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Clinical Case Study

Unexpected Hemoglobin A_{1c} Results

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CASE

A 52-year-old woman with a medical history of hepatitis B, hyperlipidemia, hypertension, anemia, and depression presented to the internal medicine clinic for a routine visit. Laboratory tests 3 months previously had revealed an impaired fasting glucose concentration of 5.9 mmol/L (106 mg/dL) [reference interval, 3.9–5.6 mmol/L (70–100 mg/dL)]. Therefore, a hemoglobin (Hb)[2] A_{1c} analysis was performed. The initial Hb A_{1c} evaluation by cation-exchange HPLC (CE-HPLC) (Hb A_{1c} Program on the VARIANT II TURBO Link System; Bio-Rad Laboratories) showed an Hb A_{1c} value of 115.8% (reference interval, 4.0%–6.0%) (Fig. 1).

In an effort to determine if the unusual Hb A_{1c} result was due to potential hemoglobinopathies, we performed an Hb variant analysis with the Bio-Rad VARIANT CE-HPLC β-Thalassemia Short Program. The analysis revealed the absence of Hb A and the presence of sickle cell Hb (Hb S) (37.4%), along with normal Hb A_2 (3.2%) and Hb F (<1.0%) (Fig. 2). Also evident was another large peak (53.0%) that eluted earlier than Hb A, which we called P2. This study suggested the presence of an Hb variant with a chromatographic retention time virtually identical to that of Hb A_{1c}, in addition to Hb S (Figs. 1 and 2). A subsequent Hb electrophoretic analysis at pH 6.0 (QuickGel Acid; Helena Laboratories) identified Hb S and another abnormal band with a mobility similar to Hb F (not shown).

PATIENT FOLLOW-UP

To identify the Hb variants, we investigated DNA sequences corresponding to the patient's β-globin genes. This analysis identified a substitution at codon 6 [GAG to GTG (Glu to Val)] on one allele, corresponding to Hb S, and a substitution at codon 1 [GTG to GCG (Val to Ala)] on the other allele, corresponding to Hb Raleigh.

QUESTIONS TO CONSIDER

1. What are the various types of methods used for measuring Hb A_{1c}?
2. How do Hb variants interfere with each of these Hb A_{1c} methods?
3. What actions should be taken when a spurious Hb A_{1c} result is present?

The presence of these hemoglobinopathies suggested that the spurious Hb A_{1c} result obtained with the CE-HPLC method was due to the elution of Hb Raleigh, which has a retention time similar to that of Hb A_{1c}. We evaluated the Hb A_{1c} result with a turbidimetric inhibition immunoassay (Dimension® Clinical Chemistry System; Siemens) and obtained an Hb A_{1c} value of 4.1%, which was not consistent with the impaired fasting glucose concentration of 5.9 mmol/L (106 mg/dL).

DISCUSSION

Hb A_{1c} is produced by nonenzymatic addition of a glucose molecule to the N-terminal valine residue on the β chain of Hb A. Glycation of the N-terminal residue changes its structure and decreases the positive charge of Hb A. The American Diabetes Association has recommended Hb A_{1c} as an indicator of long-term glycemic control in patients with diabetes mellitus and for the screening and diagnosis of diabetes mellitus; an Hb A_{1c} cutoff value of 6.5% has been suggested *(1)*.

Methods of Hb A_{1c} analysis can be divided into 2 categories: methods based on molecular charge and those based on structure. The former category includes CE-HPLC and electrophoresis, and the latter includes immunoassays, boronate affinity chromatography, and mass spectrometry *(2)*. In CE-HPLC and electrophoresis assays, Hb A_{1c} can be separated from Hb A because glycation of the N-terminal valine decreases the positive charge. Therefore, charge-based methods may be affected by posttranslational modifications (e.g., carbamylation and acetylation) *(3)* or by Hb mutations *(2)* that alter the charge. Common Hb traits such as Hb AS and Hb AC, however, do not interfere

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[2] Nonstandard abbreviations: Hb, hemoglobin; CE-HPLC, cation-exchange high-performance liquid chromatography; Hb S, sickle cell Hb.

