

Glycated hemoglobin

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Glycated hemoglobin (GHb) is formed by a post-translational, non-enzymatic, substrate-concentration dependent irreversible process of combination of aldehyde group of glucose and other hexoses with the amino-terminal valine of the α -chain of hemoglobin. The estimation of GHb has provided a dependable method of assessing glycemic control in diabetics. We modified and established the chemical method of GHb estimation described by Fluckiger and Winterhalter based upon the generation of 5-hydroxymethylfurfural from the ketoamine by treatment with oxalic acid and reacting it with thiobarbituric acid to form a coloured adduct. This method has been extensively validated in diabetics as a reliable, inexpensive and non-manipulable parameter. In 48.7% of diabetics, metabolic control judged by GHb was discrepant from blood glucose; 63% of patients showing discrepant values seemed to diet better on the day of test to produce a better blood glucose value. We have critically reviewed the other methods of GHb estimation. We studied the rate of dissipation of GHb in a group of newly diag-

nosed type 2 diabetics in whom blood glucose was lowered steadily by using glipizide. We found that lowering of GHb at 2, 4, 6, 8, 10 weeks of treatment was 1.0, 0.7, 0.5, 0.5, and 0.1 per cent respectively. This fact has subsequently been corroborated by other investigators. A large number of kinetic studies have revealed that glycemia in the recent past influences the GHb values more than the remote past. Thus, mean blood glucose of past 1 month, 2 months and 3 months contributes 50%, 40% and 10% respectively to the final result. Though the role of GHb is established in monitoring control of diabetes, its role in diagnosis of diabetes still remains questionable. We have demonstrated increased glycation in impaired glucose tolerance and stress hyperglycemia, thus indicating that these groups need to be treated. We have correlated GHb with the degree of hemolysis in patients of hemolytic anemias. GHb was significantly lower in patients of hemolytic anemia as compared to anemia of other etiologies and had a significant positive correlation ($r = 0.69$; $P < 0.001$) with erythrocyte half-life.

OVER the past several decades, diabetes mellitus has become a major health problem worldwide, reaching epidemic proportions in many developing countries as well as in minority groups in the developed world^{1,2}. Worldwide projections suggest that more than 220 million people will have diabetes by the year 2010, and the majority of these, approximately 213 million, will have type 2 diabetes³. In 1997, healthcare costs related to diabetes in the US were about \$100 billion, with one-half in direct costs; these costs also are projected to rise considerably⁴.

Type 2 diabetes mellitus is associated with increased cardiovascular and overall mortality. In fact, type 2 diabetic patients diagnosed before 70 years of age, have only 70% of the life expectancy of nondiabetic people^{5,6}. Epidemiological data suggest that classic cardiovascular risk factors, such as hypercholesterolemia, hypertension and smoking alone do not account for the excess risk of cardiovascular morbidity and mortality in type 2 diabetes mellitus. Rather, the excess morbidity and mortality is linked to the disease itself. Type 1 diabetes is accompanied by long-term micro- and macrovascular complications, the primary causes of morbidity and mortality in these patients. Diabetic nephropathy, as the single most

common cause of end-stage renal disease, accounts for more than one-third of all cases. Thus, understanding the pathogenesis and preventing and/or ameliorating these long-term complications have been major goals of research in diabetes mellitus.

The core of the issue is glycemic control. It has long been suspected that high blood glucose is harmful in a variety of ways and that all the complications whether microvascular or macrovascular, were to a larger or lesser extent linked with it. In recent times this has been well established. Amongst the various markers of glycemic control, glycated hemoglobin (GHb) has now been established as the most reliable, though many other proteins are also glycated in the diabetic and non-diabetic states.

Historical aspects

Human hemoglobin, in its structural and functional features, is the most extensively studied protein. The major component of it Hb A₀ ($\alpha_2\beta_2$) comprises over 90% the total protein, with the other two minor components being Hb A₂ ($\alpha_2\delta_2$), and Hb F ($\alpha_2\gamma_2$), with non- α units being products of different globin genes. Hemoglobin A_{1c} (HbA_{1c}) is the most abundant minor component, arising

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from post-translational modification of HbA₀ (ref. 7). While, on one hand, specific glycosylations of proteins occur under precise enzymatic control to render such modified proteins perform functions such as integrity of plasma membranes and secretions of proteins representing physiological events; on the other hand, high concentrations of free sugar in the environment, under non-physiological incubation conditions, brings about the 'browning' reaction on account of the carbonyl groups on sugars forming Schiff base adducts with amino groups of proteins, resulting in poorly soluble brown products⁸⁻¹⁰. HbA_{1c}, on the contrary, in a unique set of conditions represents the formation of a non-enzymatically glycosylated form of human hemoglobin, taking place under physiological conditions, at a specific site on the protein, viz. the NH₂-terminal of the β-chain.

As early as 1958 its occurrence was reported¹¹ in normal population at around 5% of hemoglobin, presenting as a distinct minor component chromatographically. Earliest reports on HbA_{1c} elevations in diabetics appeared in the 1960s. Huisman and Dozy¹² noted a 2 to 3 fold elevation of HbA_{1abc} in four diabetics receiving tolbutamide. In an extraordinary painstaking and careful analysis of agar gel electrophoretic data in a survey of 1200 patients at Teheran University Hospital, two abnormal patterns were traced to diabetic patients¹³. The same group quickly confirmed this by examining 47 diabetic patients and observed elevations in the same chromatographic and electrophoretic component¹⁴.

