Glycosylated Hemoglobin and Diabetes mellitus

I. Introduction:

Interest in glycosylated hemoglobin began in the 1950's with studies of the electrophoretic and chromatographic heterogeneity of hemoglobin in non-diabetics. Several hemoglobin variants were described which were present in small quantities in normal individuals. The most abundant of these fractions was later shown to be due to the addition of a hexose moiety on the β-chain and was designated HgbA1c.

In 1962, Huisman and Dozy reported an increase in the amount of "fast" hemoglobin seen in a group of diabetic patients taking tolbutamide and attributed this to an effect of the drug. Several years later in a study of 1200 patients in Tehran, Rhabar reported the appearance of an abnormal fast hemoglobin in the electrophoresis patterns of two patients, both of whom had diabetes. Further investigations by Rhabar demonstrated that this abnormal hemoglobin was identical to HgbA1c. Finally in 1971, using a macro-column cation-exchange assay, Trivelli showed a twofold increase in the levels of HgbA1 in diabetics as compared to normal controls.

In 1976, Koenig demonstrated that the level of HgbA1c was directly related to fasting blood glucose levels and that improvement in the degree of diabetic control resulted in a decrease in HgbA1c over a subsequent one to two month period. Since that time, numerous studies have demonstrated relationships between the level of glycosylated hemoglobin and: physician peak blood glucose concentrations, 24 hour glycosuria, and home monitored glucose concentrations in blood and urine.

Researchers have also studied the physiology of protein glycosylation and the potential connection between this post-translational modification and diabetic complications. Several effects of in vitro glycosylation have been observed including: enzyme inactivation, inhibition of regulatory molecule binding, increased protein cross-linking, decreased susceptibility to proteolysis, altered macromolecular recognition and endocytosis, and increased immunogenicity. Increased levels of ketoamine-linked glucose have been found in glomerular basement membranes and have been proposed as a factor in the pathogenesis of diabetic nephropathy. Glycosylation of crystalline lens protein and dermal collagen in young diabetics has been shown to increase the degree of structural protein cross-linking to levels seen in aged non-diabetics. These findings make non-enzymatic glycosylation of proteins an attractive candidate for the link between chronic hyperglycemia and the long term complications of diabetes.

II. Nomenclature:

Since their discovery, the nomenclature used to describe carbohydrate adducts of hemoglobin has become rather confusing. The designation, Hemoglobin Adducts, refers to all post-translational modifications in which a non-heme moiety has been covalently attached to the hemoglobin molecule. Within this larger group the term Glycosylated hemoglobin describes those hemoglobins in which a carbohydrate group is the added moiety. The carbohydrate may be attached at one of several sites, including the ε-amino groups of certain lysine residues and the N-terminal amino acids of
both the \( \alpha^- \) and \( \beta^- \) chains.

*Fast Hemoglobin* was originally used to describe the hemoglobin fraction which was the first to elute from cation-exchange columns and/or the fraction which rapidly migrated to the anode in gel electrophoresis. Although this term has subsequently been used as a synonym for glycosylated hemoglobin the fraction it represents is composed of a heterogeneous mixture of hemoglobin variants and adducts and the two terms are not equivalent.

The *Labile Fraction* is that portion of the glycosylated hemoglobin in which glucose is attached to the N-terminal valine of the \( \beta^- \) chains by an aldimine linkage (Schiff base). This fraction is identical to *Pre-HgbA1c* described below.

In addition to the descriptive names listed above there has also been a proliferation of abbreviations which specify various hemoglobin fractions. *HgbA* is the major adult form of hemoglobin consisting of two alpha \( (\alpha^-) \) and two beta \( (\beta^-) \) chains. Substitution of "S", "C" or "F" for "A" indicates one of the variant forms, Hemoglobin-S (Sickle), Hemoglobin-C and Hemoglobin-F (Fetal) respectively. Subscripts are then appended to these abbreviations to indicate various subfractions (usually post-translational modifications of the major hemoglobin subtype indicated). *HgbA0* indicates the major form of HgbA in which there has been no post-translational modification (or a modification exists but does not alter the characteristics of the molecule sufficiently for it to be detected by current methodologies). In contrast *HgbA1* refers to HgbA which has been modified such that there is a relatively negative charge on the molecule at a slightly acidic pH (6.7). HgbA1 is further subcategorized as *HgbA1a*, *HgbA1b* and *HgbA1c* which are all distinct fractions that can be separated chromatographically. The most studied of these fractions is *HgbA1c* which represents hemoglobin in which a glucose molecule is attached to the N-terminal valine of each \( \beta^- \) chain by a stable ketoamine linkage. *Pre-HgbA1c* is a labile precursor to *HgbA1c* in which the glucose is attached to the \( \beta^- \) chains by an aldimine linkage. The remaining subfractions of HgbA1 differ in either the specific carbohydrate attached (glucose-phosphate in HgbA1a) or the site of glucose attachment (non-N-terminal in HgbA1b). The relative percentages of each of these fractions are listed in Table 1 and this terminology is reviewed in Table 2.

