Electrospray Mass Spectrometry for Measurement of Glycohemoglobin

With the publication of the results of the landmark 9-year Diabetes Control and Complications Trial (DCCT), the importance of Hb A1c/glycohemoglobin testing in diabetes was firmly established. The DCCT showed, at least in patients with insulin-dependent diabetes mellitus, that the risk for development and progression of the chronic complications of diabetes is closely related to the degree of metabolic control as measured by glycohemoglobin determinations [1]. Generally, the same is believed to be true for patients with non-insulin-dependent diabetes mellitus, although the results of the UK Prospective Study must be awaited [2, 3].

The DCCT has confirmed that measuring glycohemoglobin in blood is an excellent tool for the long-term control of the glycemic state. Considering the high prevalence of diabetes mellitus, glycohemoglobin is one of the most important analytes in laboratory medicine today [4]. A major task of clinical chemistry should be to optimize conditions for its determination. The development of new methodologies and international standardization is an essential part of such efforts.

Despite the ongoing efforts for standardization of Hb A1c/glycohemoglobin [5], many glycohemoglobin assays are not standardized among clinical laboratories; values reported by one laboratory may not agree with those from another, and possibly neither would agree with those from the DCCT [6].

Glycohemoglobins are the products of a nonenzymatic reaction between the free aldehyde group of glucose (Hb A1c) or other sugars and the unprotonated form of free amino groups of hemoglobin. The percentage of glycohemoglobin in human blood depends on the concentration of glucose, the duration of glucose exposure to hemoglobin, and the turnover of erythrocytes. The higher the glucose concentration and the longer the exposure time, the higher the percentage of Hb A1c.

The potential glycation sites of the hemoglobin A molecule are the N-terminal amino acid valine of the four polypeptide chains (α₂β₂) and all free ε-amino groups of lysine within these chains. The predominant glycation site is the N-terminal valine residue of the β-chain, which accounts for ~60% of all bound glucose. This glycation product is generally referred to as Hb A1c. The remaining glucose is bound to one of the 44 glycation sites at the ε-amino groups within the α- and β-chains or at the N-terminal valine of the α-chain [7, 8].

Many analytical methods for measuring glycohemoglobin in human blood are currently being used by clinical laboratories, methods based on physical, chemical, or immunological differences between glycated (Hb A1c) and nonglycated hemoglobin (Hb A0). Ion-exchange chromatography (HPLC systems, minicolumns), electrophoresis, and isoelectric focusing methods exploit the charge differences between Hb A0 and Hb A1c (the attachment of glucose to the N-terminal amino group of the β-chain changes the isoelectric point of hemoglobin). However, the homogeneity of the Hb A1c peak is seriously questionable and many possible interferences are known, so the specificity of this kind of technique remains uncertain. Affinity-chromatographic methods (minicolumns, automated systems, HPLC systems) are based on the reaction of the cis-1,2-diol groups of hemoglobin-bound glucose (and other sugars) with immobilized boronic acid, forming a boronate ester. In theory, all sugar adducts of hemoglobin in human blood can be bound; hence, the amount of total glycohemoglobin is measured. Unlike cation-exchange HPLC, affinity chromatography is less susceptible to interfering substances (e.g., carbamylated hemoglobin, acetylated hemoglobin, hemoglobin variants, or degradation products). In the last few years, the spectrum of methods has been broadened by the addition of immunological methods (enzyme immunoassays, latex agglutination inhibition assays, homogeneous immunoassays), which are being used by an increasing number of laboratories. These methods use monoclonal or polyclonal antibodies directed against the glycated N-terminal group of the β-chain of hemoglobin (last 4, 6, or 8 amino acids) and are therefore specific for the type of glycation seen in Hb A1c (although glycation of many genetic variant hemoglobins produces the same N-terminal structure). As a result of these recent developments, medical laboratories can currently choose from >20 different commercially available tests for measuring Hb A1c, Hb A1, or total glycohemoglobin in human blood. In addition, there is a trend towards an increasing use of automated laboratory systems.

Unfortunately, the global application of current methods is limited by a lack of appropriate standardization. Early efforts to standardize Hb A1c/glycohemoglobin values among clinical laboratories by using a “universal calibrator” proved feasible with some methods, but did not work for several others [5, 9]. In particular, because the chemical entity to be analyzed was not defined, a reference system could not be set up. Therefore, both the AACC Subcommittee on Glycohemoglobin Standardization and the IFCC Working Group on Standardization of HbA1c proposed that the method used in the DCCT study be regarded as the “designated comparison method,” against which most if not all assay methods should be standardized, while waiting for development of a scientifically sound reference system [10].

