Hemoglobin Variants and Hemoglobin A₁c Analysis: Problem Solved?

Measurement of glycohemoglobin (GHb) is an integral component of the management of patients with diabetes mellitus (1, 2). Glycation is the nonenzymatic addition of a sugar residue to amino groups of proteins. Numerous proteins in the body are glycated, but GHb in blood is the analyte most widely used clinically to monitor glycemic control. The concentration of GHb is directly proportional to the mean concentration of glucose in the blood and the lifespan of erythrocytes (mean, 120 days). Thus, the GHb concentration represents an integrated value for glucose over the preceding 2–3 months. GHb provides an index of glycemic control that is free of the wide diurnal glucose fluctuations and is unaffected by recent exercise or food ingestion.

Two large, prospective, randomized clinical trials demonstrated a strong relationship between hyperglycemia and the development of microvascular complications of diabetes. In the Diabetes Control and Complications Trial (DCCT) and the United Kingdom Prospective Diabetes Study (UKPDS), which studied patients with type 1 and type 2 diabetes, respectively (3, 4), glycemic control was assessed by GHb. Both studies established a direct relationship between the GHb concentration and the risk of complications. These findings led the American Diabetes Association to recommend that a primary treatment goal in adults with diabetes should be near-normal glycemia with hemoglobin A₁c (HbA₁c) <7% when measured by the method used for the DCCT (2). The recommendation that HbA₁c be measured at least every 6 months in patients with type 1 or type 2 diabetes (2) has had a dramatic impact on the use of this test. The number of laboratories participating in the GHb surveys offered by the College of American Pathologists increased from 707 in 1990 to 2003. Clinical laboratories in the US currently perform >2 million GHb measurements each month (estimated from an unpublished survey by the College of American Pathologists).

Analogous to all tests widely used in patient care, GHb analysis is subject to certain limitations. Conditions that affect red cell turnover, e.g., hemolysis or significant blood loss, will alter the amount of GHb that accumulates (5). In addition, interassay variability and hemoglobin variants have restricted the use of GHb testing. Considerable progress in the standardization of GHb assays (6, 7) and accurate GHb measurement in patients with hemoglobin variants, as exemplified by two recent reports in Clinical Chemistry (8, 9), lead to optimism that the latter problems may be approaching resolution.

A brief overview of the principles of GHb analysis will facilitate comprehension of these issues. There are >30 different GHb assay methods, which are based on two general principles (10, 11). The first group of methods, which includes cation-exchange chromatography and electrophoresis, separates glycated from nonglycated hemoglobin components based on differences in their charges. The second group of assays exploit variations in structure related to the presence of the attached glucose; they include immunoassays and boronate affinity chromatography. The vast majority of GHb measurements in the US are performed by ion-exchange HPLC or immunoassay. These assays measure HbA₁c (hemoglobin glycated at the N-terminal valine of the β-chain) or total GHb (comprising HbA₁c and hemoglobin glycated at other sites).

Results reported on the same blood sample can vary substantially, depending on the analyte measured and the method used. For example, GHb values ranged from 4.0% to 8.1% in one study (12) and from 5.1% to 8.2% in another (13) on a single sample. To resolve this problem, committees were established independently under the auspices of the AACC and the IFCC to standardize GHb assays. In 1993, the AACC established a GHb standardization subcommittee, which recommended that the DCCT reference method be used as the designated comparison method for GHb standardization while reference methods and pure standards were being developed. The National Glycohemoglobin Standardization Program (now known as NGSP) was formed to harmonize GHb assays with the DCCT assay. Modeled on the Cholesterol Reference Method Laboratory Network program, the NGSP uses a network of reference laboratories and interacts with manufacturers of GHb methods to allow them to establish traceability to the Central Primary Reference Laboratory (which analyzes HbA₁c by HPLC according to the method used in the DCCT) (6). Methods and laboratories can then be certified as traceable to the DCCT according to rigorous precision and bias criteria. A different approach was adopted by the IFCC working group, which developed a mixture of purified HbA₁ and HbA₁c as a primary reference material (7). A reference method was established in which hemoglobin is cleaved into peptides by endoproteinase Glu-C. The glycated and nonglycated N-terminal hexapeptides are separated and quantified by HPLC–electrospray ionization mass spectrometry (ESI-MS) or by HPLC–capillary electrophoresis (7). HbA₁c is measured as the ratio between glycated and nonglycated N-terminal hexapeptides. The high specificity of this method yields values that are lower than those obtained with most commercial methods (7). The new reference method has been approved by the member societies of the IFCC and may ultimately provide the foundation for global standardization of GHb measurements.

Hemoglobinopathies may interfere with GHb analysis, independent of their effects on erythrocyte survival [for a review, see Bry et al. (5)]. Results may be falsely increased or decreased, depending on the particular method and the hemoglobinopathy. Hemoglobin variants that cannot be separated from HbA or HbA₁c will produce spuriously increased or decreased results by ion-exchange HPLC [see Fig. 1 in Bry et al. (5) for details]. This is not a trivial
problem: more than 850 hemoglobin variants have been identified (14). Calculations based on disease prevalence indicate that in the US in 2001 HbAS (sickle cell trait) and HbAC were present in ~250 000 and ~85 000 patients, respectively, 18 years or older with type 2 diabetes. The global impact may be substantially larger because variant hemoglobins are reportedly present in up to one-third of all diabetic individuals in other parts of the world (5).

