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SYMPOSIUM

A Review of the Challenge in Measuring Hemoglobin A1c

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Abstract

The attraction of the simple biochemical concept combined with a clinical requirement for a long-term marker of glycolic control in diabetes has made hemoglobin A1c (HbA1c) one of the most important assays undertaken in the medical laboratory. The diversity in the biochemistry of glycation, clinical requirements, and management demands has resulted in a broad range of methods being developed since HbA1c was described in the late 1960s. A range of analytic principles are used for the measurement of HbA1c. The charge difference between hemoglobin A0 and HbA1c has been widely utilized to separate these two fractions, most notably found these days in ion-exchange high-performance liquid chromatography systems; the difference in molecular structure (affinity chromatography and immunochemical methods) are becoming widely available. Different results found in different laboratories using a variety of HbA1c analyses resulted in the need for standardization, most notably in the United States, Japan, and Sweden. Designated comparison methods are now located in these three countries, but as they are arbitrarily chosen and have differences in specificity, results of these methods and the reference values and action limits of the methods differ and only harmonized HbA1c in specific geographic areas. A reference measurement system within the concept of metrological traceability is now globally accepted as the only valid analytic anchor. However, there is still discussion over the units to be reported. The consensus statement of the International Federation of Clinical Chemistry (IFCC), the American Diabetes Association, the International Diabetes Federation, and the European Association for the Study of Diabetes suggests reporting HbA1c in IFCC units (mmol/mol), National Glycohemoglobin Standardization Program units (%), and estimated average glucose (either in mg/dl or mmol/liter). The implementation of this consensus statement raised new questions, to be answered in a concerted action of clinicians, biochemists, external quality assessment organizers, patient groups, and manufacturers.

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Abbreviations: (ADA) American Diabetes Association, (ADAG) A1c-derived average glucose, (CPRL) central primary reference laboratory, (CV) coefficient of variation, (DCCT) Diabetes Compliance and Complications Trial, (eAG) estimated average glucose, (EASD) European Association for the Study of Diabetes, (HbA1c) hemoglobin A1c, (HPLC) high-performance liquid chromatography, (IDF) International Diabetes Federation, (IFCC) International Federation of Clinical Chemistry, (JDS) Japan Diabetes Society, (NGSP) National Glycohemoglobin Standardization Program, (POCT) point-of-care testing, (PRM) primary reference method, (PRMS) primary reference measurement system, (SRM) secondary reference material

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Introduction: Serendipity Again

The discovery of the deviating migration speed of sickle cell hemoglobin in an electrical field by Pauling and colleagues (in 1949) first showed the heterogeneity of the hemoglobin molecule.[1] This was a stimulus for the development of methods for hemoglobin variant detection, and using ion-exchange chromatography, Kunkel and Wallenius established the occurrence of "minor components" in normal adult hemoglobin (in 1955) in the absence of variants.[2] Detailed studies showed five subfractions, which were named hemoglobin A1a, hemoglobin A1b, hemoglobin A1c (HbA1c), hemoglobin A1d, and hemoglobin A1e, depending on the order in which they eluted.[3] As these subfractions eluted faster than the main hemoglobin A0 fraction, they were collectively named "fast hemoglobins." In 1969, Rahbar and associates[4] demonstrated that fast hemoglobins were elevated in erythrocytes of diabetes patients, and in 1971, Trivelli and coworkers were the first to suggest a relationship between fast hemoglobins, mean blood glucose concentrations, and long-term complications of diabetes patients.[5] The simple biochemical concept and the clinical need for a long-term assessment of glycemic control in the patient with diabetes contributed to the explosion in the popularity of HbA1c, resulting in the development of a broad range of analytic methods for the routine laboratory. This article reviews the two topics dominating the analytic challenge: analytic methods and standardization.

The Analytic Challenge

The development of an analytic method for the clinical laboratory relies on the biochemical properties of the analyte, the clinical requirements of the end users, and the management demands of the laboratory.