Peak name	Calibrated area, %	Area, %	Retention time, min	Peak area
A_{1a}	—	0.4	0.106	6826
A_{1b}	—	4.6	0.213	76457
A_{1c}	115.8*	—	0.405	816032
P3	—	1.3	0.770	21144
Variant window	—	4.2	0.860	70124
S window	—	40.2	0.920	665722
*Value outside of expected range			Total area:	1656306

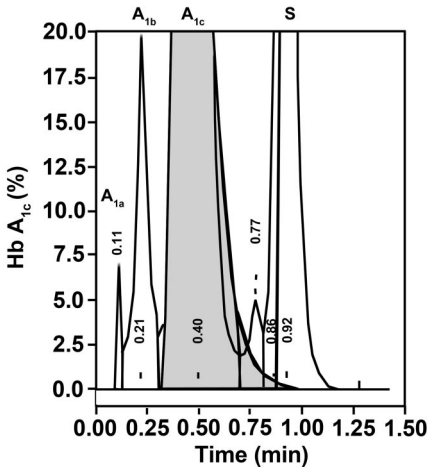

Fig. 1. CE-HPLC chromatogram for Hb A_{1c} analysis.
Hb S and an aberrant Hb A_{1c} value of 115.8% represented the predominant Hb peaks in the chromatogram.

Analyte ID	Percent	Time, min	Area
P1	1.2	0.75	33627
F	0.5	0.94	16262
P2	53.0	1.30	1542147
A_0	2.7	2.28	79887
Unknown 1	1.6	2.50	47682
A_2	3.2	3.68	108007
S window	37.4	4.45	1088414

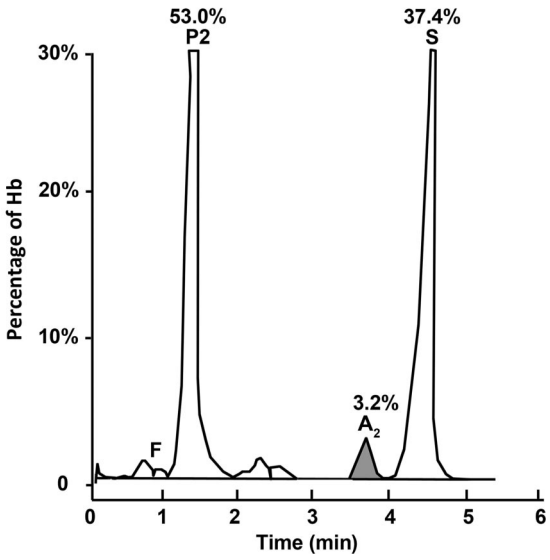

Fig. 2. Chromatogram of Hb variants analysis with the CE-HPLC β-Thalassemia Short Program.
Hb S (37.4%), wild-type Hb A_2 (3.2%), and Hb F (<1.0%) were identified, but Hb A was not detected. A large peak, which we designated P2, was detected at 53.0%.

with the CE-HPLC Hb A_{1c} assay used in this study (Bio-Rad VARIANT II TURBO) *(4)*. Immunoassays use antibodies that target N-terminal glycated amino acids on the β chain to quantify Hb A_{1c}, and the Hb A_{1c} percentage is calculated from the Hb A_{1c} and Hb concentrations *(2)*. Thus, any factor that prevents glycation or any mutation in the epitope of the N-terminal amino acids that affects antibody recognition will produce erroneous results. Additionally, patients with increased Hb F (>10%) will have a falsely low Hb A_{1c} value by immunoassay because the γ chain shares only 4 of the first 10 amino acids with the β chain of Hb A and has little to no immunoreactivity with most antibodies used in Hb A_{1c} assays *(2)*. In the boronate affinity chromatographic assay, boronic acid reacts with the cis diol groups created by glycation, thereby allowing glycohemoglobins such as Hb A_{1c} to be separated from Hb A *(2)*. On the other hand, Hb variants with excessive glycation, such as Hb Himeji, can interfere with boronate affinity chromatography *(5)*. Mass spectrometric assay, an IFCC reference method, specifically measures the glycated N-terminal valine of the Hb A β chain *(6)*, but the prohibitive cost of a mass spectrometer and the complicated nature of its installation and operation are likely to preclude its use in most clinical laboratories in the near future *(2)*.

