GLYCATED HEMOGLOBIN

1. Use

**Recommendation:** Glycated hemoglobin (GHb) should be measured routinely in all patients with diabetes mellitus to document their degree of glycemic control. Treatment goals should be based on the results of prospective randomized clinical trials such as the DCCT and UKPDS. These trials have documented the relationship between glycemic control, as quantified by serial determinations of GHb, and risks for the development and progression of chronic complications of diabetes. Laboratories should be aware of potential interferences, including hemoglobinopathies, that may affect GHb test results. In selecting assay methods, laboratories should consider the potential for interferences in their particular patient population.

Level of evidence: A

Measurement of glycated proteins†, primarily GHb, is widely used for routine monitoring of long-term glycemic status in patients with diabetes mellitus. GHb is used both as an index of mean glycemia and as a measure of risk for the development of diabetes complications (90, 122, 133). This test is also being used increasingly by quality assurance programs to assess the quality of diabetes care (e.g., requiring that health-care providers document the frequency of GHb testing in patients with diabetes and the proportion of patients with GHb values below a specified value) (134, 135).

The ADA and other organizations that have addressed this issue recommend measurement of GHb in patients with both type 1 and type 2 diabetes, first to document the degree of glycemic control, then as part of continuing care (14). The ADA has recommended specific treatment goals for GHb based on the results of prospective randomized clinical trials, most notably the DCCT (12, 133), but also the more recent UKPDS (13). Since different GHb assays can give different GHb values, the ADA recommends that laboratories use only assay methods that are certified as traceable to the DCCT GHb reference (122, 133); these results are reported as Hb A1c.

2. Rationale

Glycated proteins are formed post-translationally from the slow, non-enzymatic reaction between glucose and amino groups on proteins (136). For hemoglobin, the rate of synthesis of GHb is principally a function of the concentration of glucose to which the erythrocytes are exposed. GHb is a clinically useful index of mean glycemia during the preceding 120 days, the average lifespan of erythrocytes (90, 136-143). Although carefully controlled studies have documented a close relationship between the concentration of GHb and mean glycemia, routine determinations of blood glucose by patients or by their health-care providers are not considered as reliable as GHb to quantify mean glycemia (19, 90, 137, 138, 144-146). Concentrations of other blood-based glycated proteins (e.g., glycated serum/plasma proteins, “fructosamine”) also reflect mean glycemia, but over a much shorter time than GHb (15-30 days and 60-120 days, respectively) (90, 136-144, 147, 148). However, clinical utility of glycated proteins other than hemoglobin has not been clearly established and there is no convincing evidence that relates their concentration to the chronic complications of diabetes (90, 122).

†The terms glycated hemoglobin, glycohemoglobin, “glycosylated” (which should not be used) hemoglobin, Hb A1 and Hb A1c have all been used to refer to hemoglobin that has been modified by the nonenzymatic addition of glucose residues. However, these terms are not interchangeable. Glycated hemoglobins comprise Hb A1c and other hemoglobin-glucose adducts, while Hb A1c is made up of Hb A1a, Hb A1b and Hb A1c. Hb A1c is the major component of Hb A1c, accounting for ~80% of Hb A1c. In order to eliminate this confusing nomenclature, the term “A1c test” has been suggested. As described in the text, most of the clinical outcome data that are available for the effects of metabolic control on complications (at least for the DCCT and UKPDS) used assay methods that quantified Hb A1c. In this paper, we use the abbreviation GHb to include all forms of glycated hemoglobin.
3. Analytical Considerations

**Recommendation:** Laboratories should use only GHb assay methods that are certified by the National Glycohemoglobin Standardization Program as traceable to the DCCT reference. In addition, laboratories that measure GHb should participate in a proficiency-testing program, such as the CAP Glycohemoglobin Survey, that uses fresh blood samples with targets set by the National Glycohemoglobin Standardization Program Laboratory Network.

