

HbA1c and factors other than diabetes mellitus affecting it

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ABSTRACT

Glycated haemoglobins are haemoglobins with an attached sugar moiety. They constitute the HbA1 fraction of the adult haemoglobin HbA. HbA1c is the predominant fraction of HbA1 and gives an estimate of the blood sugar levels of an individual over the last three months. It has been observed that an HbA1c value of less than seven percent reduces the microvascular complications in diabetic patients. However, HbA1c is not affected by blood sugar levels alone. Apart from blood sugar, there are other factors that affect HbA1c. This article reviews in detail the structure, formation, methods of measurement, factors affecting HbA1c levels and their clinical significance.

Keywords: factors affecting HbA1c, glycated haemoglobins, HbA1c

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INTRODUCTION

HbA1c, a glycated haemoglobin, is formed by the glycosylation of haemoglobin. Its value represents the glycaemic status of a person over the last two to three months.⁽¹⁾ It is measured in diabetics as well as in those with impaired glucose tolerance to assess the glycaemic status over the last two to three months. According to the American Diabetes Association (ADA) Guidelines 2007, the value of HbA1c should be kept below 7% in all diabetics.⁽²⁾ According to the same guidelines, HbA1c is now referred to as A1c. Values greater than 7% indicate an increased chance of progression to diabetic complications, especially microvascular ones. Glycated haemoglobin does not include HbA1c alone. It includes other haemoglobins as well and together, these constitute the HbA1 fraction of adult haemoglobin HbA.⁽¹⁾ Among the various glycated haemoglobins, HbA1c is the predominant fraction. It appears from the abovementioned discussion that HbA1c is affected by blood glucose levels alone. However, certain studies have proven that HbA1c levels are altered in haemolytic anaemias, haemoglobinopathies and

nutritional anaemias such as iron deficiency anaemia. Even the method of assay can alter the HbA1c levels.

HISTORICAL PERSPECTIVE

Maillard has described the reaction of reducing sugars with amino acids which resulted in a stable ketoamine adduct formation.⁽³⁾ In 1958, Allen et al demonstrated that normal adult haemoglobin could be separated chromatographically on a cation exchange resin into a main component, accounting for more than 90% of the haemoglobin and three negatively charged minor components, which they designated as HbA1a, HbA1b and HbA1c, and collectively known as HbA1.⁽⁴⁾ However, they did not demonstrate the nature of these fractions. Later, Huisman and Dozy observed a two- to three-fold rise in the HbA1 fraction in four diabetic patients who were being treated with tolbutamide.⁽⁵⁾ In 1968, Rahbar reported similar findings in two diabetic patients at Tehran University and later found a similar abnormality in 57 diabetic patients.^(6,7) In a larger study of diabetic patients, Trivelli et al found a two-fold increase of HbA1c over values observed in non-diabetic subjects.⁽⁸⁾ Thus, by the mid 1970s, it was clear that HbA1 and HbA1c are elevated in humans with diabetes mellitus, although the mechanism of this abnormality was not understood.

BIOCHEMICAL CONSIDERATIONS

In 1966, HbA1c was shown to be identical to HbA, except that it has an unidentified group, aldehyde or ketone, that is attached to the N-terminal of the beta chain. The linkage was reducible by sodium borohydride (NaBH₄).⁽⁹⁾ Later, it was shown using mass spectroscopic analysis that a hexose was attached to the N-terminal valine of both the beta chains.⁽¹⁰⁾ Bunn et al subjected HbA1c to mild acid hydrolysis and isolated sugars (glucose and mannose) in a ratio of 3:1.⁽¹¹⁾ They first reduced the level of HbA1c by titrated NaBH₄ and then after acid hydrolysis, nearly all the radioactivity was recovered as (3H) formic acid. This suggested that in red cells, glucose reacts with N-terminal valine of both the beta chains to form an aldimine linkage which then undergoes an Amadori rearrangement to form a

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more stable ketoamine link. This also explains why, during acid hydrolysis, both glucose and mannose are released because racemisation at the second carbon atom gives glucose and its C-2 epimer mannose. It was initially believed that the rearrangement of aldimine to ketoamine was an irreversible process, but it was later proven to be a reversible process.^(12,13)