**Table 1**

<table>
<thead>
<tr>
<th>Name</th>
<th>Percentages</th>
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<tbody>
<tr>
<td></td>
<td>Normal</td>
</tr>
<tr>
<td>HgbA</td>
<td>&gt;90%</td>
</tr>
<tr>
<td>HgbA2</td>
<td>2%</td>
</tr>
<tr>
<td>HgbF</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>HgbA1a</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>HgbA1b</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>HgbA1c</td>
<td>4-7%</td>
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</tbody>
</table>
Table 2

<table>
<thead>
<tr>
<th>Name</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycosylated Hgb</td>
<td>Hemoglobin with a covalently attached carbohydrate moiety.</td>
</tr>
<tr>
<td>Fast hemoglobin</td>
<td>Hemoglobin fraction which elutes first from cation-exchange columns and/or migrates most anodally during electrophoresis.</td>
</tr>
<tr>
<td>Labile fraction</td>
<td>Intermediate form of glycosylated hemoglobin.</td>
</tr>
<tr>
<td>HgbA</td>
<td>Major adult hemoglobin.</td>
</tr>
<tr>
<td>HgbA0</td>
<td>Major form of HgbA in which modifications do not exist or are currently undetectable.</td>
</tr>
<tr>
<td>HgbA1</td>
<td>Post-translationally modified hemoglobin with net negative charge at pH 6.7.</td>
</tr>
<tr>
<td>HgbA1a</td>
<td>Hemoglobin with glucose-phosphate attached at β-chain N-terminus.</td>
</tr>
<tr>
<td>HgbA1b</td>
<td>Hemoglobin with glucose attached at site other than the N-terminus of the β-chain.</td>
</tr>
<tr>
<td>HgbA1c</td>
<td>Hemoglobin with glucose attached at the N-terminal valine of the β-chain by a ketoamine linkage.</td>
</tr>
<tr>
<td>Pre-HgbA1c</td>
<td>Hemoglobin with glucose attached at the N-terminal valine of the β-chain by an unstable aldimine linkage.</td>
</tr>
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</table>

III. Biosynthesis and Biology:

HgbA1c is formed in vivo as the result of a non-enzymatic post-translational modification in which the amount of end-product formed is dependent upon the average glucose concentration over the 120 day lifespan of the erythrocyte. The reaction is a two step process in which glucose initially combines with the N-terminal valine of a β-chain to form an unstable aldimine which then undergoes an Amadori rearrangement to form a stable ketoamine. The first reaction is rapid and reversible while the second Therefore, in vivo there is rapid formation and dissociation of the aldimine dependent upon the ambient glucose concentration and a slower rate of ketoamine formation. The reaction is depicted in Figure 1.
In addition to altering the charge characteristics of the hemoglobin molecule, N-terminal glycosylation also effects its oxygen carrying capabilities. The N-terminal amino acids of the β-chains are the sites of 2,3-DPG attachment and the presence of a glucose molecule blocks this interaction. This results in a shift of the oxygen dissociation curve to the left resulting in a net decrease in oxygen delivery to the peripheral tissues, however, this effect is small and not currently felt to be physiologically important.

These effects of glycosylation are apparent only with HgbA1c, the covalent attachment of glucose at other sites produces much more subtle (and poorly studied) alterations in hemoglobin characteristics.

IV. Clinical Utility of Glycosylated Hemoglobin Assays:

Since levels of glycosylated hemoglobin have been shown to correwell with other tests of glycemic control (blood and urine glucose levels) the question may be asked: "What is the need for glycosylated hemoglobin assays when other less expensive measures of diabetic control are available?". One answer lies in the central question regarding diabetic management: "Does improved diabetic control result in decreased morbidity and mortality?". Clearly, answering this question requires longitudinal studies comparing populations with differing degrees of glycemic control. Historically, assessments of "glycemic control" have been based upon intermittent blood and urine glucose determinations obtained during clinic visits and/or from patient records of home self-monitoring. The first of these methods has been shown to be unreliable due to the many factors which are known to affect glucose levels in urine and blood (ie. age, time of day, stress, meals, etc.). Additionally, it has been observed that diabetic patients may transiently improve their compliance prior to clinic visits and therefore data based upon clinic monitoring may yield biased estimates of their degree of glucose control. Home-monitoring by patients may be even less reliable. In a recent study of pregnant diabetics (a group chosen for their supposed high motivation for
compliance) it was shown that falsification of home-record entries with approximately 50% of the entries having been altered in some manner. There is a need therefore, for a reliable marker of the overall degree of glycemic control. Since glycosylated hemoglobin levels reflect the average glucose level of the preceding three to four months and are not subject to acute changes or patient "falsification" it is well suited for this research role.

