Screening for Hemoglobinopathies During Routine Hemoglobin A1c Testing Using the Tosoh G7 Glycohemoglobin Analyzer

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Abstract. Approximately 5.1% of the US population has diabetes mellitus, and hemoglobin (Hb) A1c levels are routinely measured to monitor long-term glycemic control in these patients. Many laboratories use ion exchange chromatography for such measurements, and the presence of hemoglobin variants and hemoglobinopathies often results in abnormal peaks on the chromatogram. The goal of this study was to evaluate the potential that detection of these abnormal peaks provides as a screening tool for Hb variants and hemoglobinopathies. We examined 366 specimens with abnormal peaks observed during routine Hb A1c measurements using the G7 Glycohemoglobin Analyzer (Tosoh Bioscience, Inc.). Hb variants and hemoglobinopathies were characterized by alkaline and acid electrophoresis, solubility testing for Hb S, and clinical parameters. In 252 cases, sickle cell trait was identified with a mean retention time (RT) of 1.44 (SD ±0.02) min. In 82 cases, Hb C trait was identified with a mean RT of 1.66 ±0.03 min. RTs for other Hb abnormalities, including sickle cell disease, homozygous Hb C disease, C Harlem trait, α-chain Hb variants, Hb D trait, Hb G trait, Hb J trait, Hb Raleigh, and Hb Lepore were also determined. Our results demonstrate that routine Hb A1c testing provides a potential screening tool for the detection of common hemoglobin variants and hemoglobinopathies. The previously unreported RTs for the G7 Glycohemoglobin Analyzer are provided, which can facilitate further testing in previously undiagnosed patients and confirm the cause of abnormal peaks in patients with known hemoglobin abnormalities.

Keywords: glycohemoglobin, hemoglobinopathy, hemoglobin A1c

Introduction

Blood glycohemoglobin A1c is routinely measured to monitor long-term glycemic control in patients with diabetes mellitus. Several methods are available for Hb A1c analysis, (ie, ion-exchange chromatography, electrophoresis, immunoassays, and boronate affinity) [1,2]. Many of these A1c methods have the potential to detect coexisting hemoglobin (Hb) variants or hemoglobinopathies. Over 5.1% of adult Americans are diagnosed with diabetes mellitus and it is recommended that these individuals have Hb A1c levels tested every 3-6 months [3,4]. Over 2 million Hb A1c tests are performed each month in the United States [2]. Such testing has the potential to provide cost effective screening for Hb variants or hemoglobinopathies in diabetic patients. The incidence of diabetes is higher in African-American populations and these subjects are more likely to have the most common hemoglobinopathies, including sickle cell trait (Hb AS), sickle cell disease (Hb SS), Hb C trait (Hb AC), and Hb C disease (Hb CC). It is estimated that approximately 10% of African Americans have one of these hemoglobinopathies, and that >335,000 diabetic persons have either Hb AS or Hb AC [2,5].
High performance liquid chromatography (HPLC) is commonly used to measure Hb A1c, and several manufacturers offer instruments for this purpose. Previous studies have investigated the potential of diagnosing Hb variants and hemoglobinopathies using HPLC techniques. However, these studies did not analyze specimens submitted for Hb A1c testing (most were part of newborn screening procedures), and they either used HPLC instruments not designed for Hb A1c monitoring or utilized the β-thalassemia mode programmed into the instrument [6-11]. Testing in the β-thalassemia mode is more time-consuming and thus is not routinely used for Hb A1c screening. We report the utility of routine Hb A1c monitoring as a screening tool for detecting Hb variants using the G7 Glycohemoglobin Analyzer (Tosoh Bioscience, Inc., South San Francisco, CA). Parameters are established to guide further testing for common Hb variants and hemoglobinopathies.

Materials and Methods

This research protocol was approved by the James A. Haley VA Medical Center’s Research and Development Committee and by the University of South Florida’s Institutional Review Board.

Between October 2004 and October 2006, 366 specimens analyzed for Hb A1c levels were identified at our institution as having abnormal peaks on the HPLC chromatograms. Samples were collected in Vacutainer tubes (Becton-Dickinson) containing EDTA. They were then processed on the G7 Glycohemoglobin Analyzer in the 2.2 min Variant Analysis mode, which has been certified by the National Glycohemoglobin Standardization Program (NGSP). Briefly, this analyzer utilizes non-porous ion exchange HPLC with step gradient elutions to separate Hb fractions with cation exchange groups in the column resin surface. As each Hb component is eluted, it is quantified by changes in light absorbance at 415 nm. The G7 has three modes for testing, including the 1.2 min Standard Analysis mode (used predominantly outside of the USA), the 2.2 min Variant Analysis mode (used routinely in the USA for A1c testing), and the 7.5 min β-thalassemia mode. Expected retention peaks in the Variant mode in order of elution represent glycohemoglobins A1a and A1b, Hb F, LA1c+ (representing labile Hb A1c, or the initial binding of glucose to valine in the N-terminus of the Hb β chain), SA1c (stable Hb A1c, the ketoamine form), and A0 for Hb A. Peaks after A0 that represent >5% of the total area are designated H-V0, H-V1, or H-V2, based on the retention times (RTs) and a complex algorithm specific for this instrument. Peaks eluted before A0 that do not fall within an expected RT are designated P00, P01, or PX [12]. Specimens included for this study met the guidelines for acceptability provided by the manufacturer.

