboratories and manufacturers for analysis. Due to logistical limitations, not all samples could be analyzed by all methods. Samples were obtained with IRB approval from Emory University in Atlanta, GA, the approval of the ethics review committee at DyanLIFE DX in Edmonton, Canada, and according to the principles of the Helsinki Declaration at Queen Beatrix Hospital, Winterswijk, The Netherlands.

2.2. Assay Methods
Assay methods evaluated included the Architect c Enzymatic assay (Abbott Diagnostics), Menarini HA-8180T thalassemia and HA-8180V variant modes running software version EU 1.41 (Menarini Diagnostics), D-100, Variant II NU and Variant II Turbo 2.0 (Bio-Rad Laboratories), G8 variant mode running software version 5.20, GX, GX V1.22 and G11 variant mode (Tosoh Bioscience) and Sebia Capillaries 2 Flex Piercing and Capillaries 3 Tera (Sebia). The Architect c Enzymatic is a new enzymatic assay for the Architect analyzers. The Sebia Capillaries 2 and Capillaries 3 are capillary electrophoresis (CE) methods, the
other methods are ion-exchange HPLC. Analyses were performed by the respective manufacturers with the exception of the Architect c Enzymatic assay and Variant II Turbo 2.0 (Department of Clinical Biochemistry, Toronto General Hospital, ON, Canada), the HA-8180T and HA-8180V (Queen Beatrix Hospital, Winterswijk, The Netherlands) and the Capillars 2 (Diabetes Diagnostic Laboratory, University of Missouri, Columbia, MO USA). The Trinity ultra2 boronate affinity HPLC (Trinity Biotech) at the University of Missouri-Columbia was used as the comparative method since it has been shown to be generally unaffected by hemoglobin variants (7-8). Hemoglobin variants were initially identified by ion-exchange HPLC and/or electrophoresis at the institutions where the samples were collected, and also presumptively identified using the Tosoh G7 beta thal mode (Tosoh Bioscience) and Sebia Capillars 2 Flex Piercing Hemoglobin(e) method (Sebia) at the University of Missouri-Columbia. Presumptive identification was based upon expected elution or migration time and variant peak proportion.

2.3. Data Analyses

For each method an overall test of coincidence of least-squared linear regression lines was used to determine if results for each variant were statistically significantly different (P<0.05) from those of HbAA samples. Deming regression was used to determine if the bias for each variant versus HbAA was clinically significant at 6% (42 mmol/mol) or 9% (75 mmol/mol) HbA1c; clinical significance was defined as a difference exceeding ±7% (4). In IFCC units (mmol/mol), clinical significance was defined as a difference exceeding ±11.0% at 42 mmol/mol or ±9.2% at 75 mmol/mol; these limits are equivalent to ±7% at 6 and 9% HbA1c in National Glycohemoglobin Standardization Program (NGSP)/Diabetes Control and Complications Trial (DCCT) units due to the intercept in the IFCC/NGSP master equation (9). Data analyses were performed using SAS 9.4 (SAS Institute Inc.) and Graphpad Prism 5.0 (Graphpad Software Inc).

2.4. Manufacturer Claims

For the Architect c Enzymatic assay, HA-8180T thalassemia mode, HA-8180V variant mode, D-100, Variant II NU, Variant II Turbo 2.0, GX V1.22, Capillars 2 and Capillars 3 the manufacturers claim no interference from any of the variants tested. Tosoh claims no interference from HbC, HbD or HbS for the G8 variant mode and GX; since they acknowledge interference from all four variants in the case of the G8 standard mode and G9, data from these methods were not included. Manufacturer claims for the G11 variant mode were not available at the time of this writing as it is not yet commercially available.