Biochemistry: Welcome in a Complicated Family

The development of analytic methods starts with the definition of the analyte of interest and its chemical and physical properties. Biochemical investigations showed that HbA1c is the result of a classical Maillard reaction: the *N*-terminal valine of the β chain reacts with glucose to the aldimide (Schiff base or labile HbA1c), which undergoes an Amadori rearrangement to the stable ketoamine (HbA1c).[6] This reaction also occurs at approximately 10 other sites in the hemoglobin molecule to form other glycohemoglobins in addition to HbA1c. Hemoglobin A1c makes up 60% of all glycohemoglobins (total glycated hemoglobin).[7] Glucose is not the only molecule to react with hemoglobin; in an analogous reaction, urea forms carbamylated hemoglobin.[8] Point mutation of one of the amino acids in the protein chains of the hemoglobin molecule occurs frequently in non-Caucasian ethnic groups and are called hemoglobin variants. Variant hemoglobins react with glucose in a similar way to hemoglobin A, but the electrochemical properties of both glycated and nonglycated forms are different.[9] Thus researchers have to deal with the fact that HbA1c is a member of a large and complicated family: a dominant parent hemoglobin (A0) with a twentyfold concentration, sisters (Schiff base and glycohemoglobins other than HbA1c), cousins (other derivatives like carbamylated hemoglobin), and uncles (hemoglobin variants). An additional fact is that HbA1c is not a stand-alone analyte, but is formed in quantities related to the total hemoglobin concentration. Consequently, to compensate for intraindividual and interindividual variation in the total hemoglobin concentration, HbA1c should be expressed as a ratio (HbA1c/total hemoglobin). In analytic terms, any concept of a HbA1c assay will require a dual measurement of HbA1c and total hemoglobin and therefore suffers from dual uncertainty in the outcome of the test. The ultimate challenge is to find an analytic device with good specificity and clinically relevant imprecision.

Clinical Requirements

Physicians' and patients' requirements can be summarized as reliable, interpretable, and convenient. More than any other analyte, HbA1c is a longitudinal parameter: results of a patient are monitored over years or even decades and may form the basis for change in therapy. This requires highly reproducible results over a long period of time.[10] Results should also be interpretable: numbers should relate to clinical studies, like the Diabetes Compliance and Complications Trial (DCCT), and treatment goals as defined by diabetes organizations.[11] This implies that HbA1c assays should be standardized.[12] Hemoglobin A1c testing should be convenient for the patient: no additional visit to the laboratory. And to have the optimum psychological impact, HbA1c results should preferably be available during the patient's visit to the physician.[13]

Management Demands

Hemoglobin A1c represents a high-volume request in the medical laboratory, and therefore efficiency is required. High throughput of samples, robustness of the instrument,

and low costs are prerequisites. The analytic solution chosen should also fit in the organizational structure of the laboratory: either integrated on the general chemistry analyzer or as a convenient stand-alone instrument that does not require a high degree of specialism.

Biochemistry clinical requirements and management demands set the stage for the development of an analytic system for HbA1c.

Analytic Systems for HbA1c

The requirements and demands referred to earlier have resulted in many analytic methods being developed since the 1970s, making use of the differences in electrical charge between HbA1c and hemoglobin A0 or the structural differences between glycated and nonglycated forms of hemoglobin.

Methods Based on Difference in Charge

Hemoglobins A1c and A0 have a subtle difference in isoelectrical point. This implies that, at given analytic circumstances, they have a different electrical charge, and on this basis, they can be separated. This is the basis for methods using ion-exchange chromatography, electrophoresis, capillary electrophoresis, and isoelectric focusing.[14] These methods may suffer interferences from other members of the hemoglobin family (e.g., Schiff base, carbamylated hemoglobin, and variants), so there is a need to avoid unspecificity. They have to deal with the dominating (twentyfold concentration) parent hemoglobin A0; good separation and quantitation of HbA1c and hemoglobin A0 are prerequisites. The demands of these biochemical facts have resulted in only a few methods that have survived evolution since the 1970s.

