Interference With Hemoglobin A\textsubscript{1c} Determination by the Hemoglobin Variant Shelby

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Key Words: Hemoglobin Shelby; Hemoglobin A\textsubscript{1c}; Hemoglobin variant; Hemoglobinopathy; Boronate affinity; High-performance liquid chromatography; Mass spectrometry

DOI: 10.1309/WPY5UHR424VUHG8A

Abstract

Hemoglobin variant carrier status was found in a 46-year-old African American man following detection of a falsely elevated hemoglobin A\textsubscript{1c} (HbA\textsubscript{1c}) by ion-exchange high-performance liquid chromatography (HPLC, VARIANT A\textsubscript{1c}, Bio-Rad Laboratories, Hercules, CA). Additional analysis of the hemoglobin variant using the Beta Thal Short program (Bio-Rad) revealed an unknown peak with a retention time of 4.84 minutes and a proportion of 26.3%. No mass shift in \(\alpha\)-globin or \(\beta\)-globin proteins was observed by mass spectrometry. DNA sequencing revealed a missense mutation in 1 \(\beta\)-globin allele corresponding to the hemoglobin Shelby trait. The patient was asymptomatic with a normal hemoglobin value of 13.6 g/dL (136 g/L) but had increased target cells on a peripheral blood smear. An alternative method for HbA\textsubscript{1c} determination using boronate-affinity HPLC provided a value of 3.9% (0.04; reference range, 4.0%-6.9% [0.04-0.07]), more consistent with the patient’s recent blood glucose values in the normal range.

Case Report

An asymptomatic 46-year-old African American man with normal CBC count results came to our medical center for a routine checkup. He had no history of diabetes mellitus but had borderline hypertension and a 30-year smoking history of 7 cigarettes per day. He took no medication and had quit smoking 8 months before the current visit. There was no family history of anemia and no known hemoglobinopathies. The patient felt well and consistently walked his dog 3 times a week for at least 20 minutes each time, with no fatigue or dyspnea. He was single with no children.

Determination of glycated hemoglobin (HbA\textsubscript{1c}) is routinely used to monitor long-term glycemic control in patients with known diabetes mellitus and may be used as part of a diagnostic screening panel in patients at risk for developing diabetes.\textsuperscript{1} A number of hemoglobin variants are known to interfere with HbA\textsubscript{1c} determination by ion-exchange high-performance liquid chromatography (HPLC), leading to falsely high or low HbA\textsubscript{1c} values. The use of boronate-affinity HPLC has been demonstrated as helpful in monitoring blood glucose control in patients with hemoglobin variants. This report emphasizes the need to correlate laboratory findings with associated clinical parameters and to question results that do not seem reasonable.
(HPLC, VARIANT A\textsubscript{1c}, Bio-Rad Laboratories, Hercules, CA). This method measures the stable ketoamine fraction of hemoglobin formed by the Amidori rearrangement of the labile Schiff base form, which is glycated at amino-terminal valine residues on \(\beta\)-globin chains. The chromatograph displayed a markedly elevated HbA\textsubscript{1c} peak corresponding to 12.9\% (0.13; reference range, 4.8\%-6.2\% [0.05-0.06]) and a split in the hemoglobin (Hb)A peak, which the interpretive software falsely proposed as representing HbA and HbS. The patient’s blood glucose levels from the previous month were in the normal range (96 and 89 mg/dL [5.3 and 4.9 mmol/L]; reference range, 70-110 mg/dL [3.9-6.1 mmol/L]). These values suggested an erroneous HbA\textsubscript{1c} determination because the reported level of 12.9\% (0.13) correlates with a mean plasma glucose value of more than 345 mg/dL (19.5 mmol/L) Table 1. The test was repeated using boronate-affinity HPLC (ARUP Laboratories, Salt Lake City, UT). The boronate-affinity method measures glycated hemoglobin based on the interaction of \(\text{cis}\)-glycols with boronic acid, regardless of the glycation site. The boronate-affinity analysis provided a more clinically plausible value of 3.9\% (0.04; normal range, 4.0\%-6.9\% [0.04-0.07]) albeit, somewhat lower than what would be expected based on the random glucose results already stated.

**Hemoglobin Variant Detection by HPLC**

To further elucidate the nature of the split HbA peak seen on the HbA\textsubscript{1c} chromatography, HPLC analysis was conducted using a longer ion-exchange column, which allows resolution of a variety of hemoglobin variants (VARIANT Beta Thal Short, Bio-Rad). The chromatograph displayed an “unknown” peak constituting 26.3\% of the total signal with a retention time of 4.84 minutes. Also noted was an elevated HbA\textsubscript{2} peak constituting 5.1\% of the signal Figure 2.

**Liquid Chromatography–Mass Spectrometry**

Liquid chromatography–mass spectrometry performed on a Finnigan MAT LCQ (Thermo Finnigan, San Jose, CA) using electrospray ionization showed \(\alpha\)-globin and \(\beta\)-globin protein peaks with molecular masses of 15,126 and 15,867 atomic mass units, respectively, isobaric with the normal hemoglobin constituents Figure 3.

