HbA1c Does Not Always Estimate Average Glucose

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ABSTRACT: Hemoglobin A1c (HbA1c) testing can be inaccurate in persons with elevated amounts of Hemoglobin F, or with abnormal hemoglobins found in sickle cell trait, HbC trait and HbE trait. These variants are more prevalent in African and Asian Americans, the same demographic that has an increased risk of diabetes. Variant hemoglobins might cause a false increase or decrease in HbA1c, depending on methodology and manufacturer. Case studies of two African American patients, one with and one without variant hemoglobins, are presented. The major methods used to assay HbA1c, immunoassay, HPLC and boronate affinity are described, and compared for their ability to detect variant hemoglobins. An algorithm is proposed to test new patients using the HPLC method to identify or rule out the presence of the most common variant hemoglobins. Patients with variant hemoglobins can subsequently be assigned to HbA1c methods proven to be accurate in the presence of those hemoglobins.

INDEX TERMS: Hemoglobinopathy, A1c, African-American, diabetes

Clin Lab Sci 2011;24(2):71

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Introduction

Hemoglobin (Hb) A1c measurement is the standard of care for estimating patient average glucose concentration. Point of care testing for HbA1c is recommended by the American Diabetes Association,[1] as it is rapid and allows the clinician to address the patient's status immediately, improving patient compliance.[2] Hemoglobin A1c was recently approved for use as a diagnostic tool, and an HbA1c $\geq$ 6.5% is the cutpoint for diagnosis.[1] The pre-diabetic state was cited to be an HbA1c > 5.7 to 6.4%.[1] The correlation of HbA1c to average glucose concentration was recently validated with patients who have type 2 diabetes mellitus.[3] That correlation is shown in Table 1, which indicates that a change of 1% HbA1c is equivalent to a change of ~ 1.6 mmol/L (29 mg/dL) glucose. Many clinical laboratories are incorporating this interpretation into their HbA1c results, so that the clinician and patient will see a result that includes both the HbA1c and the estimated average glucose. The estimated average glucose concentration is referred to as eAG.

Table 1. Hemoglobin A1c relates to estimated average glucose concentration

HbA1c %	Estimated average glucose mmol/L	Estimated average glucose mg/dL
5	5.4	97
6	7.0	126
7	8.6	154
8	10.2	183
9	11.8	212
10	13.3	240

Data derived from Nathan et al., 2008.

Hemoglobin A1c should be measured at least every six months in patients with diabetes, and glucose concentration should be frequently tested in patients on insulin.[1] There are no set guidelines as to how frequently a patient with type 2 diabetes mellitus who does not use insulin should perform self blood glucose monitoring, leaving that decision to the clinician and the patient. Therefore, in those six months between testing for HbA1c concentration, a patient with type 2 diabetes may measure twice a day, daily, weekly, or maybe just two weeks before visiting the physician.

When a patient reveals self glucose results that don't correlate with the HbA1c, it is not unreasonable to consider poor record keeping and noncompliance on the part of the patient. However, this is not always the case.

Two patients, two very different results

The presence of variant hemoglobins such as HbS, HbC, HbE and HbF, can affect the accuracy of HbA1c results. Data from two subjects are used here to illustrate how the presence of an unsuspected hemoglobin moiety can affect the results of HbA1c. Both of these subjects have type 2 diabetes, are ambulatory female African Americans, and control their glucose by oral medication and diet. Both subjects were part of a larger on-going study in the African-American community and both subjects provided informed consent. Subjects monitored blood glucose by fingerstick using a Bayer Contour™ glucose meter with Ascensia ™ strips (Bayer HealthCare, Daphne, AL), performing fasting glucose once a day for a week, followed by 2 hour post prandial (lunch/dinner) glucose once a day for a week over a 3 month period. A specimen for HbA1c measurement was collected at the conclusion of three months. Hemoglobin A1c was performed on a Siemens (formerly Bayer, Tarrytown, NY) DCA 2000 point-of-care immunoassay analyzer and a Bio-Rad Laboratories (Hercules, CA) D-10 analyzer. The Bio-Rad D-10 uses High Performance Liquid Chromatography (HPLC) methodology. Hemoglobinopathies were confirmed by electrophoresis (Paragon Hemoglobin electrophoresis, Acid Hemoglobin electrophoresis, Beckman Coulter, Fullerton, CA). An explanation of HbA1c methodology, immunoassay, HPLC and boronate affinity (BA), is found in the conclusion.