Nonenzymatic glycation, structural insights and chemical studies

Of the several pathogenic mechanisms by which hyperglycemia may lead to altered tissue structure and function, non-enzymatic glycation (encompassing the attachment of the free aldehyde groups of glucose or other sugars to the non-protonated free amino groups of proteins) changes the structure and function of several soluble and insoluble proteins *in vivo* and *in vitro*¹⁵⁻²⁰. Because cells and their extracellular matrix share a dynamic and reciprocal relationship²¹, modulations of matrix components by glycation leads to altered cell behaviour, including changes in cell spreading, phosphorylation of key intracellular signaling molecules, and expression of extracellular matrix proteins and their modulators²²⁻²⁴. Extracellular matrix from diabetic patients is more extensively glycosylated than extracellular matrix from nondiabetic people. In addition, the accumulation of glycation products and the accompanying structural extracellular matrix modifications correlate with the development of functional complications of diabetes²⁵⁻²⁸. These changes in tissue structure and function are slow and cumulative, producing a long time lag between the start of diabetes and the onset and progression of the complications.

Earlier chromatographic studies indicated that HbA_{1c} was identical to HbA, with an identified group linked to the NH₂-terminal valine of the β-chain; that such modification was possible through a Schiff's base was indicated by its reduction by borohydride²⁹. Analysis by mass spectrometry of the blocking group by the same authors pointed to a hexose linked to NH₂-terminal valine, with no clue then to its structure or nature of linkage. Later, mild acid hydrolysis of HbA_{1c} yielded glucose and mannose in a 3 : 1 ratio, and a total yield of 20-30%. Elegant experimental evidence proved that in the red cell, glucose reacts first with the NH₂-terminal of β-chain to form an aldimine linkage, next undergoing an Amadori rearrangement to form the more stable ketoamine linkage, came by using tritiated borohydride reduction and subsequent oxidation with periodate of the isolated β-chain, containing 95% of the radioactivity³⁰. Nearly all the radioactivity was recovered as ³H formic acid, rather than ³H formaldehyde, strongly suggestive that the second carbon atom of glucose was tritiated rather than the first. Mechanistically therefore, in the red cell, glucose forms an aldimine linkage with NH₂- of valine of the β-chain, undergoing an Amadori rearrangement to form the more stable ketoamine linkage as shown in Figure 1.

One can thus explain on the basis of racemization at the second carbon atom during acid hydrolysis, the C-2 epimer of glucose, viz. mannose being formed. Treatment of HbA_{1c} with mild acid results (by promoting the dehydration of the sugar group) in the formation of 5-hydroxymethyl furfural (5-HMF) (Figure 2).

These mechanisms were promptly confirmed³¹ and conditions determined for the establishment of a colorimetric method of utility for the determination of HbA_{1c}. Encouraged by these observations, and driven by the need to have an economical and accurate methodology for HbA_{1c} determination for monitoring glycemic control, a modified thiobarbiturate methodology was developed and tested at the Jaslok Hospital for its value in assessing management of diabetics^{32,33}.

Retrospectively viewed, it is significant to note that these studies were carried out in India in the late 1970s

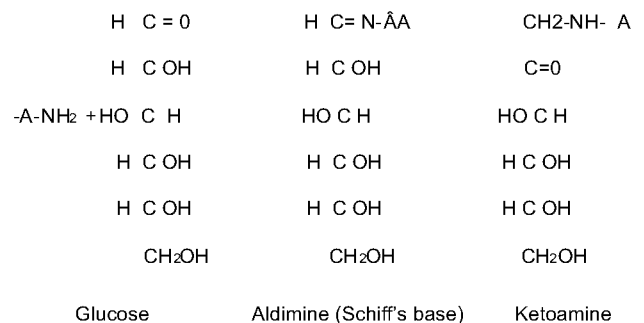


Figure 1. Amadori rearrangement of glucose molecule.

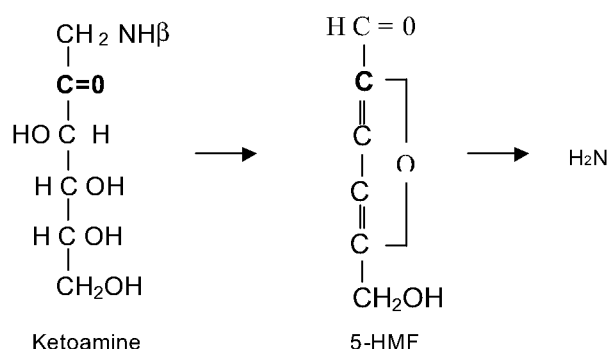


Figure 2. 5-HMF generated by treatment with oxalic acid of the ketoamine linkage by which glucose is covalently bound to hemoglobin forms a coloured adduct (absorbance maximal at 443 nm) with thiobarbituric acid.

and are among the very early efforts in the adoption of the glycohemoglobin measurement in diabetic clinical practice. It is also noteworthy that such interest in glycation of functional proteins in the diabetic was pursued to include glutathione-S-transferase and Cu-Zn superoxide dismutase^{34,35}, which led to further insights into glycation as a pathophysiological process in diabetes. Biosynthesis of glycosylated hemoglobins (HbA_{1a}, HbA_{1b}, and HbA_{1c}) occurs slowly, continuously and almost irreversibly throughout the four month life span of erythrocytes and the process is wholly non-enzymatic, as demonstrated by elegant human studies using ⁵⁹Fe-bound transferrin and measurement of specific radioactivity of the major and minor hemoglobin components during the entire life span of erythrocytes³⁶. Sugar phosphates, such as glucose-6-phosphate (G-6-P) present in red cells, can react with hemoglobin 20 times faster than glucose, in fact, with greater specificity than glucose. Fructose-6-phosphate, fructose-1,6-diphosphate, ribose-5-phosphate, ribulose 5-phosphate, and glucuronic acid but not glucose 1-phosphate or glucose 1,6-diphosphate react with hemoglobin with the rapid formation of the adduct, thus requiring an aldehyde or ketone group separated from a negatively charged COO⁻ or PO₄³⁻ group. It is very unlikely that G-6-P hemoglobin is a precursor of HbA_{1c}. Concentration of G-6-P in red cells is 1/200th that of glucose, but G-6-P reacts with hemoglobin ten times more rapidly than glucose. Structure-function relationship can be studied with considerable significance on both natural and synthetic derivatives, on account of the specificities in relation to sites of glycation. Studies on the role of potentially catalytic residues on the polypeptide (protein) which may be crucially involved in the Schiff base formation and Amadori rearrangement by bringing into spatial juxtaposition of carefully designed helical peptides, is a noteworthy step in the mechanistic understanding of protein glycation, with particular reference to the catalysis of Amadori rearrangement involved in the process^{37,38}.