**RBC Indices and Peripheral Smear**

RBC indices averaged for 7 measurements Table 2 were essentially normal with the exception of a borderline low RBC count and an increased number of reticulocytes. The patient’s peripheral blood smear showed a marked increase in the number of target cells Image 1.

**DNA Sequencing**

The complete protein coding sequence of \(\beta\)-globin with exon/intron boundaries, the proximal promoter, 5’ and 3’ untranslated regions, and intronic mutations IVS-II-654, IVS-II-705, and IVS-II-745 (common loci for \(\beta\)-thalassemia mutations) of the \(\beta\)-globin gene were sequenced bidirectionally using dye-terminator chemistry (ABI, Applied Biosystems, Foster City, CA) at ARUP Laboratories. The sample was heterozygous for a nucleic acid missense mutation of CAG to AAG at codon 131, HbA\textsubscript{1c}, glycated hemoglobin.
which confers a glutamine to lysine amino acid substitution known as Hb Shelby.5

**Discussion**

Many hemoglobin variants are known to affect HbA1c determination as measured by a number of different methods, including HPLC, isoelectric focusing, electrophoresis, and immunoassay, with falsely low and falsely high values reported.6,8 For example, common hemoglobin variants such as those found in patients with clinically silent HbS, HbC, or HbE carrier status are known to interfere with some HbA1c measurements.6

Use of charge-based quantification methods such as HPLC, isoelectric focusing, and electrophoresis leads to errors in HbA1c calculations when variant hemoglobins, and/or their adducts, coelute or comigrate with HbA or HbA1c. Immunoassays use antibodies targeted toward the glycated amino terminus of the β-globin chain to determine HbA1c levels. Although this approach overcomes charge-based interference, erroneous

**Table 2**

RBC Indices in a Hemoglobin Variant Carrier*

<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>RBC count (×10^6/µL)</td>
<td>4.46</td>
<td>4.73</td>
<td>4.42</td>
<td>4.64</td>
<td>4.85</td>
<td>4.68</td>
<td>4.57</td>
<td>4.6 (0.15)</td>
<td>4.7-5.6</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>13.5</td>
<td>13.6</td>
<td>13.0</td>
<td>13.7</td>
<td>14.4</td>
<td>13.6</td>
<td>13.1</td>
<td>13.6 (0.46)</td>
<td>12.4-17.4</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>40.3</td>
<td>43.8</td>
<td>41.2</td>
<td>40.6</td>
<td>42.0</td>
<td>40.6</td>
<td>39.5</td>
<td>41.1 (1.40)</td>
<td>39-52</td>
</tr>
<tr>
<td>MCV (µm^3)</td>
<td>90.3</td>
<td>92.7</td>
<td>93.2</td>
<td>87.5</td>
<td>86.6</td>
<td>86.9</td>
<td>86.6</td>
<td>89.1 (2.92)</td>
<td>83-93</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>30.2</td>
<td>28.8</td>
<td>29.3</td>
<td>29.5</td>
<td>29.7</td>
<td>29.0</td>
<td>28.7</td>
<td>29.3 (0.53)</td>
<td>26-31</td>
</tr>
<tr>
<td>MCHC (g/dL)</td>
<td>33.4</td>
<td>31.1</td>
<td>31.5</td>
<td>33.8</td>
<td>34.2</td>
<td>33.4</td>
<td>33.2</td>
<td>32.9 (1.17)</td>
<td>32-34</td>
</tr>
<tr>
<td>RDW (%)</td>
<td>14.0</td>
<td>14.5</td>
<td>14.3</td>
<td>14.7</td>
<td>14.5</td>
<td>15.8</td>
<td>14.5</td>
<td>14.6 (0.57)</td>
<td>13-15</td>
</tr>
<tr>
<td>Reticulocyte count (%)</td>
<td>ND</td>
<td>ND</td>
<td>3.1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>3.1 (—)</td>
<td>0.0-1.8</td>
</tr>
</tbody>
</table>

MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; ND, not done; RDW, red cell distribution width.

*Values are given as conventional units; multiplication factors for Système International units are as follows: hematocrit (proportion of 1.0), 0.01; hemoglobin (g/L), 10.0; MCH (pg), 1.0; MCHC (g/L), 10.0; MCV (µL), 1.0; RBC count (×10^6/L), 1.0; reticulocyte count (proportion of RBCs), 0.01.
results arise when amino acid substitutions occur within the target epitope, eg, in HbS, HbC, and Hb Raleigh. Affinity chromatography with boronate, while providing a reliable method for estimating HbA1c, even when hemoglobinopathies are present, does not allow recognition of variant hemoglobins. Our approach is to use a charge-based method for initial HbA1c determination (HPLC), followed by boronate-affinity chromatography in cases with aberrant peaks or unreasonable HbA1c values based on average blood glucose levels. Falsely low HbA1c values are particularly important to detect and remeasure using an alternative method because they may lead to an exacerbation of complications in patients with diabetes.

