Mini Review

IFCC standardised HbA\textsubscript{1c}: should the world be as one?

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Abstract

The central importance of HbA\textsubscript{1c} in monitoring glycaemic control was highlighted by the Diabetes Control and Complications Trial (DCCT) which showed that improved glycaemic control, as monitored by HbA\textsubscript{1c}, delayed the onset of diabetic complications. Following this publication the issue of international standardisation of glycated haemoglobin (GHb) measurements became an important objective. The lack of international standardisation resulted in several countries developing National Standardisation Programmes. The International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) Working Group on HbA\textsubscript{1c} Standardisation has established a reference measurement procedure (HPLC-MS or HPLC-CE) for HbA\textsubscript{1c} embracing the concept of metrological traceability. The reference method is anchored to a global network of 15 approved reference laboratories; this confers sustainability and a low level of uncertainty. Essential elements of a comprehensive reference measurement system additionally include the definition of the measurand and the unit of measurement. HbA\textsubscript{1c} is defined as haemoglobin (Hb) molecules having a stable adduct of glucose to the N-terminal valine of the β chain [Hb β chain (Blood) – N-(1-deoxyfructos-1-yl) Hb β chain] and that mmol/mol be used as the unit of measurement. These developments will result in improvements in inter-method and inter-laboratory agreement. Additionally, global acceptance of standardisation based on metrologically sound principles will enable clinical goals and diagnostic guidelines to be developed that can be adopted by all countries.

Keywords: diabetes; HbA\textsubscript{1c}; IFCC Network; IFCC-RMP; reference methods; standardisation.

Introduction

Diabetes affects a staggering 366 million people worldwide, with the majority of all those affected in the 40–59 age group in the low and middle income countries. It is predicted that between 2010 and 2030 there will be a 50.7\% global increase in the number of patients with diabetes. Diabetes is deadly; it is predicted to account for 4.6 million deaths in 2011, similar in magnitude to HIV/AIDS (1). Once thought of as a disease of the elderly, diabetes has shifted down a generation to affect people of working age, particularly in developing countries. This change in demographics is likely to lead to serious economic consequences.

Most worrying is that the largest percentage increase, in rates of the disease, will be in regions defined by the International Diabetes Federation (IDF) as Africa (91\%) and the Middle East and North Africa (83\%) (1). Diabetes is fast becoming one of the major pandemics of the 21st century. Diabetes, mostly type 2 diabetes, now affects 8.3\% of the world’s adult population with almost 80\% of these in developing countries. The top five countries worldwide numerically are China (90 million), India (61.3 million), USA (23.7 million), Russian Federation (12.6 million) and Brazil (12.4 million) (1). A complex interplay of genetic, social and environmental factors is driving the global explosion in type 2 diabetes. For low- and middle-income countries, economic advancement can lead to alterations to the living environment that result in changes in diet and physical activity within a generation or two. Consequently, people can develop diabetes despite relatively low gains in weight. In the developed world, diabetes is most common among the poorest communities. Either way, wherever poverty and lack of sanitation drive families to low cost-per-calorie foods and packaged drinks, levels of type 2 diabetes are exploding.

Diabetes requires continuous medical care and patient self-management in order to prevent short-term complications and decrease the risk of long-term complications (2). These complications can result in substantial increases in the total economic burden of the disease. The American Diabetes Association (ADA) has estimated that the annual cost of diabetes in the US was approximately $174 billion in 2007 (3). Previous studies have found evidence that better glycaemic control among patients with type 2 diabetes may be associated with lower requirement of health care resources and lower costs (4–6). Oglesby et al., using data from October 1, 1998, to April 30, 2003, found that diabetes-related costs were 16\% and 20\% lower for patients with good control (HbA\textsubscript{1c} less than 7\%) than (less than 7\%–9\% or less) and poor control (HbA\textsubscript{1c} more than 9\%), respectively (7).

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Whereas it may be impossible to stop the inexorable move towards pandemic levels of diabetes, we can have an affect on the glycaemic control of these patients and thereby limit the long-term complications and associated burden on the health economy. To achieve good control there needs to be an accurate and reliable means of assessing the glycaemic status of patients. HbA₁c has long been recognised as being central to achieving good glucose control, but variation in the way it has been reported globally has limited our ability to set target goals that can be utilised in all countries. If we are to limit the global effect of diabetes and its financial burden then agreement on a globally accepted standardisation system for HbA₁c measurement is vital. This will allow an international approach to the formulation and implementation on guidelines for diagnosis and monitoring of diabetes.

**HbA₁c methods**

Many methods for the measurement of HbA₁c/glycated haemoglobin (GHb) have been described over the last three decades; these methods have been based on the physical or chemical differences between the glycated and the non-glycated species of Hb. Methods have been developed making use of either the difference in charge or the difference in structure of the two species.

The many commercial methods currently available for the routine measurement of HbA₁c are based on different analytical principles, such as immunoassay, ion-exchange chromatography, and affinity chromatography/ separation.

**The need for standardisation**

Following the introduction of HbA₁c methods into routine clinical practice around 1977, it quickly became apparent that there was a significant difference in the results produced by different laboratories (8) with a between laboratory coefficient of variation (CV) of 11.2%–20.1% observed (9). It was evident that the disparate results obtained were because of the range of methods being used by laboratories, the lack of standardisation and no primary reference material. Although the new generation of HbA₁c methods now demonstrate a degree of precision that could not be imagined in the mid-1970s, comparison of results from different laboratories would still be, at best, difficult or, more likely, impossible if not for standardisation schemes.