Patient A had an average (fasting + postprandial) capillary blood glucose concentration of 5.7 mmol/L (103 mg/dL) during the three months of the study, shown in Figure 1A. The HbA1c measurement by the point of care immunoassay analyzer gave a result of 5.7%, which equates to an average estimated glucose (eAG) of 117 mg/dL. The immunoassay result of 5.7% compared well with the HPLC result of 6.2%. The HPLC chromatogram is shown in Figure 1B. Hemoglobin electrophoresis showed a normal pattern, with Hemoglobin A as the predominant hemoglobin.

Patient ASF had an average (fasting + postprandial) capillary blood glucose of 7.2 mmol/L (130 mg/dL) for the three months of the study, shown in Figure 1C. This average predicts an HbA1c of 6.1%, however HbA1c performed by the point-of-care analyzer gave a result of 4.7%. The HbA1c immunoassay result compared poorly with the HPLC result of 7.0%. The HPLC chromatogram identified 3 major hemoglobins, HbA, HbS and a large amount (15.7%) of HbF, shown in Figure 1D. This particular HPLC method is reported to be accurate in the presence of HbS;[4] therefore patients with sickle cell trait will show accurate HbA1c results. Patient ASF data was not reportable, however. The chromatogram failed the analyzer acceptance criteria due to the high HbF level. The protocol at the testing laboratory when this occurs is to send the sample to a reference laboratory that has the capability of accurately measuring glycated hemoglobin in the presence of particular hemoglobinopathies. Hemoglobin electrophoresis presumptively confirmed the presence of a low amount of HbA, the presence of HbS and an elevated level of HbF, providing a diagnosis of Sickle Beta$^+$ Thalassemia. The patient had self-reported that she had Sickle Cell Trait, but she was not aware of the Thalassemia. The point-of-care analyzer reported an erroneous result, with no error flag. The result would be interpreted as regular hypoglycemia; in reality, the patient was in good glycemic control.

The effect of unsuspected Hemoglobins on HbA1c testing

Contemporary HbA1c analyzers utilize either immunoassay, HPLC cation exchange or boronate affinity chromatography, and there are several manufacturers and analyzers on the market. The design of the method affects whether or not it is influenced by increased non-A hemoglobin, so that HPLC from manufacturer X may be affected, but HPLC from manufacturer Y may not be.[5] Similarly, immunoassay from manufacturer W may be affected, but immunoassay from manufacturer Z may not. The National Glycohemoglobin Standardization Group (NGSP) keeps an up to date table on their website[4]

which lists HbA1c methods by the instrument name and manufacturer. The table includes whether or not the method shows interference from the presence of elevated HbF, HbS trait, HbC trait, HbE trait, and carbamylated Hb. Of the 34 methods listed at the time of this manuscript, 25 methods had not been evaluated for interference by HbF, five methods reported interference by HbF, and four reported no interference. Evaluation data on HbE interference is also limited; six methods showed interference, 17 did not, and 11 had not been evaluated. The major variant hemoglobins and their effect on HbA1c accuracy is shown in Table 2.

Table 2. Major variant hemoglobins known to interfere with HbA1c accuracy

Variant Hemoglobin	Prevalence in US population	Percent of established HbA1c methods that have demonstrated no interference due to the variant hemoglobin
HbS Trait	1 in 12 African Americans	68%
HbC Trait	1 in 50 African Americans	68%
HbE Trait	1 in 30 Asian (predominant in Southeast Asia)	50%
HbF	Variable	12%

Data derived from NGSP, 2010; Harmening 2002.

Demographics of the problem

Hemoglobin A is the predominant hemoglobin in adults. Hemoglobin S is found in approximately one in 12 African Americans and one in 100 Hispanics and other races.[6] HbC is found in approximately 2% of African Americans.[6] The term "trait" refers to individuals with one normal hemoglobin gene that produces HbA, and one variant gene. Patients with HbS trait or HbC trait are rarely symptomatic, and many of these individuals do not know that they carry the abnormal hemoglobin. Hemoglobin E is associated with individuals of Asian descent, particularly southeast coastal Asia, with a prevalence of up to 30% in some areas. HbE trait is also asymptomatic. A negative patient history is unreliable in assessing the presence of hemoglobinopathies. The patient has to be tested.