Level of evidence: B

There are many (greater than 30) different GHb assay methods in current use. These range from low throughput research laboratory component systems and manual minicolumn methods to high throughput automated systems dedicated to GHb determinations. Most methods can be classified into one of two groups based on assay principle (90, 139, 149). The first group includes methods that quantify GHb based on charge differences between glycated and nonglycated components. Examples include cation-exchange chromatography and agar gel electrophoresis. The second group includes methods that separate components based on structural differences between glycated and nonglycated components. Examples include boronate affinity chromatography and immunoassay. Most charge-based and immunoassay methods quantify hemoglobin A1c, defined as hemoglobin A with glucose attached to the NH2-terminus valine of one or both beta chains. Other methods quantify “total glycated hemoglobin,” which includes both hemoglobin A1c and other hemoglobin-glucose adducts (e.g., glucose-lysine adducts and glucose-alpha chain NH2-terminus valine adducts). Generally, results of methods using different assay principles show excellent correlation, and there are no convincing data to show that any one method or analyte is clinically superior to any other. However, the reported GHb results from the same blood sample could differ considerably among methods unless they are standardized to a common reference (e.g., without standardization, the same blood sample could be read as 7% in one laboratory and 9% in another) (90, 139, 149-155).

In 1996, the National Glycohemoglobin Standardization Program (NGSP) was initiated to standardize GHb test results among laboratories to DCCT-equivalent values (154-156). The rationale for standardizing GHb test results to DCCT values was that the DCCT had determined the relationship between specific GHb values and long-term outcome risks in patients with diabetes mellitus (12, 14, 90, 122). The NGSP was developed under the auspices of the AACC and is endorsed by the ADA, which recommends that laboratories use only GHb methods that have passed certification testing by the NGSP. In addition, the ADA recommends that all laboratories performing GHb testing participate in the CAP proficiency testing survey for GHb which uses fresh whole-blood specimens (157).

The NGSP laboratory network includes a variety of assay methods, each calibrated to the DCCT reference. The DCCT reference is a high-performance liquid chromatographic cation-exchange method that quantifies hemoglobin A1c and is a NCCLS designated comparison method (140, 158). The assay method has been used since 1978 and has demonstrated good long-term imprecision (between-run CVs consistently <3%) (157). The laboratories in the network interact with manufacturers of GHb methods to assist them, first in calibrating their methods, and then in providing comparison data for certification of traceability to the DCCT. Certification is valid for one year. An important adjunct to the program is the GHb proficiency testing survey administered by CAP. Since 1996 (starting with a pilot project including 500 laboratories and expanded to all laboratories in 1998), the survey has utilized fresh whole blood samples with NGSP-assigned target values. Since initiation of the NGSP in 1996, the survey has documented a steady improvement in comparability of GHb values among laboratories, both within-method and between-method. In general, NGSP-certified methods have demonstrated less variability and better comparability to NGSP-assigned target values than non-certified methods (157). The NGSP website provides detailed information on the certification process and maintains a listing of certified assay methods (NGSP website: http://www.missouri.edu/~diabetes/ngsp.html).
A. Preanalytical

a. Patient variables

There are no clinically significant effects of age, sex, ethnicity, or season on GHb or GHb test results. The effects of age on GHb are controversial (159-161). Some studies show age-related increases in GHb, approximately 0.1% per decade after age 30 years. Other reports show little or no increase. Differences in results among the studies are probably due to differences in the selection of study subjects; when studies are restricted to participants with normal glucose tolerance (i.e., normal fasting and postprandial plasma glucose concentrations), little or no age-related increase in GHb has been found. Results are also not significantly affected by acute illness.

Any condition that shortens erythrocyte survival or decreases mean erythrocyte age (e.g., recovery from acute blood loss, hemolytic anemia) falsely lowers GHb test results regardless of the assay method (90). Vitamins C and E are reported to falsely lower test results, possibly by inhibiting glycation of hemoglobin (162, 163), but vitamin C may increase values with some assays (162). Iron-deficiency anemia is reported to increase test results (164). There is no significant effect of food intake on test results. Hypertriglyceridemia, hyperbilirubinemia, uremia, chronic alcoholism, chronic ingestion of salicylates, and opiate addiction are reported to interfere with some assay methods, falsely increasing results (139, 165-167).