Glycosylated hemoglobin assays are also useful in a clinical setting for similar reasons. Until the advent of these assays, clinicians based individual patient treatment regimens on the results of blood and urine glucose testing, patient symptoms and signs and the physician's own overall impression of the patient's disease state, motivation, etc. As noted above, this type of assessment is probably unsatisfactory for monitoring long term patient compliance, establishing therapeutic goals, and determining the effectiveness of changes in therapy. Glycosylated hemoglobin levels can provide objective data for medical decisions in each of these situations. Compliant patients in good control should have stable levels of HgbA1c which are lower than pretreatment levels and in a range 1-2% above "normal". (Attempting to actually normalize the levels of glycosylated hemoglobin in diabetic patients is difficult and should be discouraged since it may result in hypoglycemic episodes.)

Other clinical uses for these assays have been proposed, however, their utility in these settings remains to be established. In monitoring pregnancies complicated by diabetes the therapeutic goal is to control glucose levels as closely as possible and thereby reduce the risk of neonatal complications and/or malformations. To achieve such a degree of control requires that patients frequently monitor their blood glucose levels and keep accurate logs of the results. Due to questionable validity of such documentation, it has been suggested that glycosylated hemoglobin levels be assessed to provide the clinician with an additional, objective assessment of a patient's compliance and overall control. While this may be a valid use for these tests there are several arguments against routinely ordering glycosylated hemoglobin levels in this setting. Arguments against this usage are: (1) that the levels change too slowly to be of real clinical utility (2) that contamination by fetal hemoglobin can falsely elevate HgbA1c values in some assays and (3) that pregnancy alone may alter HgbA1c levels thus making interpretation difficult. The measurement of a serum protein with a shorter half-life and which is relatively unaffected by fetal blood (ie. glycosylated albumin) may be more appropriate in this situation. A second suggestion is that glycosylated hemoglobin levels could be used as a secondary screen prior to more specific testing in patients suspected of having diabetes (ie. patients with an elevated "fasting" blood glucose level). In such situations the HgbA1c could be measured and only if the results indicated long term hyperglycemia would the patient be scheduled for an oral glucose tolerance test (OGTT). Prior to developing such a program, however, it must be established that the "secondary screen" would reduce the number of specific tests sufficiently to justify its own cost. Lastly, several authors have stated that HgbA1c levels may be useful in identifying patients with "silent" hypoglycemic episodes. In these patients the level of glycosylated hemoglobin is below that which would be expected given the patient's degree of hyperglycemia. This would indicate that the individual is experiencing periods of hyperglycemia which are "compensated" for by periods of hypoglycemia such that the average blood glucose level (and therefore
HgbAlc) is lower than anticipated. While this may be true, the test is not designed to be used as a screen for patients with hypoglycemic episodes and this should not be the rationale for ordering this assay.

There are several caveats to the use of glycosylated hemoglobin assays which should additionally be observed. First, it must be realized that these tests should only be used in combination with routine blood and urine glucose determinations in the care and monitoring of diabetic patients. While levels of glycosylated hemoglobin provide information about a patient's long term glycemic status, this information cannot be used by itself to determine appropriate therapies. (This concept of only using glycosylated hemoglobin levels as an adjunct to traditional testing is supported by both the National Diabetes Data Group and the American College of Physicians.) Secondly, due to the fact that HgbA1c levels cannot readily distinguish between individuals with glucose intolerance and diabetes mellitus, the tests should not be used for (This viewpoint is also supported by the NDDG and ACP with the understanding that future test modifications and improvements may allow these assays to be used for diagnostic purposes.) The assay should, in general, not be ordered more than four times per year in a single patient. This is based on the consideration that although decreases in HgbA1c may occur in a relatively short period (1-2 weeks) after improving glucose control, the overall efficacy of therapy (and therefore changes in the regimen) should only be assessed after several months. This provides sufficient time for all factors relating to diabetic control to come into play. (ie. Patient motivation and compliance may initially be high after a change in therapy but decrease over time.) Lastly, glycosylated hemoglobin levels are essentially of no clinical value to (and should not be ordered by) individuals other than the physician primarily responsible for a patient's diabetic management.

V. Methodologies:

During the past ten to fifteen years there has been a proliferation in the types of tests available for the measurement of glycosylated hemoglobins. Due to the wide range of techniques which have been adopted for assays, there are marked differences in their utility, cost, interferences and, perhaps most importantly, in what each test actually measures. This is evident in the 1986 College of American Pathologists (CAP) survey results from laboratories offering glycosylated hemoglobin determinations. As can be seen in Table 3, the range of values for each sample is extremely wide, reflecting the dissimilarity in assays which variously measure HgbA1, HgbA1c, fast hemoglobins, glycosylated hemoglobins, etc.

<table>
<thead>
<tr>
<th>Sample</th>
<th>No. Labs</th>
<th>Mean</th>
<th>Range</th>
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</thead>
<tbody>
<tr>
<td>A</td>
<td>238</td>
<td>13.8</td>
<td>4.0-26.5</td>
</tr>
<tr>
<td>B</td>
<td>318</td>
<td>15.1</td>
<td>4.7-36.3</td>
</tr>
<tr>
<td>C</td>
<td>278</td>
<td>14.4</td>
<td>5.3-28.3</td>
</tr>
</tbody>
</table>

In 1984 the National Diabetes Data Group made a series of general recommendations for laboratories performing these tests:
(1) Desirable to have inter- and intra-assay coefficients of variation < 5%.