Methodologies to estimate GHb

There remain numerous analytical problems associated with glycated hemoglobin measurement, such as the lack of assay standardization and the problems related to its measurement in particular patient groups with hemoglobinopathies, fetal hemoglobin, renal failure (who form hemoglobin derivatives) and hemolytic diseases.

Methods of GHb assays have primarily evolved around three basic methodologies:

- (1) Based on difference in ionic charge.
- (2) Based on structural characteristics.
- (3) Based on chemical reactivity.

Methods based on differences in ionic charge

These methods are in extensive use at present. Cation exchange chromatography can either be undertaken on mini columns or in a sophisticated, automated system. The pH and temperature conditions affect the results significantly, hence, the need for a sophisticated system where the conditions can be adequately controlled. HbA_{1c} possesses less charge positivity and hence elutes faster from a cation exchange column. Pre-glycohemoglobin has similar mobility in this system and hence, it should be removed before column chromatography. Most of these systems cannot differentiate between abnormal hemoglobins, but many advanced systems possess such ability. These methods are most commonly used in clinical practice. By this method HbA_{1c} concentration in normal subjects is 4.6–6%. Diabetes is considered to be under good, fair or poor control at values of < 7%, 7–8%, and > 8% respectively.

HbA_{1c} can be separated from HbA₀ by any electrophoretic method. The most commonly used method is agar-gel electrophoresis where HbA₁ migrates to cathodic side of HbA₀. Pre-GHb migrates with GHb in this system as well and hence has to be separated in advance. Some hemoglobinopathies, like HbS or HbC trait do not, but HbF does interfere with this method.

Methods based on structural characteristics of GHb

One such method utilizes a column containing *m*-aminophenyl boronic acid coupled to agarose. GHb possesses more *cis*-diol groups, which has stronger affinity to boronic acid and hence elutes later than HbA₀. This method is influenced to a lesser extent by pH, temperature and storage conditions than cation exchange chromatography. It is also unaffected by hemoglobinopathies. However, there is a batch-to-batch variation in gel characteristics, which makes application of this method difficult. Recently, immunoassays have been developed by using a HbA_{1c} specific monoclonal antibody. An aggluti-

nator is used in the system and inhibition of agglutination of HbA_{1c} and its antibody by HbA_{1c} in the sample is quantitated. This method is not influenced by pre-GHb or hemoglobinopathies³¹.

Methods based on chemical reactivity

Chemical method of GHb estimation is based on generation of 5-hydroxymethylfurfural (5HMF) from glyco-amino groups on hemoglobin, by heating the GHb in a weak acid³³. This chemical method measures total gly-cated hemoglobin, i.e. HbA_{1c} plus glycated non-N-terminal sites. The 5HMF so generated is reacted with thiobarbituric acid and read colorimetrically. We have extensively used this method^{32,33} in a modified form. This method is laborious but least expensive. It is a very sturdy method, least affected by storage, temperature and pH conditions. It is not influenced by pre GHb, but it is advisable to remove free glucose from samples by careful wash of erythrocytes before undertaking lysis. This method is not affected by hemoglobinopathies. Instead of a GHb standard, which is difficult to device, a fructose standard can be used and results expressed as amount of 5HMF. As this method estimates HbA_{1c} as well as HbA_{1a} and HbA_{1b}, the values obtained are higher than those of anion exchange chromatography by 1–2%. By our method, good, fair and poor control is defined as GHb level of < 8%, 8–10% and > 10% respectively.

Recent advances in estimation of glycohemoglobin

Recently, procedures using electrospray ionization mass spectrometry (ESI-MS) have been developed as candidate reference methods for estimation of HbA_{1c}, following a trend for many specific reference methods in clinical chemistry to be based on gas chromatography–mass spectrometry. Roberts *et al.*³⁹ and Peterson *et al.*⁴⁰ used ESI-MS to identify those components measured as ‘HbA_{1c}’. In both reports^{39,40}, at low GHb concentrations, hemolysates from diabetic patients revealed higher β -chain to α -chain glycation, whereas at high GHb concentrations, multiple β -chain sites were glycated, accompanied by increasing α -chain glycation. In addition, Roberts *et al.*³⁹ demonstrated that even at the highest value analysed for GHb, only a single glucose molecule was added to the multiple glycation sites. They also found reasonably good linear correlations of all established methods compared with ESI-MS, although the latter method generally reported a lower percentage of GHb than the separation methods. Kobold *et al.*⁴¹ analysed endoproteinase Glu–C digests of whole-blood samples. Endoproteinase Glu–C cleaves N-terminal segments of the β -chains between the two glutamic acid residues at positions 6 and 7, with the resulting fragments containing only a single glycation site

at the β -chain N-terminal valine. By this approach, interference by carbamylated and acetylated N-terminal species and by the dimer composed of a glycated α -chain and a non-glycated β -chain are excluded. In other words, HbA_{1c} as defined by the International Federation of Clinical Chemistry (IFCC) is actually measured. However, is it more important to determine glycation on only the β -chain N-terminal valine (which has been defined as HbA_{1c} by the IFCC working group) or to estimate all possible glycation sites in hemoglobin (i.e., the total gly-cated burden) as with the ESI-MS method without Glu–C digestion of the hemolysates. Overall, measurement of glycation at the N-terminal valine of the β -chain is more specific, and a proposed reference system demands an exact knowledge of the analyte to be measured. Alternatively, measurement of all potentially glycated sites might be clinically more relevant, especially at high GHb concentrations. All of these hold promise as candidate reference methods to yield a procedure to address the problem of standardization in GHb testing. However, mass spectrometric methods may not have a primary role in routine laboratory settings in the immediate future because of the expensive and sophisticated hardware and software, the modest throughput, and the technical knowledge required for accurate and reliable results.

