GLYCATED HAEMOGLOBIN (HBA1C): AN UPDATE ON AVAILABLE METHODS

Ezegbogu, M.O.[1] and Abdulsalam, K.[2]
[1]Department of Chemical Pathology, University of Benin, Benin, Edo State, Nigeria.
[2]Department of Chemical Pathology and Immunology, Bayero University, Kano/Aminu Kano Teaching Hospital, Kano
Correspondence author: kabdussalam.cpat@buk.edu.ng; +2348039749586

ABSTRACT
Glycated haemoglobin (HbA1c) assays give a retrospective index of the integrated plasma glucose over an extended period, usually 90-120 days. This period is, however dependent on the average red cell lifespan of the individual. Since its discovery in the 20th century, improvement in the analytical techniques for HbA1c have facilitated its wide acceptance as a useful tool in monitoring glucose control, and more recently, has been recommended for the diagnosis of diabetes mellitus. This review provides an update on the methods available for estimating HbA1c levels in the light of their suitability, advantages, and drawbacks.
Keywords: Analytical Methods, Diabetes Mellitus, Glycated Haemoglobin (HbA1c)

INTRODUCTION

Measurement of glycated proteins, primarily Glycated Haemoglobin is effective in monitoring long-term glucose control in people with diabetes mellitus since It provides a retrospective index of the integrated plasma glucose values over an extended period of time and is not subject to the wide fluctuations observed when assaying blood glucose concentrations (David, 2006). This period of retrospective glucose estimation coincides with the lifespan of healthy red blood cells, which is usually between 90 and 120 days (American Diabetes Care, 2003).

Glycated haemoglobin (HbA1c) has been established as an index of long-term blood glucose concentrations as a measure of the risk of the development of complications in patients with diabetes mellitus (American Diabetes care, 2007). HbA1c assays correlate nicely with mean plasma glucose values (Rohlfing, 2002; Nathan *et al.,* 2008). In addition to being a useful tool for monitoring glucose control in known diabetics, it is now established as a useful tool for screening and diagnosing diabetes mellitus (WHO, 2009). However, misleading HbA1c values may be obtained when the average red cell lifespan is compromised (Xianthis *et al.,* 2007). These conditions include:

1. Increase in red cell turn over, such as blood loss, recent blood donation, haemolysis, haemoglobinopathies (e.g. sickling disease), red cell disorders, myelodysplastic disease, and severe malaria parasitemia (Frank *et al.,* 2000; Roberts *et al.,* 2002; Bain, 2006; Little *et al.,* 2008).

2. Interference with the test; this depends majorly on the test method employed. Common interfering molecules include haemoglobin variants such as Hb-F, S, C, D, and E (Bry and Sacks, 2001; Little and Roberts, 2009).

3. Scenarios where patients' circulating glucose values fluctuate between very high and very low values; in such cases, HbA1c values should be compared with home capillary blood glucose test charts (Gupta *et al.,* 2017).

Historical Perspective of HbA1c

HbA_{1C} was first separated from other forms of haemoglobin by Huisman and Meyering in 1958 using a chromatographic column. It was subsequently characterized as a glycoprotein by Booklin and Gallop in 1968. Its increase in diabetes mellitus was first described in 1969 by Samuel O`Rahbar *et al*. The reactions leading to its formation were characterized by Bunn and his co-workers in 1975 and its use in monitoring the degree of glucose metabolism in diabetic patients was proposed in 1976 by Anthony Cerami, Ronald Koenig, and coworkers. Barely five years on, the HbA1c assay was widely accepted in clinical practice. Consequently, several analytical methods were devised for measuring glycohaemoglobin levels, however, with varying degrees of accuracy and precision. In 2009, an international expert committee appointed by the American Diabetes Association (ADA), International Diabetes Federation (IDF) and the European Association for the Study of Diabetes (EASD) published a report that made the case for using HbA1C assay to diagnose type 2 diabetes (American Diabetes Association, 2010).