(2) Each assay run should include both normal and diabetic range controls.

(3) Each laboratory should establish its own non-diabetic reference interval and that this interval should be narrow (i.e. < 2% glycosylated hemoglobin).

(4) Procedures to remove the labile fraction should be included all assays susceptible to this interference. (see below)

A. Cation-exchange Chromatography:

In this method of chromatography, the column is packed with an inert resin support to which weakly acidic groups are attached. By varying the pH and ionic strength of the buffering system the interaction between these groups and the solute molecules allows separation on the basis of ionic charge differences.

In this procedure a sample of patient red cells is washed, lysed and the hemolysate applied to the column. At a slightly acidic pH, HgbA1 is slightly less positively charged than HgbA and can be eluted in the initial fraction by using an appropriate buffering system. Using a second buffer allows collection of the bound HgbA in a separate fraction. The hemoglobin concentrations for each fraction are determined spectrophotometrically at 415 nm and the relative percentage of HgbA1 is calculated.

This methodology is a variation of the original macro-column technique used by Trivelli et al. and is the technique to which most other assays have been compared. Several companies (Isolab, Bio-Rad, Helena) are marketing cation-exchange columns in kit form and this is currently the These kits require little additional equipment, allow multiple samples to be run simultaneously and the columns provided can be regenerated and used many times before requiring replacement. The inter-assay and intra-assay coefficients of variation for these kits are in the range of 3-10%.

There are, however, several disadvantages to cation-exchange chromatography which must be considered. The results are very sensitive to assay conditions such as buffer pH and ionic strength, rate of elution and especially temperature. In the earlier, macro-column methods, acceptable precision and reproducibility required scrupulous technique and the use of temperature controlled water jackets surrounding the column. In the current commercial kits, the use of prepackaged buffers decreases the potential error due to improper reagent preparation and most manufacturers provide nomograms for temperature corrections. Additionally these assays are susceptible to several types of interference. The results will be falsely elevated if significant amounts of Pre-HgbA1c and HgbF are present as well as with several poorly characterized hemoglobin adducts seen in uremia, chronic salicylate ingestion and alcoholism. Hemoglobin S and HgbC will conversely lower the results in some since these species elute in the HgbA fraction. Lactescent serum causes an increase in apparent absorbance at 415 nm and will overestimate the amount of hemoglobin present in a sample. Depending upon the specific procedure used this could either increase or decrease the calculated percentage of HgbA1. Finally, due to the fact that HgbA1a and HgbA1b appear to increase slowly in red cells with storage at 4°C and these are measured as part of HgbA1 by
this method, samples should not be stored as whole blood for more than 5-6 days prior to assaying. (Samples may be stored for several months as hemolysates at -20°C.)

B. Batch Chromatography

This method is a variation of cation-exchange chromatography in which a column is not utilized. Instead the hemolysate is mixed directly with the cation-exchange resin in a buffered solution to form a slurry. During incubation HgbA becomes bound to the resin particles while HgbA1 remains free in solution. The supernatant containing the "free" hemoglobin fraction is then separated from the resin by centrifugation or by the use of serum separaThe concentration of hemoglobin in the supernatant is measured spectrophotometrically and compared to an aliquot of the original hemolysate to determine the percentage of HgbA1.

This is an extremely simple assay to perform and requires only minimal equipment which makes it relatively inexpensive. The disadvantages are that it is subject to the same errors described for routine cation-exchange chromatography and has slightly higher interassay and intraassay coefficients of variation (8-12%) than the column method. (Note that these values are from a single study only and that other authors have described much higher cv's which they contend makes this technique unacceptable.)

C. High Performance Liquid Chromatography

This is essentially the same methodology described for column chromatography, however, the cation-exchange column has been incorporated into HPLC instrumentation. Several variations are currently available which differ in the degree of separation achieved and the glycosylated fraction that is measured.

This methodology is rapidly replacing routine chromatography as the reference standard due to its greatly enhanced precision and the ability to discriminate an expanded range of hemoglobin variants. Additionally this method uses much smaller sample sizes (ie. 3μl) and can be automated by the use of mechanical samplers. The primary disadvantage to this method is the requirement for an HPLC instrument which makes initial setup costs high and requires specialized technical training to operate. Interferences vary depending on the particular method used but in general are similar to those for other cation-exchange techniques. Interassay and intraassay coefficients of variation for this method are small and generally do not exceed 7%.

D. Electrophoresis / Electroendosmosis

As in the previously described assays this technique separates the various hemoglobin fractions in a hemolysate on the basis of their charge characteristics. The negatively charged sulfate and pyruvate groups within the agar gel interact with HgbA to a greater degree than HgbA1 and therefore retard its migration in an electric field. Additionally, due to the negative charge of the media, cations and associated water molecules tend to migrate to the cathode carrying solutes with them. This electroendoosmotic flow is probably more responsible for the actual separation than the direct field effect. After separation has been achieved (45-60 min.) the gels are stained and scanned with a densitometer which can be programmed to determine the relative concentration for each band and calculate the percentage of HgbA1. The results of
electrophoresis have been shown to correlate well with cation-exchange chromatography and the coefficients of variation are comparable (2-10%).