In the beginning, the focus was on shortening the time of analysis (which took days and thus was not applicable in routine use). This resulted in disposable minicolumns in the late 1970s, so 10 to 20 samples could be assayed in approximately 2 h. These systems suffered from nearly all potential problems: laborious, interference from Schiff base, and carbamylated hemoglobin (measured as HbA1c) data were not reliable due to high temperature-dependency, and the results were not standardized. In the 1980s, automated high-performance liquid chromatography (HPLC) systems were developed to solve these problems. After several generations, several systems (major suppliers: TOSOH, Bio-Rad, and ARKRAY/Menarini) have reached a high level of performance. They are highly efficient (e.g., automated, high throughput, and robust), meet the clinical requirements of reliability (use of calibrators) and interpretation (standardized at national level), and do not suffer from interference by the Schiff base or carbamylated hemoglobin. Limitations remaining are the capacity (e.g., samples are analyzed one by one and instruments often hold a maximum of 100 specimens), occasional problems with variants (although solved for most common variants, so the fact that variants are detected can also be seen as an advantage), and the fact that an HPLC is a stand-alone instrument rather than a multipurpose general chemistry analyzer.

The other members of the family, capillary electrophoresis and isoelectric focusing, were developed in the 1970s and 1980s, but they became extinct in the 1990s; analytic performance as well as throughput were insufficient and could not be improved.

Methods Based on Structural Difference: Affinity Chromatography

A structural difference between HbA1c and hemoglobin A0 results from the presence of the glucose group in HbA1c. Agents reacting specifically to this glucose moiety can be the basis for analytic methods. A method able to separate glycohemoglobins on the basis of the binding of the cis-diol groups of the glucose to m-amino phenylboronic acid cross linked on agarose was described in the early 1980s by Mallia and colleagues.[15] Glycohemoglobins bind to the affinity resin while all nonglycated hemoglobins do not. Parallel to the ion-exchange approach, the first generation consisted of disposable minicolums and largely replaced these, as they were less sensitive to temperature and interferences such as carbamylated and fetal hemoglobin. A major fundamental biochemical fact of affinity chromatography is that not only HbA1c but all glycohemoglobin binds to the resin; it is unavoidable that total glycohemoglobin is measured and thus the outcome of tests will be substantially higher than those specific for HbA1c. Fortunately, formation of glycohemoglobins is proportional, which opens the option to standardize to HbA1c units. The analytic principle has evolved in two directions: automated chemistry analyzers (Abbott) and affinity gel HPLC (Primus). The major challenge remaining for the application in automated chemistry analyzers is to achieve sufficient reproducibility. For affinity HPLC, this is the robustness of the system, especially of the gel column.

Methods Based on Structural Difference: Immunochemical Assays

An alternative approach to using the structural difference of HbA1c and hemoglobin A0 is to develop antibodies

to HbA1c; since the early 1990s,[16] the immunochemical principle has been applied to many methods. The antibody is targeted against the β *N*-terminal glycated tetrapeptide or hexapeptide group. Assay design is variable, ranging from immunoturbidimetry to latex-enhanced competitive immunoturbidimetry and enzymatic detection. There are a number of commercial assays that are applicable to a broad variety of general chemistry analyzers (including Roche, Siemens, and Vitros). Immunochemical assays are not affected by problems related to electrical charge and can be adapted easily in the routine medical laboratory. However, they all suffer with the general drawback of immunochemistry, i.e., the nonlinear calibration curve, which requires multilevel calibration. As stability of the reagent is limited (variable from test to test), relatively frequent recalibration is needed. Also, to achieve percentages of HbA1c, total hemoglobin is measured separately according to a different analytic principle that introduces additional uncertainty in the outcome. The major challenge for immunochemical tests is to achieve acceptable imprecision [coefficients of variation (CVs)], preferably with a CV below 2%, the level achieved by the best HPLCs and needed for optimum diabetes control and especially for use of HbA1c to diagnose diabetes.[17]