The method initially used to analyze the patient's Hb A_{1c} was a CE-HPLC assay. A CE-HPLC Hb variants analysis (Fig. 2), acid gel Hb electrophoresis, and DNA sequencing of β-globin genes demonstrated that the falsely increased Hb A_{1c} value was due to Hb Raleigh, which eluted in the Hb A_{1c} window (Fig. 1). Hb Raleigh is unique in that a mutation (T to C) at the second base of the codon encoding the first amino acid of the β chain changes the N-terminal valine to an alanine residue. This substitution would not necessarily induce any change in Hb A charge except that N-terminal alanines are immediately acetylated to acetylalanines soon after translation *(7, 8)*. This acetylation decreases the positive charge to one similar to Hb A_{1c}. Therefore, the retention times of Hb A_{1c} and Hb Raleigh are virtually identical; the elution peaks of these 2 Hbs fall in the same window in the chromatogram (Fig. 1). Thus, the presence of Hb Raleigh produces a falsely increased Hb

A_{1c} value when the latter is assessed by CE-HPLC. Chen et al. reported a case of falsely increased Hb A_{1c} due to Hb Raleigh in the Bio-Rad VARIANT CE-HPLC Hb A_{1c} assay *(9)*. In their case, the patient was heterozygous for Hb A and Hb Raleigh, and the falsely increased Hb A_{1c} value of 46% included a small fraction of real Hb A_{1c}. In our case, the patient was heterozygous for Hb S, in which the N-terminal valine of the β chain was glycated as Hb S_{1c}, and Hb Raleigh, in which the N-terminal acetylalanine of the β chain could not be glycated. Therefore, Hb A_{1c} did not exist in this patient. The spurious Hb A_{1c} value (115.8%) primarily represented Hb Raleigh. Other Hb variants that produce similar interferences include Hb Graz, Hb Sherwood Forest, Hb South Florida, Hb Niigata, and carbamylated Hb A *(2)*.

The turbidimetric inhibition immunoassay produced an Hb A_{1c} value of 4.1%, which represents the Hb S_{1c} percentage, an equivalent of the Hb A_{1c} percentage. Hb S_{1c} was underestimated because of the heterozygosity with Hb Raleigh. Hb S is characterized by a substitution at codon 6 (GAG to GTG) that leads to the replacement of a glutamic acid residue in the β chain by a valine residue. Although this substitution is at the sixth amino acid residue, the antibody used in the immunoassay still recognizes Hb S_{1c}. The acetylated alanine at the N terminus of the Hb Raleigh β chain, however, cannot be glycated and therefore prevents reaction with the antibody in the immunoassay. When the Hb A_{1c} percentage is calculated, the numerator includes Hb S_{1c} only, but the denominator consists of both Hb S and Hb Raleigh, as well as small amounts of Hb A_2 and Hb F. Therefore, the Hb A_{1c} percentage for this patient (as measured by immunoassay) was underestimated by approximately 50%. This issue with the Hb A_{1c} immunoassay has been discussed by Chen et al. *(9)* and Jain et al. *(10)*. Similarly, boronate affinity chromatography assays also underestimate Hb A_{1c} for patients with Hb Raleigh, because the N-terminal acetylalanine in the Hb Raleigh β chain cannot be glycated. Hb Raleigh has a decreased affinity for boronate in the column *(9)*, although the column can still interact with other glycated residues. For these patients, Chen et al. *(9)* and Jain et al. *(10)* recommended the use of fructosamine *(9)*, multiple measurements of capillary glucose throughout the day, or continuous glucose monitoring of the glycemia *(10)*. We did not perform these tests for our patient because of insufficient sample. For patients with the Hb Raleigh trait, the IFCC tandem mass spectrometric assay may be the best method, because it measures Hb A and Hb A_{1c} specifically. The IFCC reference method, however, would be useless for our patient unless a mass spectrometric assay were available to measure Hb S and Hb S_{1c}, because she had no Hb A or Hb A_{1c}.

