REVIEW ARTICLE

Challenges in Hemoglobin A1C Measurement in Patients with Diabetes having Hemoglobin Variants and Chemically Modified DerivativesDilshad Ahmad Khan¹, Sumbal Nida²**ABSTRACT**

Diabetes is considered as one of the most common metabolic disorder. It has challenged in terms of diagnosis, monitoring and management in the patients with type-2 diabetes. Glycosylated Hemoglobin (HbA1c) has recently been validated for the diagnosis diabetes in non-fasting condition, long term blood glucose monitoring and also predicting its complications in the patients. National Glycohemoglobin Standardization Program and International Federation of Clinical chemistry have been collaborating for the harmonization of HbA1c methods and directing the laboratories to maintain strict quality goals. However, standardization of various HbA1c methods being used worldwide still needs consideration especially in diabetic patients having hemoglobin variants. The main objectives are to review HbA1c methods and address challenges in its measurement methodology for the patients with diabetes mellitus having hemoglobin variants or chemically modified derivatives. Thus, to provide guidance to the clinical pathologist for selection of appropriate method for their laboratories.

Key Words: *Capillary Electrophoresis, Diabetes, Glycated Hemoglobin HbA1C, Hemoglobin Variants, HPLC.*

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Introduction

Glycosylated Hemoglobin A1c (HbA1c) measures the average level of blood glucose which is attached to hemoglobin over the past 2 to 3 months.¹ The hemoglobin is of two types, the non-glycated Hb which is the abundant part i.e 95-98%, and the glycated Hb i.e HbA1a1, HbA1a2, HbA1b and or HbA1c. HbA1c makes about 80% of glycated Hb. It is produced by non-enzymatic addition of glucose to the N-terminal valine of the haemoglobin β -chain. The formation takes place in two steps with initial formation of unstable Schiff base followed by rearranged stable ketoamine.² American Diabetes Association (ADA) included HbA1c level of > 6.5 % as a diagnostic criterion for diabetes mellitus and recommended for monitoring of average blood glucose for the patients.¹ Later the WHO also

recommended the same criteria of HbA1c for the diagnosis of diabetes.

International Federation of Clinical Chemistry (IFCC) developed a reference method in 2002 that specifically measures the concentration of only one molecular species of glycated hemoglobin: the HbA1c. This method was calibrated by primary reference material formulated by mixing pure HbA1c and pure HbA0. It is isolated, quantified by capillary isoelectric focusing, and electrospray ionization mass spectrometry.³ Later IFCC attempted to contribute to the standardization by establishing a working group.⁴ IFCC recommends two methods as the reference method, namely HPLC /electrospray mass spectrometry (HPLC-ESI/MS) and capillary electrophoresis.⁵ The IFCC has also instructed to ensure the traceability of the calibrators and controls to the reference material of higher purity. Desirable performance criteria is < 3%.

Several methods are available for the measurement of HbA1c. Clinical laboratories use highly precise commercial methods that are based on several different methodologies, i.e., ion-exchange high-performance liquid chromatography (HPLC), boronate affinity HPLC, capillary electrophoresis, immunoassay and enzymatic method.⁶ The task of

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standardization of HbA1C assays was carried out by National Glycohemoglobin Standardization Program (NGSP) following the Diabetes Complications and Control Trial (DCCT) trial.⁷ The central primary reference laboratory in NGSP still uses the in-house ion-exchange HPLC method that was used by the reference laboratory in the DCCT. NGSP emerged as the first program to carry out the standardization that led to better accuracy and precision of various assays. However, the variability persisted. To ensure an even better standardization of different methods, NGSP recently narrowed down the criteria of acceptable performance from 6% to 5% in 2019.⁸ Haemoglobinopathies are common, autosomal recessive disorder worldwide and carry an abnormal α and β chains of haemoglobin genes. The Hb variants are more prevalent in Africa and some areas of the Mediterranean, Middle East, and Southeast Asia.⁹ There are four common Hb variants that have been identified include hemoglobin S (HbS), hemoglobin E (HbE), hemoglobin C (HbC), and hemoglobin D (HbD). A survey in 2002 in Karachi, Pakistan, showed that beta thalassemia major, beta thalassemia trait, sickle cell disease, hemoglobin D Punjab, hemoglobin C and hereditary persistence of fetal hemoglobin constituted 20.6%, 13%, 5.1%, 0.76%, 0.32% and 0.22% respectively in our clinical setting.¹⁰ About 5% of Pakistani population are carriers of beta thalassemia. Hb 1Ac level may be low in normoglycemic subjects with the hemoglobin E.¹¹ Hb variants may interfere in the analysis of HbA1c, depending on the method used and may be misinterpreted the results in the diabetic patients. Moreover, many patients are misclassified due to wrong HbA1c value due to Hb variants/chemically modified derivatives by using immunoassay methods. Thus, HPLC and capillary electrophoresis methods are recommended for accurate measurement of HbA1c results in clinical practice.