Methods Based on Structural Difference: Enzymatic Assays

New enzymatic tests have been developed in the 2000s.[18] Lysed blood samples are subjected to proteolytic digestion. Glycated valines are released and serve as substrate for fructosyl valine oxidase. The produced hydrogen peroxide is measured using a horseradish peroxidase-catalyzed reaction with a chromogen. The major challenges are comparable to those of immunochemical tests.

Today's Market

Since the 1970s, we have seen the rise and fall of analytic methods. Proficiency-testing surveys provide one measure of the utilization of different commercial methods. The College of American Pathologists survey, as summarized on the National Glycohemoglobin Standardization Program (NGSP) Web site,[19] shows that, in the United States, approximately 60% of the laboratories are using an immunochemical test, 30% use automated ion-exchange HPLC, and less than 10% use a method on the basis of affinity chromatography. The European Reference Laboratory External Quality Programme[20] shows that, in Europe, there is a mirror image, with some 60% HPLC users, 35% immunochemistry users, and only a few users of affinity chromatography.

Point of Care

These methods were discussed on the basis of methodological principle. Methods could also be divided into laboratory instruments and point-of-care testing (POCT) instruments. The analytic performance of laboratory instruments is better than the performance of POCT instruments, but POCT instruments have the advantage of producing results during the patient's visit to the physician (thus meeting the clinical requirement of convenience in **Table 1**). The development of POCT instrument is a recent trend that will be covered by a separate contribution to this symposium.[21]

Standardization

With the introduction of HbA1c into routine use, it quickly became apparent that there were significant differences in the results between different laboratories as well as within laboratories when viewed over a long period. Thus results did not meet the clinical requirement of long-term reproducibility. This became an important issue after the publication of the DCCT study in 1993, a study that allowed evidence-based interpretation.[22] Differences between laboratories were due to the wide range of methods used, each with their own definition of the analyte (e.g., HbA1c, fast hemoglobins, or total hemoglobin) and specificity (e.g., hemoglobin F,

Table 1.
Conversion Factors to Derive HbA1c and eAG from IFCC and NGSP Units

	Derived from IFCC units, *X* = HbA1c in IFCC units (mmol/mol)	Derived from NGSP units, *X* = HbA1c in NGSP units (%)
HbA1c	IFCC (mmol/mol) = *X* NGSP (%) = 0.0915*X* + 2.15 JDS (%) = 0.0927*X* + 1.73 MonoS (%) = 0.0989*X* + 0.88	IFCC (mmol/mol) = 10.93*X* – 23.5 NGSP (%) = *X* JDS (%) = 1.013*X* – 0.45 MonoS (%) = 1.081*X* – 1.44
eAG	eAG (mmol/liter) = 0.146*X* + 0.93 eAG (mg/dl) = 2.63*X* + 15.0	eAG (mmol/liter) = 1.59*X* – 2.59 eAG (mg/dl) = 28.7*X* – 46.7

carbamylated hemoglobin, or incomplete separation). Standardization, on name and numbers, could overcome this and became an important objective for scientists and clinicians in the 1990s.