HbF is not a variant, nor is it a hemoglobinopathy. It is a normal hemoglobin, and the major hemoglobin in fetal life. It is found in adult blood in a low concentration. Its concentration is increased in Hereditary Persistence of Fetal Hemoglobin (HPFH), Sickle Beta Thalassemia, and other conditions.[6] In a study of 5,136 HbA1c tests performed by one reference laboratory, 1,059 were shown to have a variant hemoglobin when tested by HPLC. Hemoglobin variants were found in 30% of the samples from African Americans, with an elevated HbF being the predominant finding.[7]

In an American population, then, the patient most likely to have an inaccurate HbA1c due to a variant or unsuspected hemoglobin, is a non-Caucasian. This is important because non-Caucasians are more likely to develop diabetes, and to have more severe complications from diabetes.[8] Depending on the methodology, an HbA1c result may be higher or lower than expected in a patient with an alternate hemoglobin.[5] In either case, patient care and treatment that is adjusted based on an erroneous result can have serious consequences.

Improving the value of the HbA1c result

The National Institutes of Health provide some guidelines as to when to suspect that a patient with diabetes has a hemoglobinopathy.[9] These include 1) when the results of the self monitoring do not correlate with the HbA1c, 2) when the HbA1c is over 15% and, 3) when an HbA1c result shows a significant change from the previous result when the laboratory method changes. Unfortunately, these recommendations address investigating an incorrect result instead of preventing one. This process, combined with the ADA recommendation to utilize HbA1c as a diagnostic tool, and the emphasis on point of care testing for HbA1c, set up a system error that may affect patient diagnosis and treatment, with the greatest impact on minorities. Furthermore, it puts the responsibility for detecting the spurious result on the clinician.

It is imperative to identify early in their care the patients whose results might be affected. A simple and straightforward way is to order a hemoglobin electrophoresis on a new patient during the first appointment. Patients with normal hemoglobin

electrophoresis results can have their HbA1c tested anywhere, in a physician office, or in a reference or hospital laboratory. Patients with abnormal hemoglobin electrophoresis results should be referred to a testing facility that utilizes a method proven to be successful for that particular variant, or to an alternate method of assessing glycemic control.

An alternative and less expensive algorithm is to screen new patients for hemoglobinopathy by utilizing HPLC HbA1c. A study by Thomas et al.[10] detected 11 different hemoglobinopathies by HPLC, including HbS trait and HbC trait; the hemoglobin variants were classified by their chromatogram retention time. Several variants can be presumptively identified by certain analyzers, as shown in Figures 1D and 2B. A study of reporting practices for HbA1c indicated that a small number of laboratories provide a statement about unusual hemoglobins with their result, e.g. "Hemoglobin A1c 7.4%; patient has elevated Hemoglobin F" or "Hemoglobin variant present, suggest hemoglobin electrophoresis for additional information, as clinically indicated". Most laboratories do not provide this information.[11] A standardized result format has been proposed that includes the HbA1c result, a statement of the presence or absence of alternate hemoglobins, and the test methodology,[11,12] however this is not standard practice at the current time.

Hemoglobin A1c inaccuracies in the presence of hemoglobin variants are method dependent.[4] A patient who is tested by two different laboratories or two different methods may have clinically significantly different results. In a recent study, few laboratories included the test methodology on their reports, although several of them mentioned their methodology in their electronic and printed laboratory manuals.[11] The choice of the laboratory is frequently out of the control of the patient, such as when the patient moves, is hospitalized, or when insurance coverage changes, as insurance may dictate which laboratory a patient may use.

HPLC is useful to screen for the major non-A hemoglobins, e.g. HbS, HbE, HbC, HbF, but many other variants remain to be tested. Hemoglobin A1c may not match the average blood glucose due to other reasons as well. Biological influences to glycation, anemia – including iron deficiency and sickle cell disease, polycythemia and altered red blood cell lifespan, and pregnancy[1] can influence the accuracy of HbA1c, as can carbamylation in uremic patients and acetylation. These influences are independent of test methodology, and affect immunoassay, HPLC and boronate affinity chromatography to varying degrees. These factors are beyond the scope of this article.