3. Results

3.1. Statistical Significance

Fig. 1 shows boxplots of the differences versus ultra2 for HbAA and each variant for each method and indicates both statistically and clinically significant differences versus HbAA. The Architect c enzymatic assay showed statistically significant interference for HbAD and HbAE. The D-100, Variant II NU and Variant II Turbo 2.0 showed statistically significant differences for HbAS, HbAE and HbAD; the HA-8180V variant mode and HA-8180T showed statistically significant differences for HbAD. The G8 variant mode, GX, and G11
variant mode showed statistically significant differences for all variants; for the GX V1.22 all differences were statistically significant with the exception of HbAE. The Capillarys 2 showed statistically significant differences for HbAS and HbAD, while the Capillarys 3 showed a statistically significant difference for HbAE.

3.2. Clinical Significance

Table 1 shows the actual biases for each variant versus HbAA at 6 and 9% HbA1c (supplementary table 2 shows the biases in IFCC units at 42 and 75 mmol/mol) and along with Fig. 1 indicates which differences were clinically significant. The Architect c enzymatic, HA-8180T thalassemia mode, HA-8180V variant mode, D-100, Variant II NU, Variant II Turbo 2.0, Capillarys 2 and Capillarys 3 methods showed no clinically significant interference from any of the variants tested. The G8 variant mode and GX showed clinically significant interference from all variants. The GX V1.22 showed clinically significant interference from all except HbE; the G11 variant mode showed clinically significant interference from HbAC. Importantly, all four of the Tosoh methods are ion-exchange HPLC where inspection of the chromatogram reveals the presence of the variant.

4. Discussion

While most of the methods evaluated did not show clinically significant interference with HbA1c results in the presence of the tested variants, the four Tosoh ion-exchange HPLC methods all showed interference from one or more variants. Two methods, the G8 variant mode and GX, showed interference from all 4 variants. This can be a significant issue both in terms of clinical management and diagnosis of diabetes, especially in populations where there is a significant prevalence of one or more of these variants. As with the other ion-exchange HPLC and the Sebia CE methods, the presence of these variants can be detected by examination of the chromatograms or electropherograms and are likely to be flagged by the instrument. However, for the Tosoh methods our findings contradicted manufacturer claims in some cases, meaning that following manufacturer instructions could lead to reporting of erroneous results. The manufacturer claims no interference from HbAC, HbAD or HbAS for the G8 variant mode and GX, yet we found clinically significant interference from these variants with both methods at the 9% HbA1c level. Interestingly, we previously found no clinically significant interference with the G8 variant mode from these three variants (4). Conversely, the HA-8180V variant mode previously showed interference from HbD and HbE (4), but the manufacturer has made improvements to the method and our data confirm that with the latest version of the software (EU 1.41) there is no longer clinically significant interference. We have previously observed variations in susceptibility to variant interferences over time with ion-exchange HPLC methods, presumably due to changes in software versions or reagent lots (10). Moreover, software versions and reagent lots are not always consistent across geographic regions (e.g. the U.S. versus Europe), thus a method may show regional differences in susceptibility to variant interference. The GX V1.22 is an update to the GX that according to the manufacturer was designed to eliminate significant interference from HbAE, our findings confirm this but contradictory to manufacturer claims we still found clinically significant interference from the other three variants.
The Abbott Architect c enzymatic method did not show interference from any of the variants evaluated; this is fortunate as the presence of variants cannot be detected with this method. However, as with immunoassay and boronate affinity methods, less common variants that can affect HbA1c results due to altered erythrocyte lifespan (e.g. homozygous hemoglobin S) or altered binding of glucose to hemoglobin (e.g. hemoglobin Raleigh) also cannot be detected. Regardless of the assay method used to measure HbA1c, the possibility of interference from an undetected variant may need to be considered if a HbA1c result does not match clinical impression based on other parameters (e.g. self-monitored blood glucose). In summary, several HbA1c methods tested showed clinically significant interference from one or more of the four most common hemoglobin variants. These interferences could result in over or under-treatment of glycemia for diabetes patients with one of these variants, potentially leading to sustained hyperglycemia or hypoglycemic episodes.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**Acknowledgements**