Analytical Methods of Glycated Hemoglobin

Several analytical methods are used for HbA1c measurement in the patients with diabetes in the clinical laboratories which include enzymatic assay, immunoassay, boronate affinity assay, capillary electrophoresis and high-performance liquid chromatography. However, IFCC recommend two methods of HbA1c analysis in the clinical chemistry laboratory based on separation and chemical measurements principle; ion exchange

chromatography and capillary electrophoresis. The chemical method utilizes the reaction with the glycated N-terminal valine of the beta chain for HbA1c measurement by using the photometric method. A summary of these methods advantages and disadvantages of are briefly given in Table 1.

High Performance Liquid Chromatography (HPLC) Ion-Exchange Chromatography

The separation takes place on the basis of charge. The sample is passed through a column of resin containing oppositely charged particles as that of the analyte. The buffers of different ionic strength are used to elute the hemoglobin species, whose absorbance are measured spectrophotometrically.¹¹ The instruments based on this principal are generally robust, with better throughput and quality. The ability to identify various Hb variants is also an advantage to know its effects on Hb A1c measurements.¹² Ion-exchange HPLC assays are currently the second most common type of HbA1c method used in the clinical laboratory according to statistics from the CAP proficiency testing program.¹³ The problem is that different instruments have different resolving power even though being obtained from the same vendor. The challenge with decreased chromatographic resolution, is the increased risk of interference from the Schiff base, carbamylated Hb, or Hb variants, which may co elute with the peaks of interest and cause an erroneous HbA1c result. This method also requires a dedicated instrument and expert technicians which may not be feasible in the clinical laboratories.¹⁴

Boronate Affinity Chromatography

This is the method which pertains to the structure of the analyte and detects the cis-diol groups of glucose bound to the Hb.¹⁵ The non-glycated Hb elutes from the gel containing boronic acid leaving behind the glycated component. Which is later displaced by the sorbitol as driven by the pH of buffer and the hydrophobic interactions.

The analytical performance is dependent on the quality of the column being used. If a robust column is being used it can perform as good as an IEC. Since the detection is based on the structure and not on the charge it is immune to most of the non-glucose adducts and the Hb variants.^{16,17} This inability to detect Hb variants may actually affect the interpretation of the results in some cases.¹⁸ It measures the total glycated Hb which can give false results in certain cases.¹⁹

Table 1: Methods of measurement, advantages, and disadvantages of the HbA1C assays used in clinical laboratories.

Method	Advantages	Disadvantages
Enzymatic	<ul style="list-style-type: none"> • can be done on routine chemistry analyzer 	<ul style="list-style-type: none"> • Unable to detect Hb variants
Immunoassay	<ul style="list-style-type: none"> • No analytical interference from Hb variants in newer-generation assays 	<ul style="list-style-type: none"> • Different assays have different Antibody specificity which may cause interference with Hb variants • Immunoassay methods do not recognize non glucose adducts, which may also nonenzymatically attach to hemoglobin as a result of uremia, / high doses of aspirin • Unable to detect Hb variants
Boronate affinity	<ul style="list-style-type: none"> • Minimal interference from Hb variants • Analytical quality is as IEC 	<ul style="list-style-type: none"> • Boronate affinity chromatography measures total glycated hemoglobin via attachment of the cis -diol groups of glucose to boronic acid. Because recognition is based on structure and not charge, • It does not recognize non glucose adducts and most of variants.
Ion-exchange HPLC	<ul style="list-style-type: none"> • Separation of HbA1c is based on charge and good quality, • Fetal Hb (HbF), minor fast Hb(HbA1a/b), and carbamylated Hb (HbCarb) as well as genetic variants (e.g., sickle cell Hb) can also be visualized 	<ul style="list-style-type: none"> • Increased risk of interference from the Schiff base, carbamylated Hb, or Hb variants, which may coelute with the peaks of interest and cause an erroneous HbA1c result
Capillary electrophoresis	<ul style="list-style-type: none"> • Accurate HbA1 C result and can detect Hb variants with high resolving power between the different Hb peaks. • Can automate and increase throughput by using multiple capillaries. • Interpretation / User friendly 	<ul style="list-style-type: none"> • The runtime is longer • Technician need to do regular preventive maintenance.