The major advantage of electrophoresis as compared to column methods is that it is relatively unaffected by variations in pH, ionic strength and temperature. Variant hemoglobins such as HgbS and HgbC do not cause any interference as they migrate separately from both HgbA and HgbA1 and can actually be detected by this method. The ability to run several samples (6) and controls on each gel allows for a relatively good throughput and continuous monitoring of the assay. Additionally, the individual gels may be retained as a permanent record of the results should repeat examination or scanning be required. (This is not possible with column techniques.)

The disadvantages to this method are that it cannot resolve the HgbA1 subtypes and is subject to interference by both HgbF and Pre-HgbA1c which co-migrate with HgbA1. Electrophoresis also requires specialized equipment and training to perform and variability in gel properties between lots and manufacturers can markedly influence results. (Although this latter problem was a major source of error in the early use of this technique, recent improvements in gel preparation quality control have significantly reduced this problem.) Lastly, as with chromatographic methods sample handling and storage will affect the results and therefore samples may be held at 4°C for only a few days prior to assaying.

E. Iso-electric Focusing

While similar to agar gel electrophoresis in several respects the separation achievable by this method is greatly enhanced. This is made possible by the use of specially prepared gels in which a pH gradient is established with a mixture of ampholytes. After application of the hemolysate the gel is subjected to an electric field in a chamber similar to that used in routine electrophoresis. As the individual hemoglobins reach locations in the gel at which the pH is equal to their pi then the net charge on the molecule becomes zero and the migration stops. The individual hemoglobins are thus focused at their respective iso-electric points which allows greater separation and the quantitation of HgbA1c alone. The gel is then stained and scanned in a manner analogous to that described above for the electrophoresis procedure.

Since this method allows quantitation of HgbA1c separate from HgbA1a and HgbA1b, sample handling and storage does not have as great an effect on the results. This technique is also free from interferences by variant hemoglobins and hemoglobin adducts described previously. Pre-HgbA1c, however, has a pi which is very close to that for HgbA1c and the two bands cannot be resolved with conventional densitometers. Specialized laser scanners are able to discriminate the two bands and can be used to avoid interference from the labile fraction. These scanners are, however, expensive and greatly increase the set-up cost for this assay. As with agar electrophoresis, multiple samples (35) may be run on each gel, variant hemoglobins may be detected and quantitated, and the gels may be retained as a permanent record of the results. Specialized equipment, is the inherent difficulty in establishing the pH gradient within the gel and maintaining it for extended periods of time without degradation. If properly prepared gels are utilized, however, the interassay and intraassay coefficients of variation are approximately 3-7%.
F. Affinity Chromatography

In this column chromatographic method, separation of glycosylated hemoglobins is accomplished by the use of boronic acid residues bound to the support matrix. These residues interact with the cis-diols present on the glycosylated fraction and retard their passage while other hemoglobin variants pass through the column. This interaction is diagramed in Figure 2.

Figure 2

Immobilized boronic acid + Glycosylated hemoglobin → Boronic acid–glycosylated hemoglobin complex

The bound fraction is then eluted from the column with a sorbitol buffer and the relative percentage of hemoglobin present in this eluate is measured spectrophotometrically. As with other column methods the percentage of glycosylated hemoglobin is calculated by comparison with either the first eluted fraction or the original hemolysate.

This method measures all glucose adducts of hemoglobin regardless of the site of glucose attachment. Therefore, HgbA1c, HgbA1a, HgbA1b and other adducts are measured as a group and the results are 1-3% higher than those by assays which are based on separation by charge differences alone. This makes comparison with other assays difficult although the results tend to parallel one another. The assay is insensitive to interference from other hemoglobin variants, adducts and Pre-HgbA1c and can be modified for evaluating other glycosylated protein species. This flexibility may become an important factor in the selection of a particular assay as interest in the measuring and monitoring of other glycosylated serum proteins increases (i.e. glycosylated albumin in pregnancy). This method is relatively insensitive to pH variations, however, increasing temperature causes a decrease in the apparent concentration of glycosylated hemoglobin by 0.1-0.2% for each degree of change. As with isoelectric focusing the performance of this assay is very sensitive to the properties of the matrix (i.e. ligand concentration) and gels from different lots or manufacturers may not have the same separation characteristics. The reported interassay and intraassay coefficients of variation are generally 2-7%, however, one study reported an intraassay cv of 12.7%.

G. Phytic acid Spectrophotometry
This assay is based on the observation that when phytic acid binds to hemoglobin it alters the optical absorption characteristics of the hemoglobin molecule. The addition of a sugar moiety at the N-terminus of the β-chain blocks this binding and therefore the change in absorbance upon the addition of phytic acid is inversely proportional to the amount of HgbA1c present in the hemolysate. This is a simple assay which can be readily automated or adapted to instruments (ie Dupont ACA) already available. It is relatively insensitive to pH, ionic strength or temperature variations.