The next major advancement in the understanding of the nature of HbA1c was the discovery that it could be formed by incubating either whole blood or purified haemoglobin in the presence of glucose at 37°C.⁽¹⁴⁾ The rate of absorption of (14C) labelled glucose into haemoglobin to form HbA1c was the same whether purified or crude haemoglobin was used, suggesting that the action was not mediated by a red cell enzyme. Studies of the kinetics of conversion of HbA to HbA1c *in vivo* have lent further weight to the theory that the process is non-enzymatic.⁽¹⁵⁾ Here, injections of 59Fe-bound transferrin into a normal volunteer enabled specific radioactivity in the haemoglobin to be measured over a period of 100 days. The activity in the major part of adult haemoglobin HbA, designated as HbA0, reached a peak at 15 days and was then relatively constant for the next 80 days, consistent with normal erythropoiesis and a cell viability of approximately 100 days. The specific activities of HbA1a, HbA1b and HbA1c increased only gradually however, and continued to rise throughout the 100 days, exceeding the activity of HbA0 from approximately 60 days onwards.⁽¹⁵⁾ Several other observations were consistent with HbA1c being formed throughout the life span of the red cell as a post-synthetic modification of HbA. When human reticulocytes or marrow were incubated with radioactively labelled amino acids, the specific activity of HbA1c was much lower than that of HbA0. Young red cells, isolated by density gradient, have lower levels of HbA1c than older red cells.⁽¹⁶⁾

The amino terminus of the beta chain is not the only site of formation of glucose adducts with haemoglobin. The amino terminus of the alpha chain is similarly modified, although at an eight- to ten-fold lower rate, both *in vivo* and *in vitro*.⁽¹⁷⁾ Moreover, the modification at that site has an insufficient effect on the charge of the protein to permit separation by ion exchange chromatography in the same way as with HbA1c. There are a number of epsilon amino groups of lysine throughout the alpha and beta chains, but due to the conformational structure of the haemoglobin molecule, more reactive amino groups may be less accessible to free glucose.⁽¹⁸⁾

NOMENCLATURE

The term 'glycosylated' was used initially, but it

has been pointed out that this term strictly refers to glycosides. Therefore, the Joint Commission on Biochemical Nomenclature has proposed that the term 'glycation' is appropriate for any reaction that links a sugar to a protein, or in the particular case of a reaction with haemoglobin, the term 'glycated haemoglobin'.⁽¹⁹⁾ In the ADA Guidelines 2007, HbA1c has been referred to as A1c.

RELATION OF GLYCATED HAEMOGLOBIN TO GLYCAEMIC CONTROL IN DIABETES MELLITUS

This was first convincingly shown by Koenig et al, who examined the relationship between HbA1c and glycaemic control in five poorly controlled diabetic patients.⁽²⁰⁾ Improvement in glycaemic control caused a reduction in the levels of HbA1c after approximately four weeks. In another study of newly diagnosed diabetic patients, initially elevated levels of HbA1c were observed to decrease gradually in the weeks following the onset of dietary and insulin therapy, with a tendency to level out after approximately seven weeks.⁽²¹⁾ Reports such as these tend to substantiate the view that the measurement of glycated haemoglobin would provide an accurate and objective index of glycaemic control in diabetic patients.

LABILE HBA1C

In 1979, Svendsen et al demonstrated that the short-term (6–12 hours) exposure of red blood cells to high glucose concentrations, both *in vivo* and *in vitro*, led to significant increases in the glycated haemoglobin, as measured by ion exchange chromatography.⁽²²⁾ At the same time, it was demonstrated by Goldstein et al that HbA1c measured by high performance liquid chromatography (HPLC) increased two hours after a standard breakfast, that the increase in HbA1c correlated closely with the plasma glucose increase, and that incubating the red cell in 0.9% saline for five hours at 37°C before HbA1c assay eliminated the post-prandial increment.⁽²³⁾ This phenomenon is due to the unstable Schiff base or aldimine (also known as labile HbA1c) formed as an intermediate step in the glycation reaction, and it may be a potential source of error in any assay that relies on the effect of glycation on the charge of the molecule. In order for the original concept of glycated haemoglobin as an index of long-term integrated glycaemia to hold good, the labile fraction should be removed before such an assay is performed, and this can be achieved by saline incubation, dialysis or other chemical methods.⁽²⁴⁾ It is clear that in some patients, the perception of glycaemic control, as reflected by

glycated haemoglobin, could be altered.⁽²⁵⁾ To eliminate this fraction, the chromatographic kits now contain an additive in their haemolytic reagent that eliminates the labile fraction.