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**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CE</td>
<td>Capillary Electrophoresis</td>
</tr>
<tr>
<td>HbA1c</td>
<td>Hemoglobin A1c</td>
</tr>
<tr>
<td>HbAA</td>
<td>Hemoglobin AA</td>
</tr>
<tr>
<td>HbAD</td>
<td>Hemoglobin AD Punjab</td>
</tr>
<tr>
<td>HbAE</td>
<td>Hemoglobin AE</td>
</tr>
<tr>
<td>HbAS</td>
<td>Hemoglobin AS</td>
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<tr>
<td>NGSP</td>
<td>National Glycohemoglobin Standardization Program</td>
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<tr>
<td>DCCT</td>
<td>Diabetes Control and Complications Trial</td>
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</table>

**References**


### Highlights

- Hemoglobin variants can affect hemoglobin A1c results
- Twelve HbA1c methods were tested for interference from HbC, D, E and S traits
- Four methods showed clinically significant interference from one or more variants
- These clinically significant differences could adversely impact patient care.
Fig. 1. Box-plots summarizing the absolute differences (%HbA1c and mmol/mol HbA1c) between each test method and the comparison method for HbAA, HbAC, HbAD, HbAE and HbAS

The horizontal line in each box is the median difference between the test and comparison methods. The upper and lower limits of each box correspond to the 25th and 75th percentile of the differences, respectively. The highest and lowest horizontal bars represent the minimum and maximum differences between the test and comparison methods. Differences from HbAA that are statistically significant are indicated (#) below each bar where appropriate; clinically significant differences are indicated (*) above each bar where appropriate.
Table 1

Mean differences between test and comparative methods.\(^a\)

<table>
<thead>
<tr>
<th>Method</th>
<th>HbAA</th>
<th>HbC trait</th>
<th>HbD Punjab trait</th>
<th>HbE trait</th>
<th>HbS trait</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>6% HbAlc</td>
<td>9% HbAlc</td>
<td>n</td>
<td>6% HbAlc</td>
</tr>
<tr>
<td><strong>Enzymatic</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Architect</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enzymatic</td>
<td>25</td>
<td>55</td>
<td>-0.01</td>
<td>9% HbAlc</td>
<td>41</td>
</tr>
<tr>
<td><strong>Ion-Exchange</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>HPLC</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HA-8180T</td>
<td>39</td>
<td>55</td>
<td>-0.07</td>
<td>-0.15</td>
<td>41</td>
</tr>
<tr>
<td>HA-8180V</td>
<td>39</td>
<td>55</td>
<td>0.07</td>
<td>0.06</td>
<td>41</td>
</tr>
<tr>
<td>D-100</td>
<td>39</td>
<td>55</td>
<td>0.01</td>
<td>-0.02</td>
<td>41</td>
</tr>
<tr>
<td>Variant II NU</td>
<td>39</td>
<td>55</td>
<td>-0.02</td>
<td>-0.14</td>
<td>41</td>
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<tr>
<td><strong>Capillary Electrophoresis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Capillaries 2</td>
<td>54</td>
<td>55</td>
<td>-0.09</td>
<td>0.12</td>
<td>41</td>
</tr>
<tr>
<td>Capillaries 3</td>
<td>33</td>
<td>41</td>
<td>-0.08</td>
<td>0.03</td>
<td>37</td>
</tr>
</tbody>
</table>

\(^a^{Deming regression analysis was performed using ultra\(^2\) as the comparative method. The biases (%HbA\(_1\)c) for each method at clinical decision cutoffs of 6% and 9% HbA\(_1\)c were calculated for each variant. To correct for intermethod calibration differences, the mean biases between each test method and the comparative method for homozygous HbA samples was subtracted from those calculated for the variant samples.\(^b\)

\(^{b}\)Clinically significant differences (>0.42% or >0.63% HbA\(_1\)c at 6% or 9% HbA\(_1\)c, respectively).