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SYMPOSIUM

A Review of Variant Hemoglobins Interfering with Hemoglobin A1c Measurement

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Abstract

Hemoglobin A1c (HbA1c) is used routinely to monitor long-term glycemic control in people with diabetes mellitus, as HbA1c is related directly to risks for diabetic complications. The accuracy of HbA1c methods can be affected adversely by the presence of hemoglobin (Hb) variants or elevated levels of fetal hemoglobin (HbF). The effect of each variant or elevated HbF must be examined with each specific method.

The most common Hb variants worldwide are HbS, HbE, HbC, and HbD. All of these Hb variants have single amino acid substitutions in the Hb β chain. HbF is the major hemoglobin during intrauterine life; by the end of the first year, HbF falls to values close to adult levels of approximately 1%. However, elevated HbF levels can occur in certain pathologic conditions or with hereditary persistence of fetal hemoglobin. In a series of publications over the past several years, the effects of these four most common Hb variants and elevated HbF have been described.

There are clinically significant interferences with some methods for each of these variants. A summary is given showing which methods are affected by the presence of the heterozygous variants S, E, C, and D and elevated HbF. Methods are divided by type (immunoassay, ion-exchange high-performance liquid chromatography, boronate affinity, other) with an indication of whether the result is artificially increased or decreased by the presence of a Hb variant. Laboratorians should be aware of the limitations of their method with respect to these interferences.

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Abbreviations: (ADA) American Diabetes Association, (GHB) glycated hemoglobin, (DCCT) Diabetes Control and Complications Trial, (Hb) hemoglobin, (HbA1c) hemoglobin A1c, (HPLC) high-performance liquid chromatography, (IFCC) International Federation of Clinical Chemistry (RM) Reference Method, (UKPDS) United Kingdom Prospective Diabetes Study, (NGSP) National Glycohemoglobin Standardization Program

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Introduction

Glycated hemoglobin (GHB), reported as hemoglobin A1c (HbA1c), is a biochemical marker that is used routinely in the management of individuals with diabetes mellitus to monitor long-term glycemic control and assess the risk of developing complications.[1,2] The Diabetes Control and Complications Trial (DCCT) and the United Kingdom Prospective Diabetes Study (UKPDS) demonstrated conclusively that risks for complications are related directly to glycemic control, as measured by HbA1c.[1,2] Many diabetes organizations worldwide recommend specific HbA1c targets in terms of DCCT/UKPDS HbA1c.[3] The National Glycohemoglobin Standardization Program (NGSP) was established to standardize GHB/HbA1c results so that clinical laboratory results are comparable to those reported by the DCCT. However, the accuracy of several HbA1c methods can be affected adversely by the presence of hemoglobin variants.[4] The NGSP does not include evaluation of interferences as part of the certification program.[5]

Hemoglobin (Hb) is composed of four globin chains. Adult hemoglobin (HbA) is the most abundant form in most adults and consists of two α and two β chains. Fetal hemoglobin (HbF), which is the predominant species present at birth, consists of two α and two γ chains. HbF is a minor form in normal adults. HbA2 is minor Hb after birth and consists of two α and two δ chains. The most common Hb variants worldwide in descending order of prevalence are HbS, HbE, HbC, and HbD. All of these hemoglobins have single amino acid substitutions in the β chain. In the United States, HbC is the second most prevalent variant and HbE is the third. The presence of the HbS or the HbC trait has been shown to affect the accuracy of some HbA1c assays.[6–10] In 2004, there were 23.5 million non-Hispanic blacks aged 18 years or older in the United States.[11] Over 10% of these have either the HbC or the HbS trait.[12] The prevalence of diabetes mellitus, both diagnosed and undiagnosed, in non-Hispanic black men is 13.0% and in non-Hispanic black women is 16.3%.[13] This works out to between 305,000 and 383,000 individuals who have both diabetes mellitus and either the HbC or the HbS trait. HbE is found primarily in people from Southeast Asia and is now encountered quite commonly in the United States. HbD Punjab is found most commonly in the Punjab region of India and is also encountered in the United States.[14,15] Globally, it was estimated there were 171 million individuals with diabetes mellitus in 2000, with that number growing to 366 million in 2030.[16] It has been estimated that ~7% of the world's population are heterozygous carriers of hemoglobin disorders.[17] In 2000, an estimated 12 million individuals with diabetes mellitus worldwide also had a hemoglobin disorder. By 2030, this number is expected to increase to 26 million. These are crude estimates, but do indicate the potential size of this global issue.