Two recent reports (8, 9) address this concern. In this issue, Friess et al. (8) describe a patient who had a 9-month history of type 1 diabetes with poor glycemic control [fasting glucose concentrations up to 13.6 mmol/L (245 mg/dL)] and an inappropriately low HbA1c result of 6% (reference interval, 4.3–6.1%) as measured by ion-exchange HPLC. HbA1c measured by immunoassay was 9%, which is equivalent to an approximate mean plasma glucose concentration of 13.5 mmol/L (240 mg/dL) (2). Careful review of the HPLC chromatogram revealed an abnormal peak that interfered with the HbA1c fraction. Subsequent electrophoresis demonstrated an abnormal hemoglobin band, which was identified by DNA sequencing to have a base exchange leading to substitution of asparagine for lysine at residue 66 in the β-chain (8). Protein sequencing by mass spectrometry verified the amino acid substitution. The variant hemoglobin had decreased oxygen affinity but was clinically silent, and the patient had no evidence of hemolysis or anemia. The reduced oxygen affinity was anticipated because Lys-66 is known to be important for oxygen binding, and two other hemoglobin variants mutated at this residue had lowered oxygen binding (8). In contrast, the interference in HPLC measurements of HbA1c was unanticipated and had not been reported previously. Immunoassays for HbA1c use antibodies that recognize the first few (4–10) amino acids at the N-terminal end of the hemoglobin β-chain plus glucose attached by a ketoamine linkage (11). The substitution at residue 66 would not be expected to alter antibody binding, and the HbA1c result by immunoassay in this patient is likely to be accurate. This study is an excellent example of elucidation of discrepant laboratory data and emphasizes the importance of visual inspection of chromatograms when GHb is measured by ion-exchange HPLC.

The second report (9) describes a method to measure HbA1c by mass spectrometry that is free from interference by most variant hemoglobins. The strategy used is a modification of the ESI-MS method used in the IFCC reference method. The authors synthesized two pairs of hexapeptides corresponding to the six amino acids of the N-terminal β-chain of hemoglobin as internal standards: glycated and nonglycated unlabeled peptides, as well as deuterium-labeled glycated and nonglycated peptides (9). The innovation of the method is the inclusion of stable-isotope-labeled internal standards, which enhanced reproducibility. These isotope-labeled peptides are not incorporated in the approved IFCC reference method. In addition, univalent ions for the four peptides were selected for monitoring because they had lower noise than divalent ions, which are used in the IFCC method. This modified method exhibited linear calibration curves over a wide range of values, and results were constant among assays on different days. The ESI-MS method offers two important advantages over other GHb assays: measurement of HbA1c is unaffected by hemoglobinopathies, and variant hemoglobins can be characterized (15). Despite these valuable attributes, the equipment costs and complexity of operation currently preclude ESI-MS from widespread use in routine laboratories that measure patient samples. Nevertheless, the ESI-MS method is likely to be useful to accurately measure HbA1c in samples with hemoglobin variants that produce spurious results with commonly used methods.

Most assays routinely used in clinical laboratories for GHb analysis are subject to interference by hemoglobin variants (5, 16, 17). Although ion-exchange HPLC methods may yield erroneous results as described above, hemoglobin variants can usually be detected by examining the chromatogram. Some immunoassays avoid spurious results for several hemoglobinopathies, but interferences depend on the antibody used and vary among assays (5). Moreover, hemoglobin variants cannot be identified by immunoassays. Boronate affinity chromatography has the least interference from hemoglobin variants, but like immunoassays, it cannot detect the presence of a variant hemoglobin.

Although measurements of glycated proteins other than GHb, e.g., glycated serum albumin or fructosamine, have been used to measure metabolic control in patients with diabetes (5), their clinical utility has not been established, and there is no convincing evidence that relates their concentrations to the chronic complications of diabetes (1). GHb remains the sole glycated protein with a large body of convincing, patient-outcome data. A hemoglobinopathy should be suspected in patients with HbA1c results >15%, in patients with HbA1c results below the lower limit of the reference interval, or when the value varies substantially from other indices of metabolic control (5). In these circumstances, the sample should be analyzed by a method that uses a different assay principle that is less likely to be subject to interference.

The question posed in the title can now be addressed. As demonstrated by Friess et al. (8), inspection of HPLC tracings may yield valuable information regarding hemoglobin variants. Important unanswered questions include whether chromatograms are routinely examined visually in clinical laboratories and what action, if any, is taken if abnormalities are detected. The ESI-MS approach proposed by Nakanishi et al. (9) overcomes many of the limitations of GHb assays performed in clinical laboratories, but hemoglobin variants with amino acid substitutions or deletions in the N-terminal hexapeptides are unlikely to be measured accurately. Moreover, HbA1c results obtained by ESI-MS will not reflect mean glycemia in the presence of those hemoglobin variants that have altered rates of glycation (18) or a shortened erythrocyte lifespan (5). For these reasons, one reaches the conclusion that the answer to the question is “no”, the problem of
assessing glucose control by measurement of GHb in patients with hemoglobin variants has not been solved. Nevertheless, the two reports serve to enhance our awareness of the shortcomings associated with GHb analysis and at the same time provide reassurance that HbA1c can be measured accurately in the presence of most hemoglobinopathies.

References


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