Because the development of complications is linked to the accumulation of glycation adducts in tissue proteins, any analytical method that serves as an index of the extent of glycation should clearly be used to guide therapy in diabetes. Although the ion-exchange method does not meet contemporary standards for accuracy, immensely valuable prognostic information has been gathered with this procedure over nearly two decades of observing the 1441 and 3867 subjects, respectively, in the DCCT⁴² and the UKPDS⁴³. Other important studies, both prospective and retrospective, have used either the ion-exchange method or have used methods that correlate to the DCCT method for estimation of GHb. It is clear that near-normal glycemetic control is necessary to prevent the development and progression of complications. Moreover, because it is difficult to reverse complications, one cannot justify a clinical trial with another method to confirm the efficacy of glycemetic control on diabetic complications, as demonstrated by the DCCT and UKPDS. From the results of these clinical trials, it would be unethical to initiate a new prospective trial with treatment groups having different degrees of glycemetic control to test the efficacy of the new reference method for GHb. Therefore, regardless of the reference method(s) adopted, the HbA_{1c} concentrations measured in the DCCT and UKPDS will have to be related to values based on the new reference systems because the ESI-MS methods will yield lower values for GHb. Furthermore, this translation must be computationally and efficiently effected because adequate monitoring of glycemetic control by GHb estimation is a necessary component of management for the

Table 1. A comparison of commonly used methods for estimating glycosylated hemoglobin

	Low pressure ion-exchange chromatography (e.g. mini columns)	HPLC (e.g. Biorad's variant)	Enzyme immunoassay (e.g. DCA 2000)	Chemical method
Species measured	HbA ₁	HbA ₁ or HbA _{1c}	HbA _{1c}	Total GHb
Pre-HbA _{1c} interference	Yes	Yes (removed in some systems)	No	No
Interference in hemoglobinopathies	Yes	No	No	No
Drug interference (e.g. Aspirin, penicillin)	Yes	Yes	No	No
Interassay co-efficient of variation	2–10	2	6–8	6–8

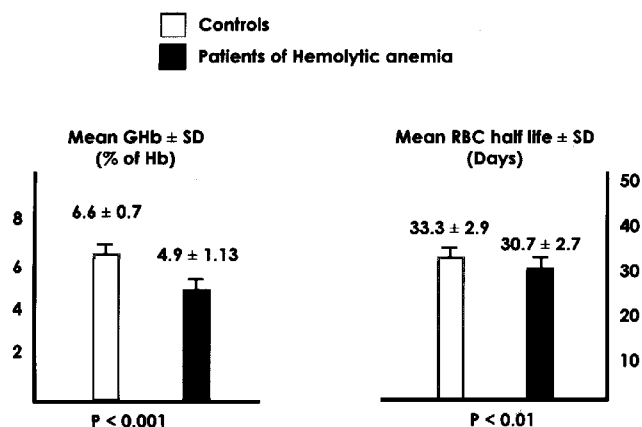


Figure 3. Comparison of glycosylated hemoglobin in patients of hemolytic anemia and controls.

patients and their healthcare providers. The transition to new standards must be completed with caution, i.e., only when the new method's efficacy can be compared with the ion-exchange method, a procedure used in two major trials and whose values reflect the contemporary clinical standard⁴⁴.

Methodological problems, selection of method and quality control

Most GHb methods are beset with a number of methodological problems (Table 1). In some of them a GHb standard is not possible, but this has now been circumvented. Interference by Schiff base or preglycohemoglobin occurs in many methods. Vitiating of results by hemoglobinopathies occurs in many methods, except the chemical method. Drugs that possess strong ionic charges, like aspirin can alter GHb in ion-exchange chromatographic methods. Last of all, a shorter erythrocyte life span will alter GHb; usually producing lower values because of a larger population of younger erythrocytes. When anemia is being treated; the rapid erythrocyte generation will also produce similar alterations. We found a lower GHb in a group of patients with hemolytic anemia⁴⁵ (Figure 3). We determined the erythrocyte half-life by serial whole blood counting following infusion of chromium (see ref. 51) tagged erythrocytes in these

patients of anemia and found a significant positive correlation ($r = 0.69$; $P < 0.001$) between GHb and erythrocyte half-life.

The ambient blood glucose is low in pregnancy and the erythrocyte generation is rapid; both these factors will tend to reduce the GHb values. Normal GHb values for a non-diabetic pregnant female are not yet established, which makes application of GHb values in this situation difficult. However, assessment of metabolic control is so important in a pregnant diabetic that monthly GHb estimations are recommended and the targets set are usually 1% below the usual values. Thus, the target HbA_{1c} value, for good control in pregnancy will be $\leq 6\%$. In renal failure, carbamylated compounds abound, which also interfere with GHb estimation. Altered rate of erythropoiesis in this situation further makes interpretation of GHb difficult.

Considering these facts, selection of a method should depend upon the infrastructure of a laboratory, workload, turn-around time desired and prevalence of hemoglobinopathies in the population it desires to serve. The chemical method that we have used extensively is the most inexpensive method, requiring minimal infrastructure and most basic reagents. However, the turn-around time for this method is about four hours. The ion exchange chromatographic methods using mini-columns are notoriously inaccurate, as the pH and temperature conditions are difficult to control. The same method by sophisticated high performance liquid chromatography is accurate and least time consuming, but is a very expensive method.