Several hemoglobinopathies (e.g., hemoglobins S, C, Graz, Sherwood Forest, D, Padova) and chemically modified derivatives of hemoglobin interfere with some assay methods (independent of any effects due to shortened erythrocyte survival) (168-170); for a review, see (171). Depending on the particular hemoglobinopathy and assay method, results can be either falsely increased or decreased. Some methods may give a value in the reference range for a nondiabetic patient with a hemoglobin variant, but this is not an assurance that no interference is present; the interference may be subtle in the reference range, but may increase steadily with increasing GHb. Boronate affinity chromatographic assay methods are generally considered to be less affected by hemoglobinopathies than methods that separate glycated and nonglycated components based on charge differences. In some instances, such as with most cation-exchange high performance liquid chromatographic methods, manual inspection of chromatograms can alert the laboratory to the presence of either a variant or a possible interference. Alternative non-hemoglobin-based methods for assessing long-term glycemic control may be useful in these situations (171).

Since interferences are method specific, product instructions from the manufacturer should be reviewed before use of the GHb assay method. In selecting an assay method, the laboratory should take into consideration characteristics of the patient population served, i.e., high prevalence of hemoglobinopathies.

b. Sample collection, handling, and storage

Blood can be obtained by venipuncture or by fingerprick capillary sampling (172, 173). Blood tubes should contain anticoagulant as specified by the manufacturer of the GHb assay method (EDTA can be used unless otherwise specified by the manufacturer). Sample stability is assay method specific (174, 175). In general, whole blood samples are stable for up to 1 week at 4°C. For most methods, whole blood samples stored at −70°C or colder are stable long-term (at least one year), but specimens, are not stable at −20°C. Improper handling of specimens, such as storage at high temperatures, can introduce large artifacts that may not be detectable, depending on the assay method.

Recently, a number of convenient blood collection systems have been introduced, including filter paper and small vials containing stabilizing/lysing reagent (176-178). These systems are designed for field col-
lection of specimens with routine mailing to the laboratory. These systems are generally matched to specific assay methods and should be used only if studies have been performed to establish comparability of test results using these collection systems with standard sample collection and handling methods for the specific assay method employed.

**B. Analytical**

**Recommendation:** Laboratories should use GHB assay methods with an interassay CV<5% (ideally <3%). At least two control materials with different mean values should be analyzed as an independent measure of assay performance. Laboratories should verify specimens below the lower limit of the reference interval or greater than 15% by repeat testing. If Schiff base (labile pre-HbA1c) interferes with the assay method, it should be removed prior to assay.

Level of evidence: C

**a. Performance goals and quality control**

Several expert groups have presented recommendations for assay performance. Early reports recommended that interassay CV be < 5% at the GHB concentrations found in apparently healthy and diabetic individuals (179). More recent reports suggest lower CVs (e.g., intralaboratory <3% and interlaboratory <5% (180)). These recommendations are reasonable; intraindividual CVs are very small (<2%) and many current assay methods can achieve CVs <3%. We recommend intralaboratory CV <3%.

The laboratory should include two control materials with different mean values (high and low) at the beginning and end of each day’s run. Frozen whole blood controls stored at –70˚ C or colder in single use aliquots are ideal and are stable for months or even years depending on the assay method. Lyophilized controls are commercially available, but depending on the assay method, may show matrix effects when new reagents or columns are introduced. It is recommended that the laboratory consider using both commercial and in-house controls to optimize performance monitoring.