Clinical utility of Glycated Haemoglobin

In the normal approximately 120-day lifespan of the red blood cell, glucose molecules react with haemoglobin, forming glycated haemoglobin. In individuals with poorly controlled diabetes mellitus, the quantities of these glycated haemoglobins are much higher than in healthy people. Once a haemoglobin molecule is glycated, it remains that way throughout its life cycle. Measuring glycated haemoglobin accesses the effectiveness of therapy by monitoring long term serum glucose regulation (International Expert Committee, 2009). The HbA_{1C}-level is proportional to the average blood glucose concentration of four weeks to three months (Nathan *et al.,* 2007). However, it has been suggested that over half of an HbA1c value is attributable to the previous month, a further quarter to the month before that, and the remaining quarter to the two months before that.

HbA_{IC} is formed by the condensation of glucose with the N-terminal value residue of each β-chain of HbA to form an unstable Schiff base (aldimine, pre-HbA_{IC}).

The Schiff base may either dissociate or undergo an Amadori rearrangement to form a stable ketoamine, HbA_{1c} (David, 2006).
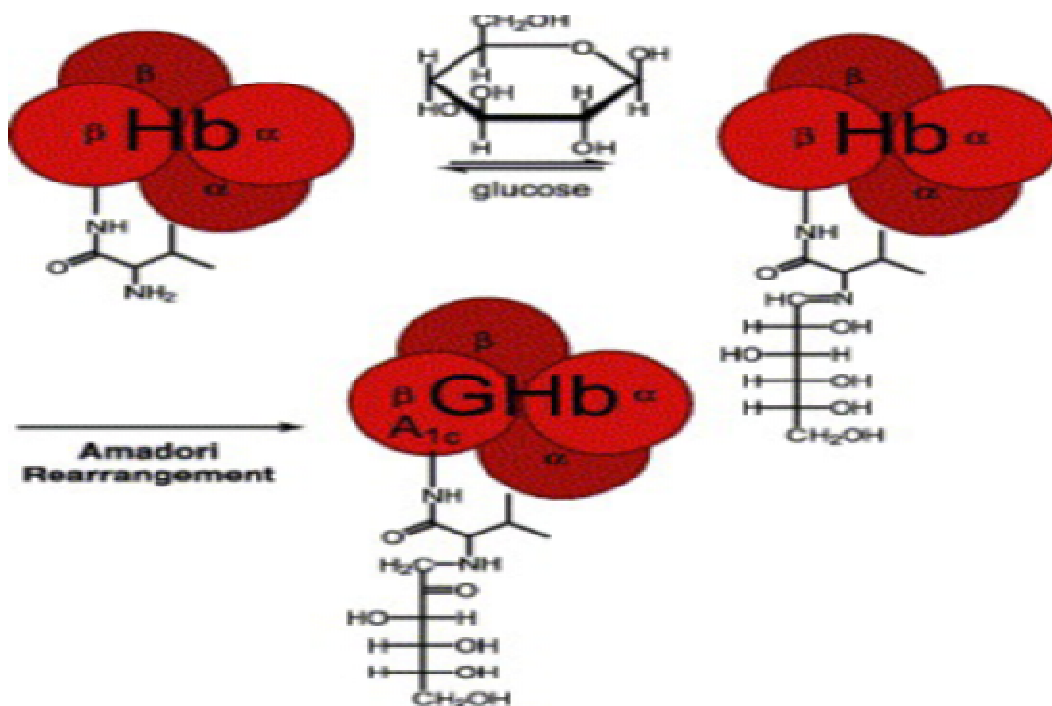
Figure 1: Chemical reaction summarizing the Formation of Glycated Haemoglobin (Burtis CA. et al 2006)