Quality control of GHb method is problematical. However, it is important to determine inter-assay and intra-assay coefficient of variation (CV) and as far as possible run a high and low standard. An acceptable intra-assay CV is 2–4% and inter-assay CV is 4–8%. In the chemical method, fructose can serve as a standard. In many other methods a standard hemolysate is now being used. Comparison of data from different laboratories is difficult, but can be set-up for study purposes. Similarly, transfer of data obtained by one method to other by various formulae is not scientific, but is being practised by many laboratories. Traditionally, HbA_{1c} has been thought to represent average glycemia over the past 12 to 16 weeks⁴⁶. In fact, glycation of hemoglobin occurs over the

entire 120-day life span of the red blood cell⁴⁷, but within these 120 days recent glycemia has the largest influence on the HbA_{1c} value⁴⁸. Indeed, theoretical models and clinical studies suggest that a patient in stable control will have 50% of their HbA_{1c} formed in the month before sampling, 25% in the month before that, and the remaining 25% in months two to four prior to sampling⁴⁹. The advantage that HbA_{1c} can give as an assessment of average plasma glucose can also be perceived as a drawback because it does not give an indication of the stability of glycemic control. Thus, in theory, one patient with wildly fluctuating glucose concentrations could have the same HbA_{1c} value as one whose glucose varies little throughout the day.

The evidence that HbA_{1c} measurement gives an indication of mean plasma glucose in the first place is not as strong as might be assumed. The initial clinical studies into HbA_{1c} in the 1970s could only compare it with the glycemic assessment that was available at the time. Thus, it was compared with 24 h glucose excursion rates⁵⁰, 'plasma glucose brackets'⁵¹, daily mean plasma glucose⁵², and the area under the curve of the glucose tolerance test⁵³. The best evidence that exists arises from the feasibility study of the diabetes control and complications trial, which compared the average of multiple HbA_{1c} measurements to the average of laboratory measured blood glucose profiles over the period of one year⁵⁴. Although there appeared to be an excellent association ($r = 0.80$), this hid the fact that an individual patient with a mean glucose of – for example, 10 mmol/l, could have an HbA_{1c} that varied from anywhere between 7% and 11%. Indeed, it has been known for several years that in any diabetic population there are a proportion of people who appear to glycate hemoglobin at a faster or slower rate than most others⁵⁵. Even within a non-diabetic reference range of 4–6%, subjects tend not to vary between 4% and 6%, but instead stay very close to their own 'set point'^{56,57}. By inference, this means that two subjects with the same degree of hyperglycemia might well have HbA_{1c} values that vary by up to 2%. The reason for these differences between 'high' and 'low' glycaters was originally thought to be the result of inter-individual differences in tissue glycation, but recent data suggest that much of these differences can be explained by the fact that high glycaters seem to have red blood cells that survive for longer than low glycaters⁵⁸. Even if there is to be a change in the HbA_{1c} set point, then it seems likely to be related to changes in red blood cell life rather than glycemia or glycation rates^{59,60}.

Of course, measures of glycemic control other than glycosylated hemoglobin exist, such as serum fructosamine⁶¹ and 1,5-anhydroglucitol⁶². Unfortunately, none of them has been investigated in as much detail as glycosylated hemoglobin and, crucially, none was measured alongside HbA_{1c} in the major complications studies. Thus, we seem destined never to know whether tests such as fructosa-

mine can predict the risk of diabetes complications any differently to HbA_{1c}.

Clinical relevance and application of GHb

Clinical relevance of GHb was established by the initial publications^{63,64} and has been re-examined extensively⁶⁴. We prospectively followed a group of type 2 diabetics with repeated blood glucose estimations and co-related the GHb values obtained after 2 months of follow-up with mean blood glucose values³³. We found a close correlation of glycemic control with GHb (Figure 4).

HbA_{1c} and diabetes complications

The clinical studies of the 1970s (refs 65–68) established the usefulness of HbA_{1c} in evaluation of long-term glycemic control. The next logical step was taken to use HbA_{1c} to evaluate whether improving glycemic control in diabetic patients could lead to a reduction in the long term small vessel (microvascular) and large vessel (macrovascular) complications of diabetes. Two seminal studies, the diabetes control and complications trial (DCCT)⁴² and the United Kingdom prospective diabetes study (UKPDS)⁴³, set out to establish the effect on microvascular complications in patients with type 1 and type 2 diabetes, respectively. It was hoped that these studies could also shed light on whether macrovascular complications could be avoided by this means.

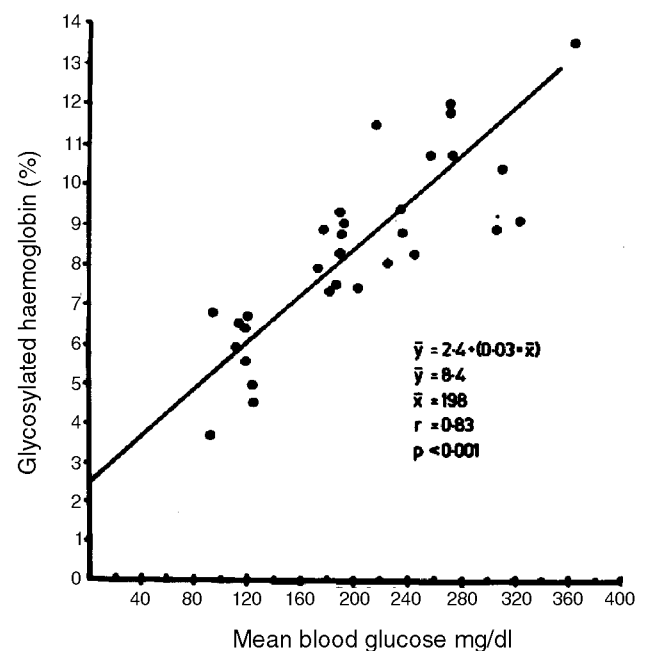


Figure 4. Relationship of mean blood glucose and glycosylated hemoglobin.