In the case of Hb Shelby, the erroneously high HbA1c value seen on ion-exchange chromatography, which has not been previously reported, is likely due to coelution of Shelby adducts (eg, glycation, carbamylation, and acetylation products) with HbA1c.

In a similar manner, the increased HbA2 seen on the Beta Thal Short program is likely to result from the presence of abnormal Shelby β-globin chains rather than representing β-thalassemia trait. An analogous situation is described for HbS, in which it is thought that coelution of HbS adducts, as well as increased δ-chain synthesis (as a result of the abnormal sickle β-chains), contributes to an elevated HbA2. Had the patient in this case been a compound heterozygote for Hb Shelby and β-thalassemia, we would expect to see abnormal RBC indices such as reduced mean corpuscular hemoglobin, reduced mean corpuscular volume, and anemia, all of which were not present.

The possibility that the propositus in this case report is a silent carrier of α-thalassemia cannot be excluded. People with a single α-globin gene deletion are often clinically and hematologically unremarkable. Hb Shelby carriers, similar to people with, eg, sickle cell trait, show a reduction in the proportion of the variant hemoglobin when there is coinheritance of α-thalassemia. Studies by Felice et al used radiolabeled globin chains demonstrated a negative correlation between average Hb Shelby proportions and α-chain abundance. Based on their findings, Hb Shelby carriers were stratified into low (11.4%), intermediate (21.7%), and high (33.3%) categories based on their percentage of Hb Shelby, which corresponds to their presumed α-region genotypes −α/−α, −α/αα, and αα/αα, respectively. In the present case, the patient’s Hb Shelby percentage was 26.3%, which may indicate a silent component of α-thalassemia because values as high as 33.8% have been reported for simple heterozygotes.

Characterization of hemoglobin variants by HPLC (based on retention time, peak characteristics, and quantity) and mass spectrometry (based on mass shift) may be used, but both methods have limitations. More than 900 hemoglobin variants are described (http://globin.bx.psu.edu/hbvar/menu.html), many of which are incompletely characterized with regard to their HPLC profiles. In this case, HbS was ruled out by a negative sickle cell solubility test and its distinct retention time (4.5 minutes for HbS vs 4.84 minutes for the present variant). Three hemoglobin variants O-Indonesia, O-Arab, and Hasharon with similar retention times were considered. The reference peaks for O-Indonesia and O-Arab provided by the VARIANT library (Bio-Rad Laboratories, version 3.0) displayed distinct conformational differences (slender peak bases vs a broad peak base) and associated glycation products not observed in our patient. Hb Hasharon was ruled out by mass spectrometry because this variant would have shown a 22 mass unit shift of the α-globin protein due to an aspartate to histidine amino acid substitution. The variant present in this case showed no shift by mass spectrometry, illustrating how certain hemoglobin variants can be overlooked by this method despite amino acid substitutions.

DNA sequencing is the “gold standard” to pinpoint the exact nature of low-incidence hemoglobin variants. In this case, sequencing revealed a missense mutation of CAG to AAG at codon 131 in one of the β-globin alleles, a variant known as Hb Shelby. The ensuing substitution of a basic amino acid (lysine) for a neutral one (glutamine) confers additional positive charge and explains the increased retention time of Hb Shelby on ion-exchange HPLC. However, because glutamine and lysine do not differ in atomic mass, no differences are observed with electrospray ionization of the intact protein.

The Shelby variant, originally thought to harbor a glutamine deletion at codon 131 and referred to as Hb Deaconess and Hb Leslie, was later shown to be the same as Hb
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SHELBY. This variant has been described in several African American families with people with the Hb Shelby trait, as well as other compound and double heterozygotes carrying Hb Shelby in combination with HbS, HbC, and α- and β-thalassemia. The mutation in Hb Shelby affects the α-β bridging of the hemoglobin molecule, making it mildly unstable and affecting its solubility, consistent with our observation of an increased number of target cells in the peripheral smear. Target cells form on air drying of peripheral blood smears in a variety of situations in which there is an increased surface-volume ratio of RBCs. Numerous hemoglobinopathies (e.g., HbS and HbC) are thought to form target cells due to poor solubility and increased precipitation of hemoglobin molecules during air drying. Indeed, oxy-Hb Shelby was found to be less stable and to precipitate more readily than oxy-HbA in vitro.

Despite mild instability of hemoglobin, the Hb Shelby trait described in this case report, other than causing a falsely elevated HbA1c, was clinically insignificant. According to the literature, other people with the Hb Shelby trait, and even Hb Shelby compound heterozygotes, as described, follow a fairly benign clinical course, although many display mild anemia. Of note, the slightly decreased half-life of RBCs in patients with the Hb Shelby trait may explain the slightly lower than expected glycated hemoglobin level as measured by boronate affinity, the borderline low RBC count, and the slightly increased reticulocyte count seen in this case.

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