Hemoglobin A1c does not always estimate average blood glucose (eAG) for every patient. The authors recommend that clinical laboratories screen for variant hemoglobins by HPLC, include information in laboratory results about test methodology, and recommend alternate methodology for patients when appropriate. An algorithm for this screening is shown in Figure 3. It is the role of the laboratory to educate physicians about the effect of variant hemoglobins on HbA1c analysis. Bio-Rad Laboratories manufactures HPLC analyzers, and is heavily invested in education for clinicians and laboratorians. An HPLC chromatogram training library is available from Bio-Rad, and can be requested by contacting the authors.

Hemoglobin A1c methodologies

The three methods used to measure glycated hemoglobin are immunoassay, high performance liquid chromatography (HPLC) and boronate affinity chromatography. Each method has its benefits and drawbacks.

Immunoassays are tests that use antibodies to detect an analyte. Immunoassays for HbA1c are often used in point-of-care testing, and the DCA 2000 (Siemens) is a popular example. Testing can be done from a fingerstick blood sample, and results are available within 10 minutes. The reagent antibody reacts specifically with hemoglobin that has a stable linked glucose at the N-terminus of the beta chain, causing precipitation. The percentage of the glucose-hemoglobin moiety as the numerator to the total hemoglobin denominator equals the HbA1c% result, i.e. HbA1c/Total Hemoglobin = A1c%

Hemoglobin S, HbC and HbE interfere with some immunoassays. The first 5 amino acids of the beta chain of HbS, C and E are identical to HbA. Several immunoassay methods use an antibody that is specific for the N-terminal glucose-valine-histidine-leucine-threonine-proline, so that all of the glycated forms, Hemoglobins A, S, C, and E are detected. An underestimation of glycation occurs in the presence of high amounts of Hemoglobin F, because its glycated sequence begins with glucose-glutamate-methionine-phenylalanine-threonine-glutamate. The reagent antibody does not recognize this sequence and omits it from the numerator, however Hemoglobin F is included in the total hemoglobin denominator. The assay does not flag inaccurate results. Many physician office laboratories and clinical laboratories use immunoassays to measure HbA1c. Automated analyzers utilizing immunoassay include Siemens Dimension, Abbott Architect, OrthoVitros, Roche Integra and Beckman Coulter Synchron Unicel.

High Performance Liquid Chromatography (HPLC) separates hemoglobin moieties by charge. The whole blood sample is hemolyzed and injected into a column filled with charged resin which binds the hemoglobins. The column is washed with buffers of different ionic strength at certain times. The various hemoglobins release or elute from the column at a specific time related to their charge and to the ionic strength of the buffer. This is referred to as the retention time. As the hemoglobin elutes, its concentration is measured by a detector. The graph of this timed process is called a chromatogram, and the axes of the chromatogram are concentration versus retention time, shown in Figure 1 and 2.

Hemoglobin A1c has a different charge from HbA, so it produces a separate peak. A normal chromatogram has two major peaks and several minor peaks. The HbA1c peak is highlighted by the instrument and its concentration is calculated relative to the total hemoglobin concentration, shown in Figure 2A. HbS, C, E and F each have a charge that is different from Hemoglobin A due to their amino acid composition. Each of those hemoglobins will elute from the column at characteristic times as well. Some analyzers identify the extra peak, such as the Hemoglobin S shown in Figure 2B. Other analyzers may flag the peak, but not specifically identify it. For example, an analyzer may identify an unknown peak as "P001" for peak 1, or it may identify it as "Hemoglobin C window". The medical laboratory scientist interpreting the HPLC follows an algorithm established by the analyzer manufacturer and the specific laboratory's protocol to decide if a report is accurate and acceptable in the presence of the variant hemoglobin. The chromatograms shown in Figure 2 are from a Bio-Rad D-10™ analyzer. The analyzer detected and quantitated HbS in the patient sample 2B. The testing algorithm for the analyzer states that the resulting HbA1c in the presence of a heterozygote AS is acceptable if the percentage of the S is less than half of the total hemoglobin. For this patient sample, the HbA1c is 11.1%. HPLC is a high complexity test, and is not CLIA approved for point-of-care testing.