Microvascular complications

The microvascular (small vessel) complications of diabetes comprise retinopathy, nephropathy, and most probably neuropathy. Patients with diabetes who develop these conditions constitute a large proportion of all subjects who develop blindness, renal failure, and/or require limb amputation. To see the effect of improved glycemic control on the development and progression of these complications, the DCCT study recruited 1441 young patients (mean age, 27 years) with type 1 diabetes, half of whom were assigned to what was 'conventional' treatment in the USA at that time (one or two daily insulin injections and daily urine/blood glucose monitoring), whereas the other half were allocated to 'intensive' treatment (three or more insulin injections/day or an external pump and multiple daily blood glucose measurements), where the aim was to achieve as near normal blood glucose concentrations as possible. Hemoglobin A_{1c} measurement was the cornerstone of glycemic assessment in these patients. Compared with a non-diabetic reference interval of 4.05% to 6.05%, the intensively treated group achieved a median HbA_{1c} value of 7%, whereas in the conventionally treated group 9% was obtained throughout the study period. After a mean follow up period of 6.5 years, the risk of developing retinopathy in the intensively treated group was reduced by 76%, the risk of developing proteinuria was reduced by 54%, and the risk of clinical neuropathy was reduced by 60% (ref. 42). Benefits extended to the slowing of retinopathy progression in patients who already had mild eye disease at study entry.

Subsequent detailed analysis showed that HbA_{1c} measurement could be used as a tool to stratify the risk of a patient developing microvascular complications because there was an exponential rise in the rate of these complications with increasing HbA_{1c} values⁶⁹. This applied to all values of abnormal HbA_{1c} and not just to the values of 7% and 9%. Further examination also showed that there was an apparent absence of a 'glycemic threshold' – short of normal glycemia – below which small vessel complications did not occur⁷⁰. Nevertheless, as with all exponential relations, there was a law of diminishing returns, whereby the absolute benefit of reducing HbA_{1c} was diminished as the starting value decreased. For example, the absolute reduction in retinopathy risk of a patient falling from an HbA_{1c} of 7% to 5.2% is the same as another patient falling from 10% to 9.7% (ref. 71). Striving for good glycemic control by aiming for as low an HbA_{1c} as possible is also not without risks because the rate of severe hypoglycemia in this study was found to increase as the HbA_{1c} concentration fell⁷². Indeed, for many patients with diabetes, the fear of experiencing an acute complication, such as hypoglycemia, is greater than the possible increased risk of developing long-term small vessel complications through having chronically high HbA_{1c} values.

After the report of the DCCT study in 1993 it was hoped, but could not be assumed, that the results from this trial into type 1 diabetes would be equally applicable to the patients with type 2 disease, who represent most of the diabetic population⁷³. It took the publication of the UKPDS in 1998 to confirm that HbA_{1c} could also be used to indicate the risk of developing small vessel complications in this group of patients⁴³. This study involved 3867 older subjects (mean age, 54 years), who were either assigned to intensive treatment, with the aim of achieving a fasting plasma glucose of 6 mmol/l, or conventional treatment, with an aim to remain free from hyperglycemic symptoms and/or keep the fasting glucose below 15 mmol/l. Again, the cornerstone of treatment evaluation was by means of HbA_{1c} measurement, using the same assay as had been used in the DCCT (Bio-Rad Diamat HPLC). The separation between the groups this time was not as impressive as in the DCCT (HbA_{1c} 7.0% v 7.9% over 10 years), but there was still a 25% risk reduction in microvascular endpoints, which was in keeping with what the DCCT would have predicted.

The UKPDS might also have inadvertently given a clue as to whether the stability of glycemic control, and not just the mean plasma glucose influences the risk of small vessel disease because some patients were treated with insulin, whereas others received sulphonylurea drugs. Because patients treated with insulin tend to have greater glucose oscillations than non-insulin treated ones⁷⁴, it might have been expected that the risk of complications at any given HbA_{1c} value would have been different between the two groups. In reality, no such differences appeared to exist.

These two recent studies proved the usefulness of HbA_{1c} measurement in predicting the risk of developing microvascular complications and, as a consequence, have led to the widespread recommendation of its increased use^{73,75-77}. However, it must be emphasized that hyperglycemia as measured by HbA_{1c} is not the sole contributor to this risk because other factors can also have an important effect. In the UKPDS, for example, a reduction in blood pressure from a reading of 154/87 to 144/82 was found to be associated with a 37% decrease in microvascular endpoints⁷⁸. There was also a clustering of microvascular disease in families participating in the DCCT, suggesting an additional genetic influence on complication development and progression⁷⁹.

Macrovascular disease

Although diabetic microvascular complications form a large proportion of the excess morbidity and mortality associated with diabetes, the main cause remains the effects of large vessel (macrovascular) disease. Diabetes is associated with a two to threefold increased risk of coronary heart disease in men, and a four to fivefold

increased risk in premenopausal women⁸⁰. The DCCT and UKPDS trials did not primarily set out to establish whether a relation between HbA_{1c} and heart disease existed, but subgroup analysis has nevertheless been performed to examine this question. In the DCCT, the cardiovascular event rate was low because of the age of the patients recruited, but there was still an excess of macrovascular events in the conventional compared with the intensive group (40 v 23), although this just failed to reach significance ($P=0.08$)⁸¹. In the UKPDS, the event rate was higher, but the HbA_{1c} separation between the two groups lower, and again the findings were statistically suggestive but not conclusive ($P=0.052$ for myocardial infarction). However, recent analysis has shown that when the whole range of UKPDS patient HbA_{1c} concentrations is taken into account there is a highly significant relation between HbA_{1c} and coronary heart disease risk in these patients⁸². Thus, HbA_{1c} appears to give an indication of macrovascular risk (additional to hypertension, smoking, obesity, sedentary habits) in patients with diabetes, and might indicate the excess risk of coronary events associated with the disease.