Homozygosity for HbS causes a serious disease (sickle cell anemia) with vascular obstruction and consequent tissue infarction. Those with HbC disease (also called HbCC disease) may have a normal hemoglobin concentration or be mildly or moderately anemic. Subjects with HbSC disease (also called sickle-hemoglobin C disease) can be affected mildly or moderately with chronic hemolytic anemia. Subjects with homozygous E disease (also called HbEE disease) are usually completely asymptomatic, and those with HbD Punjab disease may have mild hemolysis and sometimes a mild hemolytic anemia. Subjects who are heterozygous for any of these Hb variants are usually asymptomatic and have normal red cell survival.[15,18] Thus, a physician may be unaware that their patient with diabetes has one of these variants in the heterozygous form.

Since the measurement of HbA1c is dependent on a normal erythrocyte life span, it is recommended that other tests be used to estimate glycemic control in those with sickle cell, HbC, or HbD disease. Because erythrocyte survival is normal in those with heterozygous variants, HbA1c can be used as long as the Hb variant does not interfere either with the assay method itself or with glucose binding to Hb. The presence of some variants can affect either the net charge of the hemoglobin and/or the recognition of the glycated N terminus by antibodies, resulting in erroneous HbA1c values for some methods. Therefore, the effect of each variant must be examined with each specific HbA1c method. This review does not consider the possible effects of hemoglobin adducts, such as acetylated or carbamylated Hb.

HbA1c Method Types

Glycation is the nonenzymatic addition of glucose to amino groups of proteins. HbA1c is a glycated hemoglobin in which glucose is bound specifically to the N-terminal valine of the hemoglobin β chain. HbA1c constitutes the major portion of the glycated hemoglobins. Unlike other glycated hemoglobin fractions, HbA1c can be separated easily based on a difference in net charge. Total GHB is a term used to refer to all glycated Hb species. Total GHB

is composed of HbA1c, and Hb glycated with glucose at other sites, including the glycated N terminus of the α chain and the glycated ε amino groups of lysine residues. It can be measured by affinity chromatographic methods.

Four basic types of methods are used most commonly to measure HbA1c: immunoassay, ion-exchange high-performance liquid chromatography (HPLC), boronate affinity HPLC, and enzymatic assays. Most immunoassays measure HbA1c specifically; antibodies recognize the structure of the N-terminal glycated amino acids (usually the first 4–10 amino acids) of the Hb β chain. Ion-exchange HPLC separates Hb species based on charge differences between HbA1c and other hemoglobins. With boronate affinity methods, *m*-aminophenylboronic acid reacts specifically with the *cis*-diol groups of glucose bound to Hb. This method measures total glycated GHB, including HbA1c and Hb glycated at other sites, and tends to demonstrate the least interference from the presence of Hb variants and derivatives. The enzymatic method currently available measures HbA1c by using an enzyme that specifically cleaves the N-terminal valine.

Hemoglobins S, C, E, and D

Sickle cell hemoglobin, or HbS, has a valine for glutamic acid substitution at position 6 of the β chain. HbC is a different variant mutation (lysine substituted for glutamic acid) at the same site as the sickle cell mutation on the β chain. HbE contains a substitution of lysine for glutamic acid at position 26 of the β chain, and HbD Punjab (also called HbD Los Angeles), hereafter referred to as HbD, contains a substitution of glutamine for glutamic acid at position 121 of the β-globin chain. Because the S and C variants are close to the N terminus on the β chain, some (but not all) immunoassays are affected by the presence of these variants. The presence of HbE or HbD, however, with mutations much further away on the β chain, generally does not affect immunoassay methods. The presence of any of these four variants does affect the ionic charge of the Hb molecule, which may cause interference with ion-exchange methods, depending on how well the variant Hb is separated from HbA. Since boronate affinity chromatography separates total glycated hemoglobin from nonglycated hemoglobin, regardless of the hemoglobin species, there is generally no interference from most Hb variants, including HbS, C, E, and D.

In a series of publications between the years 2000 and 2008, the effects of HbS and HbC on several different HbA1c methods were described.[6–10] In 2008, a similar study was published on the effects of Hbs E and D.[19] The methodology was very similar for each of these studies. Whole blood samples collected in EDTA from individuals with homozygous HbA and heterozygous for HbS, C, E, or D were included. Single-use aliquots of each sample were stored at –70°C or colder until analysis. Samples were analyzed by several different methods. In each study the Primus boronate affinity HPLC method was used as the comparison method because results from boronate affinity chromatography are not expected to be influenced by the presence of Hb variants.[20–22] For HbAS and HbAC this was validated by comparison to a mass spectrometric reference method for HbA1c that had been shown previously to have no interference from HbS or HbC.[23] For each test method, results obtained for each type of sample (heterozygous variant and homozygous HbA) were compared to those obtained using the comparative method. An overall test of coincidence of two least-squares linear regression lines was performed to determine whether the presence of the variant trait caused a statistically significant difference in results relative to the comparative method. Deming regression analysis was then performed to determine whether the presence of the variant trait produced a clinically significant effect on HbA1c results. Given recommendations by the American Diabetes Association (ADA) of an upper reference limit of 6%, the ADA goal of 7%, and the DCCT conventional group mean HbA1c of approximately 9%, HbA1c evaluation limits of 6 and 9% were used. After correcting for possible calibration bias by comparing results from the homozygous HbA group, the method bias due to the presence of the variant trait was evaluated with a clinically significant difference being >10% relative bias at 6 and 9% HbA1c.