Name: HbA1c After All

Initially, the name "fast hemoglobins" was used. Later, according to their chromatographic properties, several trivial names were used: hemoglobin A1a, hemoglobin A1b, HbA1c (referring to their order of elution from an ion-exchange column), hemoglobin A1 (sum of hemoglobins A1a, b, and c), and hemoglobin A1c (the major component of hemoglobin A1). In the late 1970s, the terms glycosylated and glucosylated hemoglobin were introduced. In 1983, the International Union of Pure and Applied Chemistry and International Union of Biochemistry and Molecular Biology Joint Commission on Biochemical Nomenclature suggested "glycated hemoglobin" but, finally in 1986, recommended "glycohemoglobin." More recently, A1C and total glycohemoglobin have been used. Nordin proposed the more structural name "deoxyfructos (or DOF) hemoglobin." The consensus statement of the International Federation of Clinical Chemistry (IFCC), the European Association for the Study of Diabetes (EASD), the International Diabetes Federation (IDF) in 2007, and the American Diabetes Association (ADA) declares that the name should be HbA1c.[23]

Numbers

The need for standardization was felt in the beginning of the 1990s, but the lack of international efforts resulted in several countries developing standardization programs.

NGSP in the United States

After publication of the DCCT study in 1993, the American Association for Clinical Chemistry decided that all routine HbA1c results should be harmonized to "DCCT units." A subcommittee was convened and led to the formation of the NGSP.[24] The BioRex 70 HPLC of the DCCT central laboratory was chosen as the primary reference method (PRM), and the DCCT central laboratory in Minnesota was established as the central primary reference laboratory (CPRL) to set the initial calibration for the NGSP Certification Programme. To warrant continuity, there are two other primary reference laboratories operating the PRM with a single hemolysate calibrator prepared and targeted by the CPRL. Eight secondary reference laboratories work directly with the manufacturers to assist them in standardizing commercial methods and their annual (re)certification program. Secondary reference laboratories use robust routine methods calibrated against the CPRL.

Japan Diabetes Society/Japan Society of Clinical Chemistry Scheme

The national standardization scheme of Japan was developed in 1995 by the Japanese diabetes society (JDS) in collaboration with the Japan Society of Clinical Chemistry.[25] Frozen hemolysates were prepared as calibrators with the mean of the two most common HPLC systems (i.e., TOSOH and Kyoto Daiichi Kogaku) as the target. In 2000, the very specific KO500 HPLC became the reference method to assign values to new lots of calibrators, but to keep consistency, assigned values are adjusted to those initially set in 1995.

Standardization Scheme in Sweden

Mono S HPLC is used as the anchor for the Swedish standardization scheme.[26] This HPLC system is a relatively specific in separating HbA1c from most minor endogenous components, except carbamylated hemoglobin. The program started in 1998 and is used for the calibration of hospital and point-of-care instruments in Sweden.

Toward International Standardization: The IFCC Reference Measurement System

The three national standardization programs listed here are pragmatic: in all cases, a HPLC method was arbitrarily chosen as the anchor to harmonize results to comparable numbers in a geographic area. As different HPLCs produce different HbA1c numbers, it is not surprising that the national reference systems have different reference values. The NGSP reference method is the less specific of the three: approximately one-third of the chromatographic component denoted as HbA1c is not HbA1c. The Swedish reference system is the most specific, with the Japanese method somewhere in between. Consequently, the upper HbA1c level in the nondiabetes population is highest for the NGSP (6.0%), lowest for Sweden (5.0%), and in between (5.5%) for Japan. All are suitable to harmonize results, but none of them reflect the true HbA1c, and the differences hamper international comparisons. To overcome this, to achieve global standardization, and to meet the requirements of the European Union directive on *in vitro* diagnostic medical devices, the IFCC established a working group on HbA1c standardization to develop a reference measurement system within the concept of metrological traceability.[27–31] The starting point in such a system is the definition of the analyte; HbA1c is defined as hemoglobin molecules having a stable adduct of glucose to the *N*-terminal valine of the hemoglobin β chain (β*N*-1-deoxyfructosyl-hemoglobin). Pure HbA1c and pure hemoglobin A0 are isolated from human blood and

mixed in well-defined proportions to a certified primary reference material set used to calibrate the primary reference measurement system (PRMS). The PRMS values are assigned to secondary reference materials (SRMs, e.g., whole blood); on incubation with the enzyme endoproteinase Glu-C, the *N*-terminal hexapeptide of the β chain is cleaved off and glycated and nonglycated hexapeptides are separated and quantified by mass spectrometry or capillary electrophoresis.[28] The SRMs are used by the manufacturers to calibrate their instruments. A laboratory network is in place to implement and maintain the PRMS.[29]