METHODS OF MEASURING GLYCATED HAEMOGLOBIN

These methods can be divided into two groups: those that depend on the effect of glycation on the charge of the molecule and those that depend on identifying a specific property of the ketoamine linkage in glycated haemoglobin. Methods that depend on the effect of glycation on the charge of the molecule measure HbA1c or HbA1, but not the 'total' glycated haemoglobin, i.e. HbA1, plus haemoglobin glycated at sites other than the amino terminal valine of the beta chain. Since the other group of methods detects the specific property of ketoamine linkage in glycated haemoglobin, these methods measure the 'total' glycated haemoglobin.

Methods that are dependent on the charge of the molecule

(1) Ion exchange chromatography: This has been the most widely used technique. The results are expressed as the percentage of total haemoglobin. The original method utilised macrocolumns and enabled the separation of the individual fractions HbA1a, HbA1b and HbA1c, which being more negatively charged than HbA0, elute before HbA0.⁽⁸⁾ However, this is a tedious process that requires large quantities of buffer and cyanides. The pH is critical, with small changes affecting the degree of separation of the minor haemoglobins. The minicolumn system, which has now largely replaced the above system,^(26,27) has the advantage of speed and ease of handling, and is available in the form of a kit, as prepacked columns with prepared buffers, standards and additives to eliminate labile adducts. However, with the minicolumn technique, the glycated haemoglobin cannot be measured as separate fractions but as HbA1. This separation is influenced by temperature; for each 1°C rise in temperature, HbA1 increases by 0.25%.⁽²⁸⁾ Therefore, a constant temperature has to be maintained. Another problem is the presence of variant haemoglobins like foetal haemoglobin (HbF), which co-elutes with HbA1 and gives falsely high readings, whereas haemoglobin C (HbC) and sickle cell haemoglobin (HbS) co-elute with HbA and lead to the underestimation of HbA1.⁽²⁹⁾ Hence, where there is a prevalence of variant haemoglobins, this technique should be used with caution. Labile haemoglobin can also lead to high HbA1 and should be eliminated before

assay. In most assays, the range of HbA1 in non-diabetic subjects is 5%–9%, with the levels in diabetic patients ranging up to approximately 20%. The coefficient of variation is usually 2%–3% for the same day analysis, while the inter-assay variation is 4%–5%.^(26,28)

(2) High performance liquid chromatography: For HPLC, the principles of measurement of HbA1c and HbA1 are the same as for ion exchange chromatography, but the use of high flow pressures and finely divided resins results in a more constant flow rate, as well as a faster and more accurate separation.^(30–32) However, this is an expensive method.

(3) Isoelectric focusing: In this technique, haemolysate is applied to a thin-layer polyacrylamide gel containing an ampholyte with a pH level of 6–8, followed by the application of a suitable voltage to separate the haemoglobin fractions, and finally quantification by high resolution microdensitometer.^(33,34) Despite the fact that the difference in isoelectric points between HbA1c and HbA0 is only 0.02 pH units, accurate separation can be achieved. This method has the advantage of having the HbF, HbC and HbS migrate separately. The inter-assay variation is 6.9%–12.6%, which is higher than that of other techniques; however, this is also an expensive procedure.

(4) Agar gel electrophoresis: In this technique, haemolysate is applied to the agar gel at the anodic site, and after electrophoresis with a citrate buffer at 60V for 40 minutes, HbA1 is located cathodic to HbA0 and is then quantified by scanning densitometry at 420 nm after the gel has been fixed by heat drying for 20 minutes.^(35,36) It is also essential to eliminate the labile component here. HbC and HbS migrate to points anodic to HbA and do not interfere with its estimation, but HbF migrates to the same point as HbA1. The intra-assay variation is 1.6%–7.3%, while the inter-assay variation is 2.6%–7.3%.