All specimens with a variant Hb peak were further evaluated with both acid and alkaline gel electrophoresis. These tests were performed with a SPIFE 3000 Gel Electrophoresis Analyzer (Helena Laboratories) according to the manufacturer’s recommendations. Specimens were also tested for the presence of a sickling Hb variant using the Pacific Hemostasis Sickling Hemoglobin Screening Kit (SickleScreen, Fisher Scientific Co.). Briefly, red blood cells were lysed using surfactant and the released Hb was reduced with sodium hydrosulfite and tested for insolubility in concentrated phosphate solutions.

Results

Data for all specimens are summarized in Table 1. The majority of the specimens (n = 252, 69%) were consistent with Hb AS. Diagnostic criteria for Hb AS included expected migration patterns on acid and alkaline electrophoresis and positive Sickle-Screen testing. The mean RT was 1.44 (SD ±0.02) min. An H-V-0 peak was seen in all but 3 specimens, which had HV-0 peaks. These 3 cases occurred in the same month, suggesting either a problem with a specific reagent batch or a calibration error. Electrophoresis did not demonstrate a coexisting additional Hb abnormality in these cases, although it is possible a silent second mutation was present. As expected, RTs for the 2 cases (0.55%) of Hb SS were 1.42 and 1.44 min, similar to Hb AS.

Hb AC (n = 82, 22%) was the second most common hemoglobinopathy in this study. Diagnostic criteria included expected migration patterns on acid and alkaline electrophoresis and
Two cases of Hb C Harlem trait were identified (a father and son). Sickle solubility testing was positive in each case. The electrophoresis migration patterns, which are unique to Hb C Harlem, were consistent with this diagnosis [13]. Hb C Harlem is a rare β-chain variant that results from 2 amino acid substitutions, one identical to Hb S and the other identical to Hb Korle-Bu [13]. There is increased propensity for sickling in this disorder due to the Hb S mutation. As expected, the RTs (1.50, 1.52) were between those for Hb C and Hb S [13].

<table>
<thead>
<tr>
<th>Hb variant</th>
<th>N</th>
<th>Mutation (7)(8)</th>
<th>Migration pattern (7)(8)</th>
<th>Sickle test</th>
<th>Mean RT</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb AS</td>
<td>252</td>
<td>β 6Glu→Val</td>
<td>Alkaline/Acid: A + S position</td>
<td>+</td>
<td>1.44</td>
<td>0.02</td>
</tr>
<tr>
<td>Hb SS</td>
<td>2</td>
<td>β 6Glu→Val</td>
<td>Alkaline/Acid: S position</td>
<td>+</td>
<td>1.43</td>
<td>-</td>
</tr>
<tr>
<td>Hb AC</td>
<td>82</td>
<td>β 6Glu→Lys</td>
<td>Alkaline: A + A₂ Acid: A + C</td>
<td>-</td>
<td>1.66</td>
<td>0.03</td>
</tr>
<tr>
<td>Hb CC</td>
<td>2</td>
<td>β 6Glu→Lys</td>
<td>Alkaline: A₂ Acid: C</td>
<td>-</td>
<td>1.67</td>
<td>-</td>
</tr>
<tr>
<td>Hb SC</td>
<td>1</td>
<td>β 6Glu→Val</td>
<td>Alkaline: S + A₂ Acid: S + C</td>
<td>+</td>
<td>1.40, 1.62</td>
<td>-</td>
</tr>
<tr>
<td>Hb C Harlem trait</td>
<td>2</td>
<td>β 6 Val, 73Asn</td>
<td>Alkaline: C + S Acid: S</td>
<td>+</td>
<td>1.51</td>
<td>-</td>
</tr>
<tr>
<td>α chain variant 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1</td>
<td>α 75Asp→Tyr</td>
<td>Alkaline: anodal to S Acid: A</td>
<td>-</td>
<td>1.32</td>
<td>-</td>
</tr>
<tr>
<td>α chain variant 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4</td>
<td>Various</td>
<td>Alkaline: A + S Acid: anodal to S</td>
<td>-</td>
<td>1.52</td>
<td>0.02</td>
</tr>
<tr>
<td>D trait&lt;sup&gt;c&lt;/sup&gt;</td>
<td>9</td>
<td>β 121Glu→Gln</td>
<td>Alkaline: A + S Acid: A</td>
<td>-</td>
<td>1.26</td>
<td>0.05</td>
</tr>
<tr>
<td>G trait&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4</td>
<td>α 68Asn→Lys</td>
<td>Alkaline: A + S Acid: A</td>
<td>-</td>
<td>1.27</td>
<td>0.05</td>
</tr>
<tr>
<td>Hb J variant (Baltimore)</td>
<td>4</td>
<td>β 16Gly→Asp</td>
<td>Alkaline: Fast migration Acid: A</td>
<td>-</td>
<td>1.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Hb Raleigh</td>
<td>2</td>
<td>β 1Val→ac-Ala</td>
<td>Alkaline: A Acid: A + F</td>
<td>-</td>
<td>0.66</td>
<td>-</td>
</tr>
<tr>
<td>Hb Lepore</td>
<td>1</td>
<td>Fusion, β and δ genes</td>
<td>Alkaline: S Acid: A</td>
<td>-</td>
<td>Not detected</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup> Consistent with Hb Winnipeg.
<sup>b</sup> Consistent with Hb Hasharon, Titusville, Memphis, or Setif.
<sup>c</sup> Differentiated on laboratory/clinical parameters.
Hb D and Hb G are β- and α-globin variants, respectively. Both typically migrate to the Hb A position on acid agar and to the Hb S position on alkaline agar. Although often difficult to identify, Hb G also has an Hb G₂ peak seen near the carbonic anhydrase position on alkaline electrophoresis [13]. Typically, these two Hb variants are distinguished based on clinical parameters and laboratory values including the relative percentage of the Hb variant present. Average RTs for Hb D (n = 9) and Hg G (n = 4) variant traits were 1.26 and 1.27, respectively.