The major problem with this assay is its susceptibility to interferences from Pre-HgbA1c, HgbF and other hemoglobins which are altered at the amino ends of the β-chains. This includes the normal interaction of 2,3-DPG at this site and since 2,3-DPG is not included in the reference standards the level of glycosylated hemoglobin can be over estimated. There is also a requirement for EDTA anticoagulated blood since heparinized samples yield results which are approximately 2% higher. The reported coefficients of variation generally range from 2-6%, however, in one comparison study this method was judged unacceptable due to an interassay cv of 15%. (Boucher et al, 1973) H. Thiobarbituric acid Colorimetry

In this method the hemolysate is heated, either by boiling or in an oven, with oxalic acid to release 5-hydroxymethylfurfural (5-HMF) from any hexoses present. The remaining proteins are precipitated with trichloroacetic acid leaving the 5-HMF in the supernatant. The 5-HMF is then reacted with thiobarbituric acid to yield a colored complex which can be measured spectrophotometrically at 443 nm. Since all hexoses are reactive this technique measures hemoglobin glycosylation at any site and requires meticulous removal of free glucose prior to assaying to prevent falsely elevated results. Aldimines do not react with this method and therefore there is no apparent interference from the labile fraction. There is also no interference from other non-hemoglobin adducts or variant hemoglobins.

The major advantages to this method are its lack of major interferences, that it requires litde specialized equipment, and that it can be easily modified to detect glycosylation of other protein species. Additionally, thiobarbituric acid colorimetry can be automated and because it is a chemical method, fructose may be used as an assay standard rather than true glycosylated hemoglobin standards which are difficult to maintain for long periods.

The disadvantages are that this method requires strictly controlled and standardized assay conditions to provide consistent results. One of the most critical conditions is the length of time the sample is heated with oxalic acid. The production and the destruction of 5-HMF are both accelerated by heat as is shown in the graph in Figure 3 where the net amount of 5-HMF produced from several fructose standards (µmol/l) is plotted against the length of time the samples were heated. As can be seen, the maximum yield for all samples was achieved at approximately two hours and that this amount decreased with additional heating. The yield of 5-HMF is also dependent upon the amount of protein (hemoglobin) present in the sample and most variations suggest 10 mg/ml as the optimum concentration.
The results are expressed as nanomoles of 5-HMF per 10 mg Hgb which makes the results of this assay difficult to compare with other methods which express the results as percentages. With strict attention to these factors, however, the performance of this assay can be very good with coefficients of variation of 1-9%. I. Periodate Oxidation Fluorometry

The principle of this assay is very similar to that of the colorimetric procedure. Carbohydrate moieties are oxidized by periodate with the formation of formaldehyde. This is then reacted with acetylacetone and ammonia to form a fluorescent condensation product 3,5-diacetyl-1,4-dihydrolutidine (DDL) which can be measured fluorometrically. All compounds which are periodate sensitive must be removed prior to the reaction so that the results are not falsely elevated. This requires the use of acid tetrahydrofuran (THF) since both hemin and acetone (used in other assays to prepare purified globin) interfere with the fluorometric detection step. Additionally, a step to remove any excess periodate must be included (ie. zinc precipitation) since this interferes with the formation of DDL.

As with the thiobarbituric method, this assay measures glycosylation at any site on the hemoglobin molecule, it is free from interferences from other hemoglobin adducts and variants and it can be modified to measure other glycosylated proteins. This assay does, however, measure the labile fraction since the reactis not influenced by the type of hemoglobin-carbohydrate linkage. Additional disadvantages are the
requirement for specialized equipment and that the results are expressed as the number of glycogroups per milligram of hemoglobin which makes comparisons to other assays difficult.

J. Radioimmunoassay

This assay utilizes a polyclonal sheep anti-HgbA1c which is added to a patient hemolysate and allowed to incubate. Radio-labelled HgbA1c is then added, allowed to incubate and the immune complexes are precipitated with ammonium sulfate. The residual radioactivity of the supernatant is quantified and is proportional to the amount of patient HgbA1c originally present in the sample. The antibody preparation used in the assay is preabsorbed against HgbA and there is essentially no cross-reactivity with this form of adult hemoglobin. There also appears to be little cross-reactivity with other genetic hemoglobin variants or adducts. In the initial studies, however, there was cross-reactivity with HgbA1a and HgbA1b and therefore sample handling and storage may affect the results. The antibody cross-reactivity with Pre-HgbA1c was not evaluated in the original study, however, it is likely that it is significant. The assay is not commercially available at this time and due to requirement for radioactive materials there is little clinical interest in adopting it over other assays. The clinical performance is unknown.

K. Laser Nephelometry

This immunologic method utilizes a rabbit anti-HgbA1 which exhibits nearly 100% cross-reactivity with HgbA. Therefore, the initial step in the procedure is the removal of HgbA from the patient hemolysate with a cation-exchange slurry as in the batch chromatography method. An aliquot of the supernatant is then incubated with anti-HgbA1 and the relative increase in light scattering due to the formation of immune complexes is measured with a laser nephelometer. Interferences from the labile fraction, other adducts and variant hemoglobins are undetermined. Due to requirement of removing HgbA, there is little interest in this methodology and the performance has not been assessed clinically.