In the DCCT study, initial feasibility data measured HbA_{1c} and seven-point blood glucose profiles quarterly for a one-year period in 278 type 1 diabetics, bringing out a close linear relationship between glycaemic control and HbA_{1c} (ref. 54). In this study each 1% increase in HbA_{1c} corresponded to an increase of average blood glucose by 30 mg/dl (Table 2). Clinical laboratories for reporting the mean blood glucose based on the HbA_{1c} value often use this relationship. It is obvious that it does not have a sound scientific basis, but serves some purpose in practice, as patients are still used to interpreting the glycaemic control by blood glucose values.

GHb cannot be used as a measure of hypoglycemia, but in DCCT study, as a group, the patients in the intensive group had a three-fold increase in hypoglycemic episodes and as pointed out above, this group had a significantly lower HbA_{1c}.

Discrepancies between GHb and blood glucose values reported by patients are expected, as self monitoring of blood glucose is not yet an accurate procedure and falsification of data is possible by patients. We measured

Table 2. Correlation of GHb with blood glucose⁵⁴

HbA _{1c} (%)	Mean blood glucose (mg/dl)
4	60
5	90
6	120
7	150
8	180
9	210
10	240
11	270
12	300

GHb and blood glucose values in 4203 patients and interpreted them as indicative of good, fair and poor control by both the parameters⁸³. The interpretation by these two parameters was in agreement in 51.3%, one-step different (e.g. good by one method, showing fair by other method) in 43% and two-step different (e.g. good by one method showing poor by other method) in 5.7% of patients. In 63% of patients showing discrepant values, GHb showed poorer control than post-prandial blood glucose values, thus indicating that a large number of patients diet a little better on the day of testing to produce better blood glucose values. This brings out the importance of GHb as a non-manipulable and reliable parameter in assessing metabolic control as compared to SMBG or one-point blood glucose estimations.

In early period of GHb use, this parameter was presumed to reflect metabolic control over the past 120 days, this period representing the life-span of erythrocytes. A large number of kinetic studies have revealed that glycemia in the recent past influences the GHb values more than the remote past⁸⁴. Thus, mean blood glucose of past 1 month, 2 months and 3 months contributes 50%, 40% and 10% respectively to the final result. By mathematical modeling the $t_{1/2}$ of GHb is estimated to be 35.2 days⁸⁵. This means that half of glycation seen during estimation has occurred in the previous 35.2 days. We studied the rate of dissipation of GHb in a group of newly diagnosed type 2 diabetics in whom blood glucose was lowered steadily by using glipizide. We found that lowering of GHb at 0–2, 2–4, 4–6, 6–8, 8–10 weeks of treatment was 1.0, 0.7, 0.5, 0.5 and 0.1 percent respectively⁸⁶ (Figure 5).

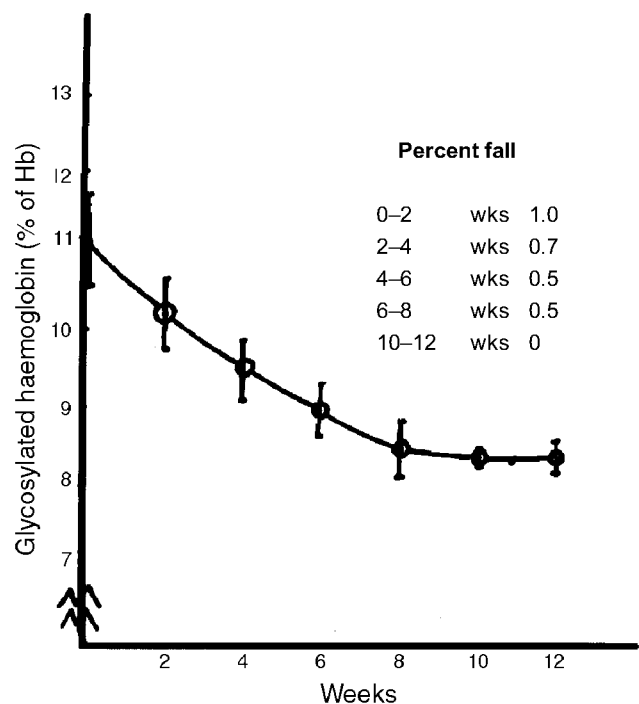


Figure 5. Rate of fall of glycosylated hemoglobin over time.

Kinetic studies⁸⁵ of GHb reveal that short-lived postprandial glycaemic excursions do not influence its results as significantly as long-lasting hyperglycemia, as produced by elevated fasting blood glucose. However, we do not know as yet whether or not such glycaemic excursions determine the long-term complications of diabetes. Same study⁸⁵ reveals that a normal fasting blood glucose coupled with elevated post-prandial blood glucose is not associated with an elevated GHb.

Targets for patients with diabetes

The publication of the DCCT and UKPDS has led to a reappraisal of the glycaemic control targets that should be aimed for in the treatment of patients with type 1 and type 2 diabetes. Previously, some guidelines tried to account for the lack of standardization in glycosylated hemoglobin measurement by comparing patients using the number of standard deviations (SDs), their HbA_{1c} result lay from their particular assay's non-diabetic mean value. However, the SD targets were necessarily rather arbitrary and the use of SDs could lead to discrepancies in patient classification, depending on which glycosylated hemoglobin assay was used⁸⁷.