Five additional alpha chain variants were identified. Based on acid and alkaline electrophoresis results and clinical criteria, one case was consistent with Hb Winnipeg. The other 4 cases were not specifically identified, but based on their electrophoretic mobility were consistent with Hb Hasharon, Titusville, Memphis, or Setif. Solubility testing for Hb S was negative in each case. Their RTs are listed in Table 1.

Two variants were identified that are known to falsely elevate Hb A₁c levels measured with HPLC. Sickle solubility testing was negative in these cases. Hb J variants (Hb J Baltimore being most common) typically affect the β globin chain resulting in fast migration on alkaline electrophoresis [13]. Our Hb J variant cases (n = 4) produced PO peaks between 1.00-1.02. Hb Raleigh is also a β globin chain variant. Our cases of Hb Raleigh (n = 2) demonstrated unusually large peaks with RTs of 0.65 (LA1C+ peak, 46.7%) and 0.66 (SA1C peak, 54.7%).

One case of Hb Lepore was evaluated for Hb A₁c analysis. Hb Lepore represents a fusion between the β and δ genes [13]. On alkaline electrophoresis it migrates with Hb S, but it does not migrate in the Hb S window on HPLC. Our case was detected initially with Hb electrophoresis, and Hb A₁c testing was done out of curiosity. The HPLC chromatogram was normal. As expected, Hb A₁c screening would not have detected this case of Hb Lepore.

Discussion

In this study, we demonstrated that HPLC testing for Hb A₁c provides a reliable screening tool for the most common hemoglobinopathies and Hb variants. Furthermore, we report previously unpublished RTs for variant peaks for the G7 Glycohemoglobin Analyzer in the Variant mode, which is routinely used in the USA for Hb A₁c testing. Previous investigations have demonstrated the benefits of HPLC to diagnose hemoglobinopathies. One study, utilizing the Bio-Rad Variant II in β-thalassemia mode to diagnose Hb variants and hemoglobinopathies, concluded that in approximately 75% of cases confirmatory alkaline and acid electrophoresis was unnecessary [8], although others have questioned this conclusion [14]. As there are over 900 Hb variants currently known, definitive diagnosis with HPLC alone would be difficult in some cases [15]. However, this fact is also true for differentiating many hemoglobinopathies using alkaline and acid electrophoresis. While some hemoglobin variants would be silent with Hb A₁c testing, as was Hb Lepore in this study, the common variants yielded reproducible abnormal peaks. We have shown that the RTs for Hb S, Hb C, common alpha chain variants, Hb D and G variants (combined), Hb J variants, and Hb Raleigh demonstrate little or no overlap.

Previous studies examined the influence of hemoglobinopathies and Hb variants on the A₁c test results [1,2,5]. Certainly, it is important to determine if these abnormalities affect the Hb A₁c measurements. However, we are unaware of investigations describing the additional abnormal peaks produced by Hb variants during A₁c testing. The G7 analyzer and similar instruments are currently not approved as diagnostic tools for hemoglobinopathies. However, the abnormal retention peaks detected during routine Hb A₁c monitoring should not be ignored in patients without a previous diagnosis of a Hb variant or hemoglobinopathy. Our results establish a basis for referral of individuals for further testing when abnormal peaks are identified. Furthermore, the RTs presented can be used to corroborate abnormal peaks detected in patients with known Hb variants and hemoglobinopathies. Future investigations should focus on what specific additional testing might be indicated to establish a diagnosis of each specific Hb variant/hemoglobinopathy based on the values established in this report and the patient's
clinical history. Also, tests should be made to
determine any additional diagnostic benefits by
using the G7 analyzer in the β-thalassemia mode
compared to the Variant mode described here.

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