Methods of detecting a ketoamine linkage⁽¹⁸⁾

(1) Weak-acid hydrolysis: This is one of the oldest methods, where glycated haemoglobin is hydrolysed by a weak acid and the amount of 5-hydroxymethyl furfural (5-HMF) released is quantified colourimetrically after reaction with thiobarbituric acid. This is an inexpensive method, but has some disadvantages. 5-HMF is destroyed as it is being released and its production is non-stoichiometric. Glucose itself interferes with the colour formation in proportion to its concentration, and the hydrolysis step lasts for several hours. To overcome these disadvantages, scrupulous adherence to the rigid assay conditions is required. By performing the

hydrolysis in an autoclave at increased temperatures and pressures, the yield of 5-HMF is enhanced and is more constant over a much shorter period of time. The use of fructose or glycated haemoglobin standards helps to correct the variation in hydrolysis between assays. These precautions and modifications lead to an intra- and inter-assay coefficient of variation of less than 2% and 3%, respectively, as well as shorten the procedure to less than two hours. The advantages of this method are its ability to detect glycation at all sites and non-interference from the labile fraction or haemoglobin variants.

(2) Affinity chromatography: For this method, aminophenyl boronate that is immobilised on cross-linked agarose provides a suitable matrix for affinity chromatography columns. Glycated haemoglobin adsorbs to the affinity gel, while non-glycated haemoglobin passes through the system. The adsorbed fraction is then removed by elution with a competing ligand at high concentrations, enabling the calculation of the percentage of total glycated haemoglobin. This method is very precise, with the coefficient of variation at 2%–2.6%. It is sensitive to temperature changes, and there is conflicting evidence on the interference by labile HbA1. Therefore, it is advisable to remove the labile HbA1. It is less sensitive to pH changes, and there is no interference from variant haemoglobins.

CLINICAL USE OF HBA1C

The HbA1c test has been used in diabetics, as HbA1c is formed over a period of two to three months and reflects the glycaemic status of a patient over the past two to three months.⁽³⁷⁾ In the ADA guidelines 2007, HbA1c has been referred to as A1c. These guidelines recommend that A1c should be performed at least twice a year in patients who are meeting their treatment goals (and who have stable glycaemic control), and quarterly in patients whose therapy has changed or who are not meeting their glycaemic goals. The availability of the A1c results at the time when the patient is seen (point-of-care testing) has been reported to result in the frequency of intensification of therapy and improvements to glycaemic control.^(38,39) There is a correlation between the A1c levels and mean plasma glucose levels on multiple testings over two to three months.⁽⁴⁰⁾ For example, an A1c value of 6% corresponds to a mean plasma glucose level of 135 g/dl.

The ADA has also recommended that the lowering of A1c reduces the risk of microvascular and neuropathic complications and possibly, macrovascular complications. A1c should thus be kept to less than 7% for patients in general and to less than 6% for individual

patients. A1c is the primary target for glycaemic control. The guidelines also suggest that post-prandial glucose may be targeted if the A1c goals are not met despite reaching pre-prandial glucose levels.⁽²⁾

CONFOUNDING MEDICAL CONDITIONS

Accepting glycated haemoglobin as a reflection of integrated glycaemia presupposes two basic assumptions: first, that the patient has a normal red cell life and second, that the particular assay method is specific for the non-enzymatic adduction of glucose to haemoglobin. The various confounding medical conditions are as follows:

(1) Acute and chronic blood loss: This decreases the red cell survival rate, which results in a decrease in the A1c levels.^(41,42)

(2) Haemolytic anaemias: Similarly, due to decreased red cell survival, the A1c is lowered.⁽⁴³⁾

(3) Haemoglobin variants: These can alter the A1c levels, as discussed previously.

(4) Blood urea: de Boer et al have shown that in patients with uraemia and normal glucose tolerance, glycated haemoglobin measured by ion exchange chromatography was significantly elevated, with seemingly no correlation with the degree of glucose intolerance.⁽⁴⁴⁾ This is due to the excessive amount of cyanate derived from the urea, which causes carbamylation at the N-terminal valine residue, and this carbamylated haemoglobin results in an increase in the HbA1(a + b) and hence, the increased levels of HbA1.⁽⁴⁵⁾ Agar gel electrophoresis and weak acid hydrolysis should be used in cases of elevated blood urea levels as they do not detect the carbamylated haemoglobin.⁽⁴⁶⁾ In chronic renal failure patients, haemolysis and sometimes the gastrointestinal loss of blood lowers the A1c levels. Hence, the effect of urea on A1c levels varies.