Capillary Electrophoresis (CE)

Capillary electrophoresis is a method of HbA1C analysis that separates different types of Hb peaks based on ion charge /physical properties in the chromatogram. The separation of Hb takes under the net effect of electromotive and electroosmotic force under applied voltage. HbA1c has an additional peak separated from Hb A0 due to loss of one positive charge amino group from HbA1c by the attachment of the glucose moiety.²⁰ The common Hb variants that usually interfere have little to no interference in this method.²¹ It also has high resolving power

between the different Hb peaks. HbA1c peak can easily be differentiated with Hb variants (Figure-1). Because the reproducibility is well within the criteria set by the IFCC, bias and interference are well within the criteria set by the NGSP, and robustness is demonstrated over a longer period in patients whole blood samples, capillary electrophoresis method is recommended for diagnosis and monitoring of patients with diabetes mellitus. Nowadays instruments are available with automation and high through analysis due to multiple capillary option in the instruments.

Fig 1. Chromatogram of HbA1c determination on CE of Hb variants' samples. (a) A2A: Normal; (b) β -thalassemia trait; (c) Homozygous HbE;

Immunoassays

This method uses antibodies that specifically bind to the glycated amino terminus of the Hb β chains. Commercially available methods use antibody-mediated turbidimetric inhibition immunoassay for measurement of HbA1c in blood. HbA1c is expressed as a percent after Hb is measured photometrically and the ratio HbA1c/Hb is determined.²² It can be performed on automatic chemistry analyzers. The earlier first-generation assays had antibodies with lower specificity resulting in interference of the Hb variants which may cause misleading Hb A1c values for patients with Hb variants (ie, HbSS, HbCC, HbSC, or HbS β -thalassemia).²³ Detection of Hb variants remained a challenging task in south east countries, where thalassemia is a common disease. This results in underestimation through immunoassay analyzers with subsequent lower detection of diabetic patients.²⁴ The method also does not recognize the adducts that may occur in uremia, high dose of aspirin and alcohol.²⁵⁻²⁷ However, the new immunoassays have improved specificity. Analysis of HbA1c on the point of care instrument are not standardized with IFCC and NGS for most the devices. The results may be the normoglycemic range at immunoassay in the diabetic patients and reduction in follow up for diabetes prevention. We do not recommend the POC device for diagnosis and monitoring of the diabetic patients.

Enzymatic Method

Fructosyl valine oxidase acts on the glycated valine in the Hb. Hydrogen peroxide is formed that reacts with a chromogen giving a color. It becomes proportional to the amount of glycated Hb. Total as well as glycated Hb are measured photometrically.²⁸ This, can be done on routine chemistry analyzers. However, the separate measurement of total and glycated Hb may decrease the analytical quality of the test. Since the method involves two steps, it is not very cost effective. Its inability to detect the Hb variants makes it a lesser option in our part of the world due to the high prevalence of various Hb variants. Newer methods are being developed called the direct enzymatic assays that may prove to be useful in future.²⁹

Effect of Hemoglobin Variants on Measurement of HbA1c

Haemoglobinopathies and various biological factors can affect the accuracy of HbA1c measurements

depending upon the type of method used as shown in Table 1. All methods have their limitations. Most often, reporting of analytically inaccurate results can be avoided if the consumer follows carefully the instructions of the manufacturer.

The concept of glycated Hb is vital to understand in order to avoid interferences in the method. It is the molecule formed after the non-enzymatic glycation of the valine amino acid at the N terminal of the beta chain of the Hb represented as HbA1c. Total glycated Hb include glucose being attached to alpha chain amino group of lysine residue as well. Out of the methods discussed the boronate affinity measure the total glycated Hb not HbA1c. Since the Hb variants such as Hb S, Hb D, Hb E and Hb C involve substitution of amino acids at different positions, they may affect the immunoassay-based methods. They also alter the total charge on the molecule and may cause interference in the ion exchange chromatography.

The patients with hemoglobin variants affect the result by causing falsely high or low levels depending on the method being used. Patients with Hb SS, Hb CC, and Hb SC should be interpreted with caution due to the effect of anemia and altered cell life span.³⁰ The presence of Hb F has been observed to co elutes with other peaks. However, the current ion exchange chromatography can separate the two peaks. Understanding this concept of HbA1c is vital to understand the interpretation of the chromatograms.¹⁵

In a study the effect of Hemoglobin variants on four methods were observed. It was identified that the Hb F had significant interference at concentration of 5%,10-15%,15% and 30% by TINIA, capillary electrophoresis, Boronate affinity and Ion exchange HPLC respectively. The Hb E was however easily picked up by capillary electrophoresis as compared to HPLC.³¹