Monitoring and Diagnosis of Diabetes Mellitus

Numerous studies support the use of HbA_{1c} as an objective measure of glycaemic control (Peacock, 1984; Sacks, 1994; Cagliero *et al.,* 1999; Xanthis *et al.,* 2007; Reynolds *et al.,* 2007; Nathan *et al.,* 2007; Trapp *et al.,* 2008; Gallagher *et al.,* 2009). HbA1c concentrations are therefore a valuable and widely used adjunct to blood glucose determinations for monitoring long-term glycaemic control. In addition, the HbA1c assay is a measure of the risk for the development of complications of diabetes (Sacks *et al.,* 2012). These risks are associated with the likelihood of the development of Advanced Glycation End-products as is commonly seen in diabetics, eventually leading to the development of micro- and macrovascular diabetic complications such as coronary heart disease (Stratton *et al.,* 2000; Selvin *et al.,* 2007), retinopathy (DCCT, 1995; Miyazaki *et al.,* 2004; Trapp, *et al.,* 2008), nephropathy, and neuropathy (Larsen *et al.,* 2010).

Accurate assessment of glycaemic control using HbA1c assays has helped eliminate the confusion clinicians face in monitoring their patients as a result of the erratic fluctuations accrued to glucose-based methods. For instance, quite different conclusions may be drawn from blood taken at 9.30am soon after breakfast (When blood glucose might be over 20mmol/l) and at the end of an afternoon clinic (when

blood glucose in the same patient may be lower than 3mmol/L). Of course, simply measuring HbA1c cannot by itself realize the standards of good glucose control, but the result can indicate whether or not success has been achieved.

Since the wide acceptance of HbA1c assays in clinical medicine, researchers have made a case for its application on the diagnosis of diabetes mellitus. In July 2009, an international expert committee (appointed by the American Diabetes Association (**ADA**), International Diabetes Federation (**IDF)** and the European Association for the Study of Diabetes (**EASD**) published a report supporting the use of HbA1C assay to diagnose type 2 diabetes. Use of HbA1c to diagnose diabetes has been considered by the expert panels in the past, but the idea was roundly rejected (American Diabetes Association, 1997; American Diabetes Association, 2003). The primary obstacle was a lack of standardization of the assay, but that is no longer the case (Consensus Committee, 2007). In fact, A1C is better standardized than other measurements of glucose (The International Expert Committee, 2009). Other advantages of HbA1C include the fact that it is a better indication of overall glycemic exposure over time and that there is substantially less day-to-day variability.

From a practical standpoint, HbA1c is much easier to measure because it does not require fasting or timed samples, and it is currently used to manage diabetes. The attractiveness of a single test to both diagnose and manage diabetes, especially while the test is easy to administer and largely reproducible, suggests that greater reliance on the A1C assay is inevitable. The WHO Consultation concluded that HbA1c can be used as a diagnostic test for diabetes, provided that stringent quality assurance tests are in place and assays are standardized to criteria aligned to the international reference values, and there are no conditions present which preclude its accurate measurement. An HbA1c of 6.5% is recommended as the cut point for diagnosing diabetes. A value less than 6.5% does not exclude diabetes diagnosed using glucose tests. The expert group however refrained from making any formal recommendation on the interpretation of HbA1c levels below 6.5% as a result of insufficient scientific data at the time (WHO, 2011).

Glycated Haemoglobin in Pregnancy
Pregnancy is associated with many physiological derangements including hyperglycaemia which may tend to gestational diabetes mellitus especially in pre-disposed patients if left unattended. In view of the undoubted impact of diabetic control on foetal well-being, it is especially important to aim for and achieve normoglycaemia during gestation (and indeed before conception). Home blood glucose monitoring has allowed women to stay at home up to the time of delivery, overcoming the need for admission to hospital for several months to check blood sugar which used to be routine practice. In these circumstances, it is reassuring to carry out HbA1c measurements.