The First Dilemma

The availability of the IFCC reference measurement system raised the classic dilemma associated with a change from familiar old units to unfamiliar new units, as seen before for mmol/liter versus mg/dl, kilometers versus miles, and Celsius versus Fahrenheit. The advantages of the new method were clear: metrological traceability and worldwide harmonization of HbA1c values. But the disadvantages were also clear: change would cause confusion and require extensive prolonged and expensive education for all parties involved (e.g., clinical chemists, physicians, patients, and manufacturers). In the discussion on old and new HbA1c values, a new element was introduced: why not express HbA1c in average blood glucose units?[32] The IFCC, the IDF, the EASD, and the ADA came to a consensus statement, a typical compromise to end the discussion and to have the best of two worlds: HbA1c should be reported in both IFCC (mmol/mol) and derived NGSP (%) numbers as well as in estimated average glucose (eAG) values (mmol/liter or mg/dl) if the A1C-derived average glucose (ADAG) study reached its *a priori* targets, which were not known at that time;[23] the study has now been published.[32] Two other issues are more straightforward: the name should be HbA1c and the IFCC reference system is the only valid analytic anchor.

The Second Dilemma

The consensus statement raised new discussions; report of the outcome of a laboratory test in three numbers is unpractical (for laboratories, for physicians/patients, and for manufacturers), and in countries not using the NGSP-numbers before (Japan), there is no reason to report them now. The consensus statement was agreed upon before the outcome of the ADAG study; clinical and scientific societies now need to evaluate the outcome of the study and decide whether to implement eAG or not. The relationship between HbA1c and glucose might be different for different groups (i.e., age, ethnicity, geography, and pregnancy).[33]

Concerted Action

The analytic basis for HbA1c is irrefutable; the IFCC reference system represents the only valid anchor from which all other units in which HbA1c might be expressed are derived (**Table 1** and **Figure 1**). Which numbers will be used is a political issue, and a decision has to be made in a concerted action of all who are involved: clinicians, biochemists, external quality assessment organizers, patient groups, and manufacturers.

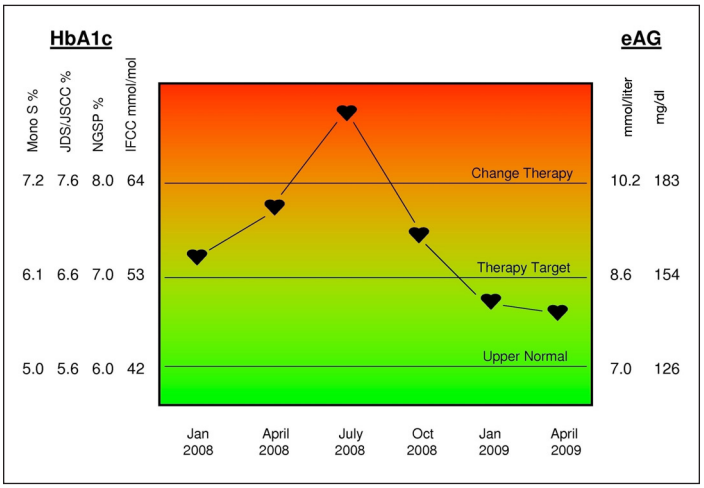

Figure 1. Multilingual patient chart. The monitoring of HbA1c of a virtual patient at six points in time from January 2008 until April 2009 is shown, expressed in all HbA1c units on the left-hand *y* axis and eAG units on the right-hand *y* axis, with indication of the upper normal level, therapy target, and level for change therapy. JSCC, Japan Society of Clinical Chemistry.

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