The DCCT and the UKPDS has allowed a more 'evidence based' approach to be taken to the recommendations, and the fact that they both used the same HbA_{1c} method has allowed SD targets to be dispensed with. The European diabetes policy group guidelines (as part of the International Diabetes Federation) now recommend that both patients with type 1 and type 2 diabetes aim for a DCCT or DCCT equivalent assay value of <7.5% to reduce the risk of microvascular complications^{88,89}. In the USA, it is recommended that a value of <7.0% be achieved, with values >8% suggesting that additional action should be taken⁹⁰. As mentioned above, the absolute value of these targets might change if the proposed HbA_{1c} IFCC standard becomes adopted, but many clinicians seem to favour continuing with the current *de facto* DCCT standard.

HbA_{1c} as a screening test for diabetes

There remains considerable interest in extending the use of glycosylated hemoglobin measurement to include the diagnosis besides the monitoring of diabetes. Most studies have brought out the fact that glucose tolerance test (GTT) is a more sensitive diagnostic method and impaired GTT may occur with normal GHb. Using the 1985 WHO oral glucose tolerance test (OGTT) criteria for diagnosing diabetes⁹¹, a meta-analysis of 34 studies has found HbA_{1c} to be of limited value as a screening test because of the large number of subjects who have either impaired glucose tolerance or frank diabetes, but have HbA_{1c} values that are within the non-diabetic reference

interval⁹². Thus, a raised HbA_{1c} would appear to be specific for diagnosing diabetes, but the test is not particularly sensitive. We reported GTT and GHb values in 196 patients suspected of having diabetes⁹³. In our study, GHb was found to be as specific but only 57% sensitive as compared to GTT. Many authors have suggested a postprandial blood glucose along with a GHb estimation to improve the sensitivity in diagnosis of diabetes. Our study showed GHb values of $5.99 \pm 1.1\%$, $6.96 \pm 1.2\%$ and $8.8 \pm 2\%$ in non-diabetics, impaired glucose tolerance and diabetes groups respectively. Each group differed significantly ($P < 0.01$) from each other. This is in contrast to studies mentioned above⁹². Increased glycation in IGT group in our study, supports current data describing significant vascular complications in IGT group. Even when using the proposed new diabetes diagnostic criteria^{94,95}, which define diabetes as a fasting plasma glucose value >7 mmol/l, the same limitation in diagnosing type 2 diabetes by using GHb is found⁹⁵.

Some authors support the idea that HbA_{1c} testing is likely to be a more physiological assessment of glucose intolerance than the artificial conditions of the OGTT, and so believe this should be the preferred diagnostic test. Certain studies have shown HbA_{1c} to be as good a predictor of microvascular disease as fasting or two-hour post-OGTT glucose values^{96,97}, although not all studies have reached this conclusion⁹⁸. One group took a more pragmatic approach to diagnosis, stating that subjects with a HbA_{1c} below 7.0% are not likely to require pharmacological treatment of their condition and so need not be classified as diabetic, although the meta-analysis from which the value of 7% was derived did not convincingly take account of differences in HbA_{1c} methods between constituent studies⁹².

In addition to the difficulties arising out of methodologies used in estimating GHb, a few additional clinical considerations may influence the GHb values. It has been demonstrated that HbA_{1c} can be high with normal GTT⁹⁹ and high values can occur in non-diabetics¹⁰⁰. A genetic polymorphism has been described which influences the rate of glycation¹⁰¹ but probably the prevalence of such polymorphism is low.

It is unlikely that HbA_{1c} will ever be a reliable test for the diagnosis of type 2 diabetes for the following reason. If hyperglycemia, rather than glycation, is the true cause of diabetic complications (and it continues to be the means of diagnosing diabetes) then HbA_{1c} is fundamentally limited by the fact that two individuals with the same degree of glucose tolerance can have HbA_{1c} values that differ by nearly 2% (ref. 87). Thus, a subject with a HbA_{1c} value of 4% would need to increase his/her glycation rate by 50% to match another non-diabetic subject with a HbA_{1c} value of 6%. It is therefore not surprising that there can be overlap between the HbA_{1c} values of patients with diabetes and those of subjects without the disease. Even if glycation is thought to be the underlying

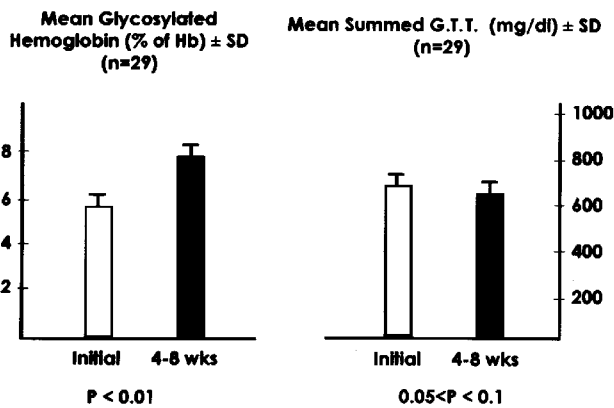


Figure 6. Glycosylated hemoglobin and summed GTT initially and 4-8 weeks after a vascular episode. Initial GTT shows stress hyperglycemia associated with increased glycosylation.

reason for complications, we have to be sure that glycation of hemoglobin gives an accurate reflection of glycation in small vessels. Because it is known that HbA_{1c} values can be affected by factors that are independent of glycemia or glycation rates^{58,80,100}, this assumption may not be valid.

Currently, guidance from the USA recommends against using HbA_{1c} in the diagnosis of type 2 diabetes¹⁰², but recent European recommendations find a role for the test although, curiously, this is only if confirmatory glucose testing is also performed⁸⁸.

In a group of 29 patients with acute vascular events and stress hyperglycemia as determined by an initial and follow up GTT, we found a significant elevation of GHb. This lends support to the fact that such transitory hyperglycemia is important and needs to be treated¹⁰³ (Figure 6).

In conclusion, within the past ten years, studies using HbA_{1c} have answered positively the fundamental question as to whether glycemic control influences the outcome of patients with diabetes. Therefore, despite its inherent limitations, HbA_{1c} seems destined to continue to be the most valuable parameter for assessing glycemic control.

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