Certainly, HbA_{1c} concentrations during pregnancy show a positive correlation with birth weight which is consistent with established experience that large babies are associated with poorly controlled diabetes. There are changes in glucose tolerance during pregnancy even in non-diabetic women, so that "normal" for HbA_{1c} in pregnancy needs to be established separately (Diabetes care, 2007; Yu *et al.*, 2014; Ye *et al.*, 2016). However, Soumya *et al* (2015) suggested 5.3% as the diagnostic cut-off for diabetes mellitus in cyesis.

Laboratory Methods for Glycated Haemoglobin
There are nearly one hundred different methods available for measuring glycated haemoglobin (Sacks, 2011). Commercial assays to measure HbA1c became available in 1978 (Davis *et al.,* 1978; Beccaria *et al.,* 1978), and the test gained popularity during the 1980s. The first mention of glycated hemoglobin by the World Health Organization was in 1985 when the potential value of its measurement in diabetes was indicated (WHO, 1985). Three years on, the American Diabetes Association (ADA) suggested that HbA1cdetermination should be performed at least semiannually for routine monitoring of patients with diabetes (American Diabetes Association, 1985). The commercialization of HbA1c assays led to the development of a plethora of methods to measure glycated hemoglobin. The general concept underlying these methods is to separate the glycated from the non-glycated hemoglobin and quantify the amount of each (Sacks, 2012).

Laboratory techniques that have been used to achieve this are based on:
Charge differences:
a. Ion-exchange chromatography
b. High performance liquid chromatography (HPLC)
c. Electrophoresis
d. Isoelectric focusing
Structural differences
a. Affinity chromatography
b. Immunoassay
Chemical analysis: (Now obsolete)
a. Photometry
b. Spectrophotometry (David, 2006).

1.   Ion-Exchange Mini-columns.
Ion-exchange chromatography separates haemoglobin variants on the basis of charge. The cation exchange resin (negatively charged), packed in a disposable minicolumn, has affinity for the positively charged haemoglobin. The patient's sample is haemolyzed and an aliquot of the haemolysate is applied to the column. A buffer is applied and the eluent collected. The ionic strength and pH of the eluent buffer are selected so that GHbs are less strongly bound to the negatively charged resin and thus are eluted first. The GHbs - $A_{Ia} + A_{Ib} + A_{IC}$, expressed collectively as HbA, are measured using a spectrophotometer. A second buffer of different ionic strength can be added to the column to elute the more positively charged main haemoglobin fraction. This is read in the spectrophotometer and GHb is expressed as a percentage of total Haemoglobin (Burtis *et al.*, 2006). Alternatively, only the HbA, is eluted and a separate dilution of the original haemolysate is made, against which the HbA, is compared. In all ion-exchange column methods, it is important to control the temperature of the reagents in order to obtain accurate and reproducible results (David, 2006).

2.   High Performance Liquid Chromatography (HPLC)
This method uses an ion exchange or affinity column to separate HbA1c molecules from other hemoglobin molecules (Hamwi, 1995; Camargo and Gross, 2004). The HbA1c content is measured based on the ratio of HbA1c peak area to the total hemoglobin peak areas.

Assays require $5 \mu l$ of whole blood and finger stick samples can be collected in a capillary tube for analysis. Anticoagulated blood is diluted with a haemolytic agent containing borate. Samples are incubated at 37^0C for 30 minutes to remove Schiff base and inserted in the autosamplers. One major advantage of this technique is the rapid turn-around time, which is usually as short as 3-5mins (David, 2006).

Electrophoresis
Agar gel electrophoresis on whole blood haemolysates at pH 6.3 provides good resolution of HbA and HbA_1 (Menard *et al.,* 1980).

The gel contains negatively charged moieties that interact with the haemoglobin. After 25-35 minutes, the HbA1c separates on the cathodic side of HbA. Quantification is performed by scanning densitometry at 415nm. Results generally agree well with those obtained by HPLC or column chromatography, but are less precise (Menard *et al.*, 1980; David, 2006).