Reference intervals: The laboratory should determine its own reference interval according to NCCLS guidelines (NCCLS Document C28A) even if the manufacturer has provided one. Test subjects should be nonobese and have FPG <6.1 mmol/L (110 mg/dL). For NGSP-certified assay methods, the SD for the reference interval is generally 0.5% GHB or less, resulting in a 95% CI of 2 % GHB or lower (e.g., mean hemoglobin A1c +/- 2 SD = 5.0 +/- 1.0%). For assay methods that are NGSP-certified, reference intervals should not deviate significantly (e.g., > 0.5%) from the 4-6 % range. Note that ADA target values derived from the DCCT and UKPDS (9), not the reference values, are used to evaluate metabolic control in patients.

**b. Out-of-range specimens**

The laboratory should repeat testing for all sample results below the lower limit of the reference interval and, if confirmed, the physician should be informed to see if the patient has an abnormal hemoglobin or evidence of red cell destruction. In addition, sample results greater than 15% GHB should be repeated and, if confirmed, the possibility of a hemoglobin variant should be considered (171).
c. Removal of labile GHb

Formation of GHb includes an intermediate Schiff base which is called “pre-A1c” or labile A1c (181, 182). This material is formed rapidly with hyperglycemia and interferes with some GHb assay methods, primarily those that are charge-based. For methods that are affected by this labile intermediate, manufacturer’s instructions should be followed for its removal.

3. Interpretation

A. Laboratory-physician interactions

The laboratory should work closely with physicians who order GHb testing. Proper interpretation of test results requires an understanding of the assay method, including its known interferences. For example, if the assay method is affected by hemoglobinopathies (independent of any shortened erythrocyte survival) or uremia, the physician should be made aware of this.

An important advantage of using an NGSP-certified assay method is that the laboratory can provide specific information relating GHb test results to both mean glycemia and outcome risks as defined in the DCCT and UKPDS (12, 90, 122). This information is available on the NGSP website. For example, each 1% change in GHb is related to a change in mean plasma glucose of approximately 2 mmol/L (35 mg/dL).

Some studies suggest that immediate feedback to patients at the time of the clinic visit with GHb test results improves their long-term glycemic control (183). However, additional studies are needed to confirm these findings before this strategy can be recommended. It is possible to achieve the goal of having GHb test results available at the time of the clinic visit by either having the patient send in a blood sample shortly before the scheduled clinic visit or by having a rapid assay system convenient to the clinic.

B. Clinical application

Recommendation: Treatment goals should be based on ADA recommendations which include maintaining GHb concentrations <7% and reevaluation of the treatment regimen for GHb values > 8%. (Note that these values are applicable only if the assay method is certified as traceable to the DCCT reference.) GHb testing should be performed at least biannually in all patients and quarterly for patients whose therapy has changed or are not meeting treatment goals.

Level of evidence: B

Treatment goals: GHb measurements are now a routine component of the clinical management of patients with diabetes mellitus. Based principally on the results of the DCCT, the ADA has recommended that a primary goal of therapy is a GHb value < 7%, and that physicians should reevaluate the treatment regimen in patients with GHb concentrations consistently >8% (9, 10). These GHb values apply only to assay methods that are certified as traceable to the DCCT reference, with reference interval approximately 4-6% HbA1c or HbA1c-equivalent. In the DCCT, each 10% reduction in GHb (e.g., 12 vs. 10.8% or 8 vs. 7.2%) was associated with approximately 45% lower risk for the progression of diabetic retinopathy (184). Similar risk reductions were found in the UKPDS (133). It should also be noted that in the DCCT and UKPDS decreased GHb was associated with increased risk for serious hypoglycemia.

Testing frequency: There is no consensus on the optimal frequency of GHb testing. The ADA recommends (14): “that for any individual patient, the frequency of GHb testing should be dependent on the judgment of the physician. In the
absence of well-controlled studies that suggest a definite testing protocol, expert opinion recommends GHb testing at least two times a year in patients who are meeting treatment goals (and who have stable glycemic control) and more frequently (quarterly assessment) in patients whose therapy has changed or who are not meeting glycemic goals.” These testing recommendations are for patients with either type 1 or type 2 diabetes. Diabetes quality assurance programs (e.g., ADA Provider Recognition Program and HEDIS 2000 (134, 135)) have generally required documentation of the percentage of patients with diabetes who have had at least one GHb determination during the preceding year. Studies have established that serial (quarterly for one year) measurements of GHb result in large improvements in GHb values in patients with type 1 diabetes (185).