Elevated HbF

Hemoglobin F is the major hemoglobin during intrauterine life. HbF contains two α chains and two γ chains. At birth, the level of HbF is between 60 and 95%. During the first year of life, the percentage of HbF falls to values close to adult levels. The upper limit of normal is generally taken as 1% in adults. Elevated HbF levels can occur in patients as a result of pathologic conditions (e.g., leukemia, anemia, thalassemia) or a hereditary persistence of fetal hemoglobin.[18] Individuals with the most common form of hereditary persistence of fetal hemoglobin can have HbF levels of up to 30%, and because they are generally asymptomatic, patients and their physicians may be unaware of the existence of the condition.[15] A few studies have examined the effects of elevated HbF on HbA1c methods with variable results, but these studies did not

use a comparative method known to be free of interference from HbF. However, the International Federation of Clinical Chemistry (IFCC) Reference Method (IFCC RM) for HbA1c measures glycated and nonglycated hexapeptides from HbA β chains. Because HbF has no β chains, HbF does not cause interference with the IFCC RM because only the HbA terminal hexapeptides are measured. Therefore, a few commercial HbA1c assay methods have been evaluated using the IFCC method for comparison.[24]

In the past, many of the ion-exchange column methods (including minicolumns that measured HbA1) and electrophoresis methods could not separate HbF from HbA1c or HbA1; HbF comigrated or coeluted with HbA1c. Most of the current ion-exchange HPLC methods separate normal levels of HbF into a separate peak. Some of these methods (as indicated in **Table 1**) can also separate HbF when HbF levels are elevated.

Interference from HbAS, AC, AE, and AD and Elevated HbF

Results of these studies on HbS, C, E, and D and elevated HbF are summarized in **Table 1**. Methods are grouped by method type (immunoassay, ion-exchange HPLC, boronate affinity, and enzymatic) and within method type are listed in alphabetical order by the manufacturer. For certain research studies, criteria for clinical significance that have been selected may be too wide. For HbS, C, E, and D the exact biases and differences at 6 and 9% HbA1c can be found in the publications from which **Table 1** is derived.[7–10,19]

As shown in **Table 1**, there are clinically significant interferences with some methods for each of these variants. It is also important to consider the possible risks of sending out an inaccurate result due to Hb variant interference. Immunoassay methods provide a result with no indication of a possible interference unless the result is outside of the clinical range, i.e., the sample may only be repeated and/or investigated if the result is unusually high or low. Unfortunately, interference from Hb variants is not always dramatic enough to raise these concerns but it may be biased enough to cause errors in treatment; a few immunoassay methods show this type of interference from HbS and HbC. Fortunately, there is no interference from Hb variant D or E with any of the immunoassay methods that have been evaluated.

Interferences from Hb variants using ion-exchange HPLC methods can usually be detected in the chromatograms. Results may be either biased high or low depending on the specific method. It is important for users of these methods to be aware of these interferences and to know what to look for in the chromatogram in deciding whether or not the result is acceptable. In most (but not all) cases, reporting of inaccurate results can be avoided if manufacturer instructions are followed carefully. For some methods, however, manufacturer instructions alone do not provide sufficient information for making the correct decision about reporting results.[19]

For some time it was assumed that boronate affinity methods do not show interference with elevated F, as all glycated hemoglobins are measured regardless of the Hb structure. In an early paper by Weykamp and colleages,[6] glycated hemoglobin results from a manual boronate affinity column method were low for subjects with a hereditary persistence of HbF but the conclusion was that these results were likely correct. A later study using the IFCC RM as the comparative method showed that boronate affinity results are low in samples with elevated HbF and the results are related to the amount of HbF.[24] This interference could be a consequence of a lower glycation rate for HbF compared with HbA. Approximately 60% of HbA glycation occurs at the amino-terminal valine residue of the β chain.[15] HbF has two γ chains, for which the terminal residue is glycine, in place of β chains. The N-terminal residue of the γ chain presumably is glycated at a slower rate, leading to a lower concentration of GHB for a given plasma glucose concentration. Because boronate affinity measures the ratio of glycated to nonglycated hemoglobin regardless of species, the presence of elevated HbF results in a false lowering of the HbA1c result.