Another study compared the results of HbA1c of patients, which screened negative for haemoglobinopathies on HPLC and Capillary electrophoresis-based instruments. The results showed significant correlation ($R^2 = 0.99$, $P < 0.0001$). However, the analysis of patients with known HbS variant showed underestimation of HbA1c by HPLC compared to capillary electrophoresis. Similar results were seen with a patient with HbD variant. It was however successfully detected by capillary

electrophoresis.³²

In another study good correlation existed between the results of HPLC and capillary electrophoresis ($r=0.935$). Precision study had a total CV of 1.22 % and 1.15 % by HPLC and capillary electrophoresis respectively in the diabetics patients. Similarly considering the threshold values of Icterus, Hb F, labile Hb and carbamylated Hb on the two methods showed Capillary electrophoresis, to be a better choice.³³ A recent study compared results between HPLC and immunoassay of HbA1c revealed not good correlation ($r: 0.368$), with a mean bias of $-0.50 \pm 1.62\%$ ($-5.5 \pm 17.7 \text{ mmol/mol}$).³⁴ The NGSP evaluates the effects of Hb variants and periodically updates on the web site. It should be consulted, to be well aware for certain interferences before dishing out the results.²¹

Effect of Chemically Modified Derivatives on measurement of HbA1c

Carbamylated hemoglobin is formed in patients of chronic kidney disease. It is due to high urea that results in increased binding of isocyanic acid to the molecule. Labile fraction is formed at early stages of glycation process and depends on the blood glucose concentration.³⁵ There is incomplete separation between carbamylated hemoglobin and HbA1c in HPLC. HbA1c is not a reliable indicator of glycemic control in patients of end stage renal disease. Levels of carbamylated hemoglobin may be upto 2% of total Hb in uremic patients.³⁶ Glycated albumin is a better option in such cases. The effect of carbamylated and the labile fraction on the result also varies with the method at hand. It was observed, that an in vitro exposure of sample to potassium cyanate resulted in increase of HbA1c from 5.2 to 5.9 % on Ion-exchange chromatography. But had no effect on results by capillary electrophoresis. A study showed that increase in labile fraction of more than 4% and carbamylated Hb of more than 2% resulted in decrease of Hb HbA1c results by ion-exchange chromatography.^{37,38}

Several other conditions including blood transfusion, hemorrhage, anemia affect HbA1c measurements.³⁹ Iron deficiency is one of the major causes of anemia which affects the HbA1c levels.⁴⁰ Increased cell survival also tends to increase the HbA1c levels such as in iron, folate and VitB12 deficiency anemia. The exact cause of this is still unclear. It is postulated that decreased production of reticulocytes is balanced by

decreased destruction of red blood cells. Thus, ensuing maintenance of total RBC population.⁴⁰ On the other hand the conditions that lead to decreased cell survival results in lower values of HbA1c.⁴¹⁻⁴³ The effect of common interferences in the have been discussed in table 2.

Table 2: Factors affecting the results of HbA1C

Factors	Causes	Effect on HbA1C result	Rectification
Erythropoiesis	<ul style="list-style-type: none"> Increased red cell life span e.g. Splenectomy Iron deficiency anemia, Vit B12 deficiency 	Increase in HbA1C	Glycated Albumin
Erythrocyte destruction	<ul style="list-style-type: none"> Decreased life span e.g. Splenomegaly, Hemolytic anaemia, Drugs 	Decrease in HbA1C	Glycated albumin
Haemoglobinopathies	<ul style="list-style-type: none"> Hb F, Met Hb Increased bilirubin, carbamylated hemoglobin, aspirin, alcoholism Increased triglycerides and bilirubin 	Increase or decrease Increase in HbA1C Decreased in HbA1C	Capillary electrophoresis HPLC Capillary electrophoresis HPLC Capillary electrophoresis
Assay related	<ul style="list-style-type: none"> Hemoglobinopathies 	Variable HbA1C	Capillary electrophoresis

Conclusion

In conclusion, the selection of the HbA1c method require technical expertise for diagnosis of diabetes in the patients having haemoglobin variants or chemically modified derivatives. Familiarity with optimum specificity, sensitivity, interferences and limitation of HbA1c assays are essential for diagnosis and monitoring of average blood glucose in the patients with diabetes. HPLC assay has relatively decreased chromatographic resolution with increased risk of interference from the Schiff base and Hb variants, which may co elute with the peaks of interest. Capillary electrophoresis seems to be the recommended method with minimum interference and better resolution especially for countries where haemoglobin variants are more prevalent.

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