Interpretation: GHb values in patients with diabetes are a continuum; they range from normal in a small percentage of patients whose mean plasma glucose concentrations are close to those of non-diabetic individuals, to markedly increased values, e.g., two- to threefold increases in some patients, reflecting an extreme degree of hyperglycemia. Multiple comparisons between the two systems are required to generate this equation, which will be used only by manufacturers (not by individual clinical laboratories) to establish traceability. Proper interpretation of GHb test results requires that physicians understand the relationship between GHb values and mean plasma glucose, the kinetics of GHb, and specific assay limitations/interferences (90). Small changes in GHb (e.g., +/- 0.5% GHb) over time may reflect assay variability rather than a true change in glycemic status.

4. Emerging Considerations

A. Use of GHb for diabetes screening/diagnosis

At present, the ADA does not recommend GHb for diabetes screening or diagnosis (186). There is considerable controversy surrounding this issue and further studies are needed to determine if GHb is useful for screening and/or diagnosis of diabetes (187-190). Harmonization of GHb assays has obviated one of the most commonly stated reasons for not using GHb for screening and/or diagnosis. Optimal clinical utility of GHb for screening and/or diagnosis will also require highly precise assay methods.

B. Use of other glycated proteins including advanced glycation end-products for routine management of diabetes mellitus.

Further studies are needed to determine if other glycated proteins such as fructosamine are clinically useful for routine monitoring of patients’ glycemic status. Further studies are also needed to determine if measurements of advanced glycation end-products (AGEs) are clinically useful as predictors of risk for chronic diabetes complications (191). None of these analytes was evaluated in the DCCT or UKPDS.

C. Global harmonization of GHb testing

In 1995, the International Federation of Clinical Chemistry (IFCC) formed a Working Group on HbA1c Standardization (IFCC-WG). This committee, which includes members from the NGSP Steering Committee and Laboratory Network, has been evaluating several candidate reference methods and purified GHb materials (purified HbA1c) that potentially could provide firm links between the NGSP and GHb standardization programs in other countries (192). Such a scheme is particularly attractive since it would allow GHb test results worldwide to be comparable to those in the DCCT and UKPDS. The IFCC has established a laboratory network using both mass spectroscopy and capillary electrophoresis as candidate reference methods. The candidate reference material is a mixture of highly-purified HbA1c and HbA0 (193-195). Initial comparisons between samples analyzed by the IFCC Laboratory Network and the NGSP Laboratory Network are encouraging; there appears to be a linear relationship between the two refer-
ence systems (personal communication from Kor Miedema, Chairperson IFCC-WG, 17 January, 2000). If further studies confirm a consistent relationship between the two networks, it will be possible to use one of the IFCC reference methods to replace the current NGSP anchor (a designated comparison method with far less specificity for HbA1c than either the mass spectroscopy or capillary electrophoresis methods). Assuming that the IFCC reference system is adopted by the NGSP and other standardization programs, an important issue that would need to be addressed is the different values obtained between the networks. The IFCC reference system yields GHb concentrations lower than those measured in the DCCT and UKPDS. Therefore, the question is whether the lower IFCC-based values should be adopted along with the new reference system or should the current values, which are traceable to the DCCT and widely used, be retained? In the latter event, the results obtained with the IFCC reference system would be converted into DCCT-equivalent concentrations by an equation. Multiple comparisons between the two systems are required to generate this equation, which will be used only by manufacturers (not by individual clinical laboratories) to establish traceability. Proper resolution of this important question will require international consensus with a process that includes both clinicians and laboratorians.