Other Hb Variants

Less common Hb variants have also been evaluated in terms of their effects on HbA1c measurements.[4] There are several ways that a Hb variant can cause some kind of interference with some methods. If the Hb substitution causes a change in the net charge of the Hb (as with Hb variants S, C, D, and E), then it may cause interference with methods such as ion-exchange HPLC or electrophoresis. In some cases the variant Hb (both glycated and nonglycated) may coelute or comigrate with HbA1c. If there is a substitution at a glycation site, this could alter the rate of glycation and affect certain methods. One example is Hb Raleigh where there is a Val→Ala substitution at the β chain terminus, which produces substantial acetylation, thus preventing glycation at this position and falsely lowering results

Table 1.
Interference of Heterozygous Variants S, C, D, E, and Elevated HbF with Specific HbA1c Methods

Manufacturer	Method	Interference from				
		HbAS	HbAC	HbAE	HbAD	↑ HbF
Immunoassay						
Abbott	Architect/Aeroset	Yes ↑	Yes ↑	—[b]	—[b]	—[b]
Bayer (Metrika)	A1cNOW	Yes ↑	Yes ↑	No	No	—[b]
Beckman	Synchron System	No	No	No	No	—[b]
Dade	Dimension	No	No	No	No	—[b]
Olympus	AU system	Yes ↑	Yes ↑	No	No	—[b]
Ortho-Clinical	Vitros	No	No	No	No	—[b]
Point Scientific	HbA1c on Modular P	No	No	No	No	—[b]
Roche	Cobas Integra	Yes ↑	Yes ↑	—[b]	—[b]	—[b]
Roche	Cobas Integra Gen.2 (Tina Quant)	No	No	No	No	—[b]
Roche/Hitachi	Hitachi (Tina Quant)	No	No	No	No	—[b]
Siemens (Bayer)	Advia	Yes ↑	Yes ↑	—[b]	—[b]	—[b]
Siemens (Bayer)	DCA 2000	No	No	No	No	Yes[c]
Ion-exchange HPLC						
Bio-Rad	D-10 (short)	No	No	No	No	—[b]
Bio-Rad	D-10 (extended)	No	No	No	No	—[b]
Bio-Rad	Variant A1c	No	No	No	Yes ↓	—[b]
Bio-Rad	Variant II A1c	No	No	No	No	No
Bio-Rad	Variant II Turbo A1c	No	No	Yes ↑	Yes ↑	—[b]
Menarini	HA8140 (diabetes mode)	Yes ↑	No	—[b]	—[b]	—[b]
Menarini	HA8160 (diabetes mode)	No	No	Yes ↓	Yes ↓	—[b]
Menarini	HA8160 (TP mode)	No	No	No	Not quantified	—[b]
Tosoh	A1c 2.2 Plus	No	No	Yes ↓	No	Yes[c]
Tosoh	G7	No	No	Yes ↓	No	No[d]
Tosoh	G8	—[b]	—[b]	Yes ↓	No	—[b]
Boronate affinity						
Axis-Shield	Afinion	No	No	No	No	—[b]
Primus	Boronate affinity HPLC	No	No	No	No	Yes[c]
Other						
Diazyme	Direct enzymatic A1c	No	No	No	No	—[b]

[a] An ↑ or ↓ indicates an artificial increase or decrease in results.
[b] Not evaluated.
[c] HbF levels above 15% cause a clinically significant low bias.
[d] Offline manual recalculation must be performed if the HbF peak is mislabeled as labile HbA1c (LA1C).

for immunoassay and likely boronate affinity methods. If the variant causes a reduced erythrocyte life span, the HbA1c (or total GHB) would be falsely lowered, regardless of the method used. Each variant Hb must be evaluated to determine the extent of the interference with each method.

Conclusions/Summary

In summary, Hb variants can interfere with HbA1c methods for a variety of reasons. Depending on the patient population for a particular laboratory, this can be a significant concern. All HbA1c methods are inappropriate for the assessment of glycemic control in patients homozygous for HbS or HbC, with HbSC disease, or with any other condition that alters erythrocyte survival. Laboratorians should be aware of the limitations of their method with respect to interference from the most prevalent Hb variants. They can also select new methods that are less likely to have interference. If an ion-exchange HPLC method is used, then careful inspection of chromatograms may identify the presence of aberrant peaks produced by variants. In this way unacceptable results can be detected and, in most cases, alternate HbA1c methods can be used. As with any laboratory test, any result that does not fit the clinical picture should be investigated further with the clinician.

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