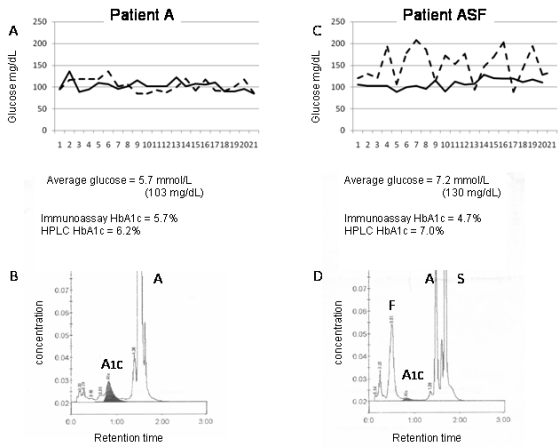

Figure 1. Variant hemoglobins affect HbA1c result accuracy.
Three month composite fingerstick glucose from fasting (solid lines) and 2 hour post dinner (dashed lines), and corresponding HPLC chromatograms. Major hemoglobins are noted by their identity. A: Three month glucose readings of Patient A with average glucose concentration of 103 mg/dL. B: HPLC chromatogram of Patient A shows HbA1c peak in black and HbA as the major hemoglobin. HbA1c is 6.2% of the total hemoglobin. C: Three month glucose readings of Patient ASF with average glucose concentration of 130 mg/dL. D: HPLC chromatogram shows Patient ASF has three major peaks, HbF, HbA and HbS. HbA1c is shown in black, and is 7.0% of the HbA.

Boronate Affinity Chromatography is not affected by most alternate hemoglobins,[4,13] and is used in many reference laboratories as well as in point-of-care testing. It does not directly measure HbA1c. Recall that

immunoassay and HPLC measure hemoglobin that has a glucose at the N-terminus of the beta chain. Boronate affinity measures hemoglobin that has been glycated at any of its attachment sites, including other amino acids on the beta chain, as well as amino acids on the alpha chain. The result is reported as "glycated hemoglobin". The benefit of this method is that normal and variant glycated hemoglobins are measured, so HbS, HbC, and HbE do not chemically interfere with the result. One drawback is that results are given as 'glycated hemoglobin', which can be confusing to the clinician, but the corresponding (calculated) HbA1c is also provided. The Ultra2™ by Primus is an example of an analyzer which utilizes boronate affinity.

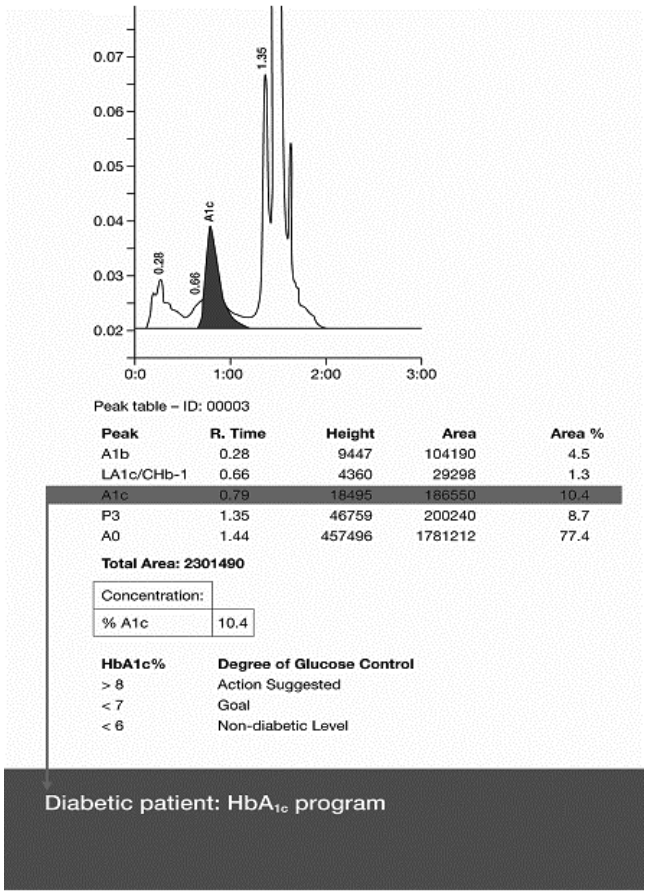

Figure 2A. HPLC chromatogram with interpretation.
Hemoglobin fractions separate as peaks that are identified based on their retention (R) time on the column. The HbA1c peak is shaded. Patient HbA1c is 10.4% of the total. Chromatogram courtesy of Bio-Rad Laboratories.