L. Summary of Available Methods

The methods described above are reviewed in Table 4 and are divided by double horizontal lines into several categories depending upon how the separation and/or measurement of glycosylated hemoglobin is achieved: (1) Charge differences - cation-exchange iso-electric focusing, (2) Structural differences - affinity chromatography, phytic acid spectrophotometry, (3) Chemical differences - thiobarbituric acid colorimetry, periodate oxidation fluorometry, and (4) Immunologic differences - RIA, laser nephelometry.
<table>
<thead>
<tr>
<th>Assay</th>
<th>Interassay cv</th>
<th>Intraassay cv</th>
<th>Comments</th>
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<tbody>
<tr>
<td>Cation exchange</td>
<td>3-10%</td>
<td>3-7%</td>
<td>Original method, widely used, measures HgbA1, sensitive to conditions, many interferences.</td>
</tr>
<tr>
<td>Batch chromatography</td>
<td>10%</td>
<td>8-12%</td>
<td>Simple, measures HgbA1, sensitive to conditions, many interferences, little clinical experience.</td>
</tr>
<tr>
<td>HPLC</td>
<td>2-9%</td>
<td>6-10%</td>
<td>Small samples, &quot;gold standard&quot;, automatable, measures HgbA1 or HgbA1c, special equipment needed, low throughput, interferences.</td>
</tr>
<tr>
<td>Electrophoresis</td>
<td>2-11%</td>
<td>2-7%</td>
<td>Insensitive to conditions, no HgbS/HgbC interference, measures HgbA1, permanent record, special equipment needed, gel variability.</td>
</tr>
<tr>
<td>Iso-electric focusing</td>
<td>?</td>
<td>?</td>
<td>Measures HgbA1c, no interferences, special equipment needed, gel variability.</td>
</tr>
<tr>
<td>Affinity chromatography</td>
<td>2-9%</td>
<td>1-6%</td>
<td>Insensitive to conditions, little interference, measures glycosylation at multiple sites, gel variability, modifiable for other proteins.</td>
</tr>
<tr>
<td>Spectrophotometry</td>
<td>4-6%</td>
<td>2-4%</td>
<td>Insensitive to conditions, automatable, interferences, anticoagulant effects.</td>
</tr>
<tr>
<td>Colorimetry</td>
<td>2-9%</td>
<td>1-2%</td>
<td>Little interference, automatable, measures glycosylation at multiple sites, modifiable for other proteins, use chemical standards, sensitive to assay conditions, difficult units.</td>
</tr>
<tr>
<td>Fluorometry</td>
<td>3.8%</td>
<td>2.2%</td>
<td>Automatable, little interference, measures glycosylation at any site, modifiable for other proteins, use chemical standards, special equipment needed, difficult units.</td>
</tr>
<tr>
<td>RIA</td>
<td>?</td>
<td>?</td>
<td>Little interference (?), special equipment needed, radioactivity, cross-reactivity (?).</td>
</tr>
<tr>
<td>Nephelometry</td>
<td>?</td>
<td>?</td>
<td>Interferences, cross-reactivity, special equipment needed, requires initial removal of HgbA.</td>
</tr>
</tbody>
</table>

VI. Interpretation of Results:

The position paper of American College of Physicians (1984) regarding the use of glycosylated hemoglobin in the management and diagnosis of diabetes mellitus states:

"The primary risk of the test is the adverse effects that may result from changes in diabetic therapy based on inaccurate or misinterpreted test results."

Therefore explicit knowledge of the assay type, its potential in vivo and in vitro interferences and the interaction of glycosylated hemoglobin levels with other disease states is of paramount importance to the physicians utilizing these tests.
The fact that the formation of HgbA1c proceeds through a labile intermediate (Pre-HgbA1c) has consequences regarding both the measurement and interpretation of HgbA1c levels. The charge characteristics of the intermediate are essentially the same as for HgbA1c, therefore the results of assays which depend on charge differences for separation will be "falsely" elevated if there is a significant amount of Pre-HgbA1c present. Generally, in normal individuals and in well controlled diabetics the amount of this intermediate is low (1-2% of total glycosylated hemoglobin), however, the level of Pre-HgbA1c may increase rapidly during short periods of hyperglycemia. Samples taken during such episodes may contain Pre-HgbA1c at levels corresponding to 10-25% of the total HgbA1c. Therefore, the higher the glucose level at the time of sampling, the more likely the labile fraction will interfere with the HgbA1c result. In order to prevent this, a pre-analytical step to remove Pre-HgbA1c has been incorporated into most assays which are susceptible to this interference.