David (2006) opined that since HbF migrates to the same region as HbA, it may cause a falsely increased HbA. This position was further butressed by Litttle*et al* (2009). However, Doelman *et al., (*1997) working independently surmised that HbA1c assays using capllary electrophoresis is free from interference by haemoglobin variants (HbF, HbC, and HbS).

3. Isoelectric Focusing
The haemoglobin variants separate on isoelectric focusing on the basis of their migration in a gel containing a pH gradient (Spicer *et al* 1978; Cossu*et al.,* 1984). Ampholines on pH range of 6-8 establish the gradient in 1mm-thick acrylamide gel slabs. On completion of isoelectric focusing, the gels are fixed and then scanned on a high-resolution integrating microdensitometer. HbA_{ic} is adequately resolved from HbA_{Ia}, HbA_{IC}, Hbs, and HbF (Koval*et al.,* 2011).

4. Affinity Chromatography
Affinity gel columns are used to separate GHb, which binds to the column, from the non-glycated fraction. m-aminophenylboronic acid is immobilized by cross-linking to beaded agarose or another matrix (e.g. glass fiber). The boronic acid reacts with the cis-diol groups of glucose bound to haemoglobin to form a reversible five-member ring complex, thus selectively holding the GHb on the column. The non-glycated haemoglobin does not bind, and Sorbitol is used to elute the GHb. Absorbance of the bound and non-bound fractions, measured at 415nm, is used to calculate the percentage of GHb

5. Immunoassay
Assays of HbA_{IC} have been developed using antibodies raised against the Amadori product of glucose (Ketoamine linkage) plus the first few amino acids at the N-terminal and of the β-cahin of haemoglobin (Engback *et al.,* 1989). The latex enhanced immunoassay method is based on the interactions between antigen molecules (HbA1c) and HbA1c specific antibodies coated onto Latex beads. The agglutinator, a synthetic polymer containing multiple copies of the immunoreactive portion of HbA_{IC} blocks the anti- HbA_{IC}monoclonal antibody that is attached to latex beads. This agglutination produces light scattering, measured as an increase in absorbance. HbA_{1c} in the patient's sample competes for the antibody on the latex, inhibiting agglutination, thereby decreasing light scattering (Jaisson, *et al.,* 2014; Maurice, 2015). The antibodies do not recognize labile intermediates or other Glycated Haemoglobins (such as HbA_{1a}, HbA_{1b}) because both the ketoamine with glucose and the specific amino acid sequences are required for binding. Similarly, other haemoglobin variants, such as HbF, HbA_2, HbS and carbomylated haemoglobin are not detected (Bry *et al.*, 2001).

Recent innovation has yielded a Direct Enzymatic HbA1c Assay which uses a single channel test and reports %HbA1c values directly, without the need for a separate Total Haemoglobin test or a calculation step (Liu *et al.,* 2008). The Direct Enzymatic HbA1c Assay™ procedure is simple and straight forward. In addition, the reagents do not contain latex particles, and hence do not coat analyzer cuvettes and lines. Most importantly, enzymatic HbA1c assays have the highest specificity among all HbA1c assays with a linearity range from 4 to 16% (Gupta *et al.,* 2017).

Table 1: A comparison of commonly used methods for estimating HbA1c (Chandalia and Krishnaswamy, (2002)

Analytical Method	Ion-Exchange Chromatography	HPLC	Immunoassay	Chemical Method
Species measured	HbA1C	HbA1C	HbA1C	Total GHb
Pre-HbA1C interference	YES	YES (removed in some)	NO	NO
Drug interference	YES	YES	NO	NO
Inter-assay CV	2-10	2	6-8	6-8

Table 2: A Summary of the Advantages and Disadvantages of commonly used methods for estimating HbA1c