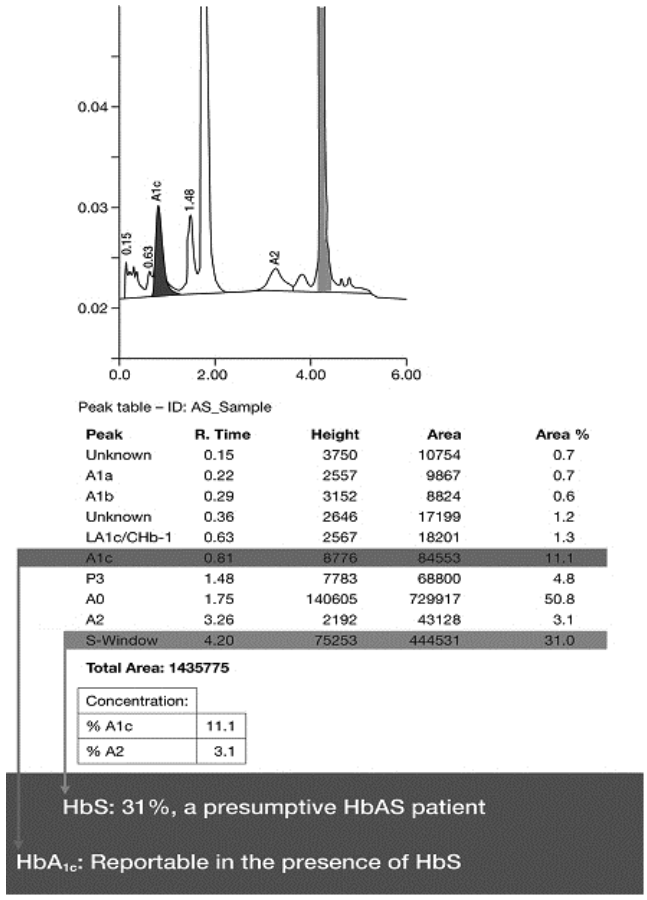

Figure 2B. HPLC chromatogram with interpretation.
The HbA1c peak is shaded. HbS (shaded peak with R. time of 4.20) is identified as a well defined peak by this method, and quantified as a percent. Hemoglobin S does not interfere with the HbA1c test. Patient HbA1c is 11.1%. Chromatogram courtesy of Bio-Rad Laboratories.

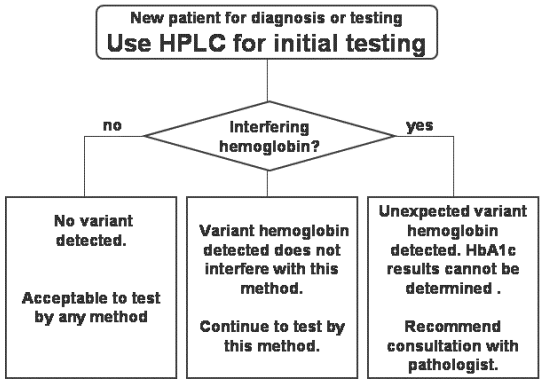

Figure 3. Use of HPLC to screen new patients for variant hemoglobins.
Patients will either be classified as normal (no variant), variant hemoglobin with acceptable results, or variant hemoglobin that interferes with analysis.

Acknowledgements:

This project was funded by the American Society for Clinical Laboratory Science and approved by the Institutional Review Board at the University of West Florida. Contour glucose meters and strips were kindly provided by Bayer Diagnostics. We thank West Florida Hospital in Pensacola, Florida for the HPLC analysis, Arthur Williams at Diabetes Technologies Inc. and Jon Davis at Primus Diagnostics and Dr. Mark Kummer for discussion, and Mark Matthew De Luna for hemoglobin electrophoresis.

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