Several studies have demonstrated that the amount of glycohemoglobin measured by cation-exchange chromatography, affinity chromatography, and immunoturbidimetric assays equally well reflects the degree of glycemic control in diabetics. Correlation studies between cation-exchange vs boronate-affinity chromatography have shown that, in the lower range of percentage glycation values, the affinity methods measure lower glycohemoglobin values because the heterogeneity of the Hb A1c
peak in cation-exchange chromatography results in the reporting of greater values [11, 12]. In an uncontrolled diabetic, however, the affinity values measured are generally higher than the cation-exchange values because multiple sites are glycated in both the α- and β-chain of hemoglobin. To end this uncertainty of which analyte to measure, the IFCC Working Group on Hb A1c standardization has chosen the specific Hb A1c epitope [β-N-(1-desoxyfructosyl) hemoglobin] as the analytical entity to measure and develop a standardization procedure for Hb A1c.

In this issue, Roberts et al. [14] describe the use of electrospray mass spectrometry (ESMS) as an alternative method for quantifying glycohemoglobins. This technique has been recognized for many years as one of the most valuable tools for the characterization of variant hemoglobins. Because the mass spectrometer separates components according to their molecular mass, the α- and β-chains, and their glycated and nonglycated derivatives, can easily be separated. However, ESMS cannot differentiate between the different sites of glycation because all the glycated species that exhibit single (or double) glycation on a given chain have an identical mass.

The ESMS method described by Roberts et al. is a new and excellent approach to the measurement of glycohemoglobins. The method is, in principle, capable of measuring the extent of glycation in both the α- and β-chains and can be applied directly to diluted and hemolyzed samples without the interferences that have hampered other reported methods.

Several challenges must be considered in defining the role of mass spectrometric techniques for glycohemoglobin. First, the reproducibility, both within and between runs, needs to be refined and documented by evaluations such as that in the NCCLS EP5-T2 protocol [15] for glycohemoglobin assay method certification as used by the American National Glycohemoglobin Standardization Program, which set a target value of CV <3% for secondary reference methods. Also, because ESMS instrument performance is known to vary from day to day, the performance of this method should be monitored for at least 6 months. Samples used for this monitoring process should be kept stable at −70 °C, thus avoiding the disturbance of the ESMS pattern by degradation products (e.g., cysteinylated β-chain) that form in significant amounts when whole-blood samples are stored at 2–4 °C for several weeks.

Second, more work is needed to determine whether the percentage glycation is best calculated as peak height or peak area. Because the ratio of signals observed for equimolar amounts of α- and β-chain is ~4:3, and assuming that the glycated and nonglycated forms would respond similarly, use of the ESMS technique may require inclusion of a reference standard.

Third, the detection limit of the method requires attention. Roberts et al. found that ESMS did not detect multiply glycated hemoglobin. Multiple glycation events in both the α- and β-N-valines and the ε-lysine residues have been demonstrated beyond doubt [7, 8], and Roberts et al. concluded that diglycated hemoglobin was not detected in their method because their concentration is <2% of the total nonglycated hemoglobin, below the detection limit of the assay.

Finally, the reference range requires further study. Considering that ESMS measures total glycation without being affected by interferences, one would expect low ESMS values in nondiabetic subjects. The reference range stated by Roberts et al., averaging ~3.0% in nondiabetic subjects, is only slightly lower than those obtained by the most specific cation-exchange methods, e.g., polyCat A and MonoS, where values <3.5% are considered normal [16–18]. These low values were also confirmed by affinity chromatography [11]. The agreement of ESMS values [14] with results by several affinity methods is good; however, the affinity methods used are all internally matched or standardized with the BioRex 70/DCCT-designated comparison method, somewhat complicating interpretation of the agreement [19].

ESMS may not have its primary role in routine laboratory settings. The sophisticated (and expensive) analytical instruments and computer software to analyze the data sets produced by ESMS in a relatively short time will probably remain out of reach for most routine clinical laboratories. For use as a Reference Method, ESMS methodology will need to distinguish the glycation of lysine residues in the central region of the α- or β-chains from glycation of α-terminal and β-terminal valines. Reference systems require an exact knowledge of the analytes to be measured; for Hb A1c, glycohemoglobin, standardization through the IFCC will focus on Hb A1c as defined as β-N-(1-desoxyfructosyl) hemoglobin [13]. Refinements of mass spectrometric techniques have the potential to distinguish these analytes, and the report of Roberts et al. represents a welcome contribution to stimulate such refinements. The future promises to be interesting as candidate methods evolve to provide improved standardization in this clinically important area.

References


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