Advantages	Method of Testing	Disadvantages
HbA1c overestimation leads to aggressive glucose management, resulting in more frequent hypoglycaemic episodes (Hamwi*et al.,* 1995).	Chromatography based HPLC assay	a. Altering the normal process of gly cation of HbA to A1C. b. Causing an abnormal peak on chromatography, making estima tion of A1C unreliable. c. Making the red blood cell more prone to hemolysis, thereby de creasing the time for glycosylation to occur and producing a falsely low A1C result (Carmago and Gross, 2004).
Reduces the scattering of light and the absorbance(Maurice, 2015).	Antibody based immunoassay	a. Time required to complete the analysis. b. Technical skills required for han dling. c. High price of reagents.
a. Enzymatic assay proved to be a robust and reliable method for HbA1c measure element suitable for routi ne practice in clinical chemistry laboratories (Jaisson*et al.,* 2014). b. The assay is designed to report %HbA1c values directly without need for a separate measurement of total hemoglobin and is not adversely affected by interferences from com mon hemoglobin variants in samples (Liu *et al.,* 2008).	Enzyme-based enzymatic assay	A disadvantage of the enzymatic method is its relatively high cost

Standardization of HbA1c Assays

One of the major drawbacks of the clinical use HbA1c was its lack of Standardization as different test methods reported their values using different formats. This not only confused clinicians, but diabetics as well using mmol/L or mg/dl for self-monitoring of their day-to-day glucose control found it difficult to understand when their results were discussed in terms of percentages (Hoelzel *et al.,* 2004; Little *et al.,* 2001). In 2007, the International Federation of Clinical Chemistry (IFCC) recommended that HbA_{1c} results be expressed as $mmolHbA_{1c}/mmolHb$ instead of HbA_{1c} percentage (Diabetes care, 2007).

To eliminate confusion and streamline these discrepancies, a consensus statement on the worldwide standardization of haemoglobin A_{1c} measurement was adopted in May 2007 by the American Diabetes Association (ADA), European Association For The Study Of Diabetes (EASD), International Diabetic Federation (IDF) And The International Federation Of Clinical Chemistry (IFCC). It states that new IFCC reference system is the only valid anchor for implementing the standardization of the measurement of HbA_{1c}. In addition, HbA_{1c} results were to be reported worldwide in IFCC units (mmolglycatedHb/mol total Hb) and derived NGSP units (%). Using the IFCC-NGSP master equation, the 25 – 42 (mmol/mol) range would indicate non-diabetics as the similarly derived NGSP units of the non-diabetic range were 2.5 – 4.2% (HbA_{1c}) (Diabetes Care, 2007).

Table 3: Relationship between the HbA1C units

Current HbA1c (%)	New HbA1c (mMol/Mol)
6.0	42
6.5	48
7.0	53
7.5	59
8.0	64
9.0	75

IFCC-HbA1c (mmol/mol) = [**DCCT**-HbA1c (%)-2.15] x 10.929 (Gallagher *et al.*, 2009)

CONCLUSION

Diabetes mellitus, a metabolic disease characterized by the inability of an individual to properly handle glucose load is a major disease burden worldwide, especially in low and middle-income countries and is predicted to increase from 240 million to 380 million by 2025 (Shaw *et al.,* 2010). Experts have recommended that early diagnosis and effective monitoring of diabetics will significantly improve treatment outcomes. Since the introduction of Glycated Haemoglobin in clinical medicine in the 20th century, it has roundly been accepted as an indispensable tool in monitoring diabetics, and more recently, has been included into the protocol for diagnosis diabetes mellitus (American Diabetes Association, 2010).

This review is aimed at providing an update on the laboratory methods available for HbA1c estimation as well as their merits and drawbacks. HbA1c assays are commonly executed using Capillary electrophoresis, HPLC, and Immunoassay, although all have their inherent limitations. The immunoassay technique has greater prospects for routine use due to its relatively low cost, rapidity, sensitivity, and specificity. Moreover, it is easy to use and has been adapted as a reliable Point of Care testing Device.

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