Another factor that must be kept in mind when interpreting the results of these assays is the "averaging" effect caused by the relative slowness of the Amadori rearrangement. As stated above, the major effect of a short period of hyperglycemia will be an increase in the amount of Pre-HgbA1c. Due to the fact that the reaction constants for both the formation and dissociation of the labile intermediate exceed the reaction constant for the Amadori rearrangement, a short period of hyperglycemia followed by a return to "normal" glucose levels will have only a small net effect on the actual level of HgbA1c. (Note: The net effect will be further minimized if a period of relative hyperglycemia is followed by a hypoglycemic episode) Therefore, a diabetic patient with marked swings in blood glucose could have the same level of HgbA1c as a patient in whom there is a relatively stable level of glycemia.

Since the measurement of glycosylated hemoglobin presupposes that the major hemoglobin present in an individual is HgbA, interpretation of HgbA1c levels in patients with hemoglobin variants can be very difficult. For example, the presence of HgbS or HgbC (which elute with HgbA) will falsely lower the apparent HgbA1 level when assayed by cation exchange chromatography. Conversely, HgbF which elutes with the glycosylated hemoglobins will falsely elevate the HgbA1 level with this method. (Note that elevated levels of HgbF may be present in: hereditary persistence of fetal hemoglobin, β-thalassemias, normal newborns and pregnant women after a fetal-maternal bleed.) For these reasons hemoglobin studies should be performed in patients either suspected of having a variant hemoglobin or with glycosylated hemoglobin levels which do not coincide with their level of glycemia.

Acute alterations of an patient's hemoglobin pool due to hemorrhage, hemolysis or transfusion can also have profound effects on glycosylated hemoglobin values. All of these conditions may cause a decrease in HgbA1c due to the loss of older erythrocytes (which contain the highest concentrations of glycosylated hemoglobin) and their replacement by either younger cells from the patient's marrow or transfused normal erythrocytes. Therefore, to prevent erroneous interpretation of "improved control" due to an apparent decrease in HgbA1c, it must be established that a patient's hemoglobin/hematocrit have been stable over the preceding three to four months. In chronic conditions in which the average life-span of the erythrocyte is reduced (i.e. hypersplenism, red cell enzyme deficiencies, uremia, prosthetic cardiac valves, etc.) the amount of HgbA1c is also decreased and this may be falsely interpreted as indicating a
better degree of control than is actually present. If the red cell life-span, although shortened, remains relatively constant then glycosylated hemoglobin levels may still be of value as long as the results are referenced to the individual’s own "baseline" and not to published values.

VII. Labile Fraction Removal

Since the recognition of Pre-HgbA1c as a source of error in glycosylated hemoglobin assays, various methods for the removal of the labile fraction have been proposed. The first of these was the incubation of the patient’s red cells in a glucose-free isotonic saline solution at 22°C. This technique requires approximately 10-14 hours of incubation and therefore adds a day to the turn-around-time for the assay. Increasing the temperature to 37°C shortens the incubation time to 5-7 hours, however, this is still much longer than the time required for the assay itself. In 1981 Nathan et al. reported that by using a solution at pH 5.0 containing 12 mM aniline as a catalyst and 30 mM semicarbazide as a glucose trap the incubation could be reduced to 30 minutes at 38°C. The use of such toxic reagents, however, limited the interest in this method. Finally, in 1982 Bannon showed that the use of these chemicals was unnecessary and that merely lowering the pH of the incubating solution to 5.5 was sufficient to allow 99% removal of the labile fraction in 30 minutes at 37°C. Some commercial kits are now being marketed with a buffered lysing reagent (0.1% saponin, 0.05% EDTA) containing 0.05M potassium biphthalate at pH 5.0. The washed erythrocytes are lysed/incubated in this solution for 15 minutes at 37°C which results in the removal of approximately 99% of the labile fraction. An additional measure to reduce the potential interference from Pre-HgbA1c is to obtain samples for these assays after the patient has fasted since it is the in vivo level of glucose which accelerates labile fraction formation.

VIII. Conclusions

Measurement of glycosylated hemoglobin in patients with diagnosed diabetes mellitus is a useful adjunct to routine blood and urine glucose measurements and provides an indication of long term control for both clinical and research settings. These assays may also be useful in other settings (ie. pregnancy, diagnosis/screening, etc.), however, the cost effectiveness and utility of such testing has not been established. The tests should not be ordered more than four or five times a year in an individual patient and their use should be restricted to those physicians primarily responsible for diabetic management.

There are numerous methodologies now available for measuring glycosylated hemoglobin levels and they vary with respect to cost, interferences and the hemoglobin fraction measured. If properly performed most of these methods have acceptable coefficients of variation and there is no one assay which is clearly superior to the others (although the immunologic methods and batch chromatography appear clearly inferior). The selection of a particular method must be based upon familiarity with the technique, availability of instrumentation, cost and throughput requirements.

The results of these tests should be interpreted with a knowledge of the patient’s status, the limitations of the particular assay and the influence of other disease states on the level of glycosylated hemoglobin. Erroneous results or improper interpretation can lead to potentially detrimental changes in therapy and must be avoided.
Bibliography


Mechanism and speed of reactions between haemoglobin and glucose; consequences for the measurement of glycosylated haemoglobins in patient material. *Clin Chim Acta* 1982;125:341-350