POINTS TO REMEMBER

- Hb A_{1c} assays can be divided into methods that use molecular charge (CE-HPLC and electrophoresis) and methods that use molecular structure (immunoassays, boronate affinity chromatography, and mass spectrometry).
- Hb variants (or their glycated forms) may interfere with Hb A_{1c} assays based on CE-HPLC and electrophoresis by coeluting/comigrating with Hb A and/or Hb A_{1c}. If an Hb variant's amino acid substitution occurs within the N-terminal β-chain epitope recognized by an anti–Hb A_{1c} antibody or if a patient has a greatly increased Hb F percentage, Hb A_{1c} immunoassays will be affected. Hb variants with decreased or increased glycation will interfere with boronate affinity chromatography. Mass spectrometry is the IFCC reference method and generally appears to be unaffected by the presence of genetic or chemical modifications to the Hb A molecule.
- When a spurious Hb A_{1c} result is obtained, the possibility of interference by Hb variants should be considered, and the interpretation of Hb A_{1c} results should be based on the patient's medical history and other laboratory results. In addition, efforts should be made to identify the Hb variant, and alternative Hb A_{1c} methods that are free of the interference should be used. If there is no appropriate method for a particular Hb variant, fructosamine, daily multiple testing of capillary glucose, or continuous glucose monitoring may be used to monitor glycemic control. These alternative tests may also be used for patients who have an altered erythrocyte life span and changes in the degree of glycation. Hb A_{1c} testing cannot be used for these individuals.

The present case is an example of how Hb variants can interfere with Hb A_{1c} assays to produce spurious results. When an aberrant Hb A_{1c} value is generated and/or the value does not match the clinical impression, the possibility of interference by Hb variants should be considered, and the interpretation of Hb A_{1c} values should be based on the patient's medical history and other laboratory results. Efforts should be made to identify the Hb variant, and alternative Hb A_{1c} methods free of the interference should be selected to monitor the patient's glycemic control.

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Commentary

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The 4 most common hemoglobin (Hb) variants worldwide and in the US are Hb S, Hb E, Hb C, and Hb D. In addition, there are many other less common variants. These variants frequently go unrecognized in the heterozygous form (e.g., the Hb S trait) because they are usually clinically silent, but such a variant might still be clinically important if its presence can lead to erroneous test results. A large number of patients with diabetes have clinically silent Hb variants that may interfere with Hb A_{1c} measurement by some methods. Most of the commonly used Hb A_{1c} methods have been evaluated with the 4 most common Hb variant traits (see http://www.NGSP.org), and although one or more of these variants interfere with some methods, others do not. When an Hb variant causes a change in the erythrocyte life span or actually produces a change in Hb glycation, Hb A_{1c} measurement may not give clinically useful results, regardless of the assay methodology. In the case of the patient described by Sofronescu et al., there were actually 2 Hb variants present, Hb S and Hb Raleigh. Because there is no Hb A, one cannot directly measure Hb A_{1c}. One could consider using boronate affinity to measure the total glycated Hb, which would include glycated Hb S and Hb Raleigh, except that Hb Raleigh is glycated to much less of an extent (compared with Hb A) because of the specific amino acid substitution. A small number of cases require other measures of glycemic control, such as fructosamine or continuous glucose monitoring, instead of Hb A_{1c} measurement, but such cases are rare. For the vast majority of patients with diabetes (and for those being tested for the presence of diabetes), Hb A_{1c} measurement is the best way to assess long-term glycemic control, as long as an appropriate methodology is used. It is the responsibility of all manufacturers to clearly state their methods' limitations, and it is up to each laboratory to know—and to convey to their clinicians when appropriate—these limitations for the methods they use to measure Hb A_{1c}.

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