(5) Pregnancy: Studies have shown that HbA1c levels decrease during the second trimester of a normal non-diabetic pregnancy and rise during the third trimester.⁽⁴⁷⁻⁴⁹⁾ These changes are very slight, amounting to less than 1% of total haemoglobin. When using glycated haemoglobin as an aid in assessing glycaemic control in diabetic pregnancies, these 'physiological' changes should be kept in mind.

(6) Other anaemias: Brooks et al assessed the HbA1 values in 35 non-diabetic patients with iron deficiency anaemia before and after treatment with iron.⁽⁵⁰⁾ They observed that the HbA1 values were significantly higher in iron deficiency anaemia patients and that the levels decreased after treatment with iron. The mechanism leading to increased glycated HbA1 levels was not clear.

It was proposed that in iron deficiency, the quaternary structure of the haemoglobin molecule is altered, and that glycation of the beta globin chain occurs more readily in the relative absence of iron.⁽⁵⁰⁾ Sluiter et al attempted to provide an explanation for the above findings, and they were of the view that the formation of glycated haemoglobin is an irreversible process and hence, the concentration of HbA1 in one erythrocyte would increase linearly with the cell's age.⁽⁵¹⁾ In patients with normal blood glucose values and red cells that are younger than usual, after the treatment of iron deficiency anaemia, the HbA1 concentration falls. However, if the iron deficiency has persisted for a long time, the red cell production rate would fall, leading not only to anaemia but also to a higher than normal average age of circulating erythrocytes and therefore, of an increased HbA1.⁽⁵¹⁾

In 1980, Mitchell et al⁽⁵³⁾ commented on the study conducted by Brooks et al, in which they calculated the absolute amount of HbA1 in each red cell, i.e. the mean corpuscular HbA1, and found that there was no difference in the HbA1 level before and after iron treatment. They also analysed the study conducted by Sluiter et al and were of the view that red cell age was unlikely to be a significant factor in explaining the change in HbA1 during the treatment of iron deficiency anaemia.⁽⁵²⁾ Later, Heyning et al also reported no change in the HbA1c concentrations compared to controls among non-diabetic patients with iron deficiency anaemia before and after treatment. They proposed that the reported differences in the HbA1c concentrations before and after iron supplementation are due to differences in the laboratory methods used for measuring HbA1c.⁽⁵³⁾ However, Rai et al investigated the different methods, and observed that no difference was detected among colourimetric, ion exchange chromatography and affinity chromatography.⁽⁵⁴⁾ Hansen et al demonstrated that there was no significant difference in HbA1c concentration levels among iron deficient patients, vitamin B₁₂ deficient patients and controls.⁽⁵⁵⁾ They were of the opinion that in iron deficiency anaemia, the erythrocyte survival rate is normal, while in vitamin B₁₂ deficiency, the red cell survival rate is decreased, whereas the haemolytic component is often minor and affects both mature and immature erythrocytes. Hence, normal levels of glycated haemoglobin are to be expected. The HbA1c levels decreased on treatment of anaemia, which was probably due to increased bone marrow erythropoiesis on treatment, leading to the production of new immature erythrocytes.⁽⁵⁵⁾ Further studies showed that the HbA1c levels were higher in patients with iron deficiency anaemia and that the levels decreased significantly following treatment with iron.^(56,57) The

probable explanation of elevated HbA1c in iron deficiency anaemia is that supposing serum glucose is accepted to remain constant, a decrease in the haemoglobin concentration might lead to an increase in the glycated fraction. The exact mechanism remains elusive.

NORMAL VALUE

The normal value of HbA1c is 3.8%–6.4%.⁽⁵⁸⁾

CONCLUSION

HbA1c is not affected by blood sugar levels alone, and there are various confounding factors when measuring HbA1c. It is hence prudent to rule out all other confounding factors before making a therapeutic decision based on the HbA1c levels. Also, the effects of iron deficiency and vitamin B₁₂ deficiency on HbA1c should be studied in greater detail.

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