Standardisation with common calibration was first proposed in 1984 by Peterson et al., with these investigators examining factors, such as inter-method correlation and reproducibility between laboratories (10). However, it was only after the publication of the Diabetes Control and Complications Trial (DCCT) study in 1993 that the issue of international standardisation of GHb measurements became an important objective for scientists and clinicians (11). The lack of international standardisation resulted in several countries developing national standardisation programmes. The most widely recognised is the National Glycohemoglobin Standardization Program (NGSP) (12) which utilized the method that was used to harmonise HbA₁c results throughout the DCCT (13).

There were two main problems with the NGSP solution: the results reported were not true HbA₁c concentrations, but rather the best estimates that HbA₁c analyser technology from the 1980s [when the DCCT and the UK Prospective Diabetes Study (UKPDS) were conceived (14)] could deliver and there was no primary reference material. This meant that, although most laboratories were now reporting similar values with the same sample, the similar values were not the true HbA₁c concentration. This was also the case with other national programmes. There was a need to be able to establish the ‘true’ concentration of HbA₁c in samples by first defining what was meant by HbA₁c and then by determining a means of measuring this as accurately as possible using a ‘reference method’ of analysis. Sweden and Japan also developed standardisation programmes, based on optimised high performance liquid chromatography (HPLC) methods.

**National standardisation programmes**

The NGSP was established in 1996 in the USA. The NGSP Laboratory Network consists of Primary and Secondary Reference Laboratories (PRLs and SRLs, respectively) and an Administrative Core.

The Central Primary Reference Laboratory (CPRL), using an optimised HPLC method (13), is the anchor for the programme (as it was during the DCCT). The CPRL sets the initial calibration for the NGSP and is the laboratory to which all other laboratories in the NGSP network are compared. The three other primary reference laboratories also utilise the same method and serve as a back up to the CPRL.

SRLs analyse HbA₁c by routine methods that are calibrated to match the CPRL, and are generally less labour-intensive compared to the CPRL method (15).

The performance of the SRLs is monitored using monthly measurement of 10 pooled frozen whole blood samples spanning a range of 20–86 mmol/mol HbA₁c (4%–10%). In order to pass and maintain reference laboratory status the estimate of the SD of the difference between sample replicates must not be >2.5 mmol/mol (0.229%). In addition, the mean of the differences between the individual network laboratories and the CPRL must not be >3.3 mmol/mol (0.35%) HbA₁c. If a laboratory falls outside of the limits prescribed by the CPRL then it may not participate in certification processes until the problems have been addressed and the next monitoring exercise has been passed (16).

NGSP SRLs interact with manufacturers of HbA₁c methods to assist them, first in calibrating their methods and then in providing comparison data for certification of traceability to the DCCT.

Work towards achieving inter-laboratory standardisation of HbA₁c in Japan was started in 1993 with the establishment of an HbA₁c Standardisation Committee of the Japan Diabetes Society (JDS) (16). National calibrators were the basis of the standardisation scheme of the JDS and the Japanese Society for Clinical Chemistry (JSCC).
Standardisation of HbA1c measurements was initially performed by using common calibrators (two calibrators available for use) with HbA1c values of 37 mmol/mol (5.5%) (MCA) and 91 mmol/mol (10.5%) (MCB) assigned by the JDS. The calibrators consisted of lyophilised haemolysates. The target values were assigned using the consensus mean for HbA1c reported by the outcome of 100 laboratories using HPLC methods of proven quality.

In 2000 the JSCC developed a high-resolution ion-exchange HPLC method, termed the KO500 (17), and in 2001 a set of national calibrators (five levels of deep-frozen haemolysate) called JDS/JSCC Calibrator Lot 2 the values of which were adjusted to maintain the values of MC calibrator to avoid clinical confusion. The measurements in following intercomparison studies used the KO500 HPLC method calibrated with JDS Calibrator Lot 2 and the subsequent Lots. Measurements are performed by three domestic network laboratories using the KO500 method. Inter-laboratory differences in the daily measurement of HbA1c are now <3% CV.

The Swedish scheme utilised a Mono S ion-exchange system as the method of choice (17).

The role of the International Federation for Clinical Chemistry and Laboratory Medicine in standardisation

Studies have shown that harmonisation of test results obtained by different HbA1c assays is feasible if all of the methods are calibrated with the same set of calibrators (18, 19) and/or are adjusted to a designated comparison method (DCM) (20). These principles have been used in previous national initiatives for the harmonisation of HbA1c results.

All of the national initiatives described were important steps toward improvement of the comparability of HbA1c test results, but national standardisation programmes based on different DCMs cannot replace uniform worldwide standardisation anchored on a metrologically sound international reference measurement system comprising (21):

1) A clear definition of the analyte based on its molecular structure;
2) A primary reference material containing the analyte in a pure form;
3) A validated reference method that specifically measures the analyte in human samples;
4) A global network of reference laboratories that is sustainable and guarantees that the reference method is performed with the necessary analytical quality and is capable of assigning reliable values to matrix-based secondary reference materials and calibrators.

In 1995 the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) established a working group (WG) with the remit to achieve a uniform international standardisation of GHb (22). The aim of the IFCC initiative was to eventually supersede national standardisation activities, which have taken different approaches to standardisation, and result in an international standardisation scheme. The key aims have been addressed as follows.

Definition of the analyte

In the case of GHb the IFCC WG decided to base the standardisation process on the identification of HbA1c as the stable adduct of glucose to the N-terminal amino group of the β chain of HbA0. The rationale was, that by only measuring this specific adduct a reliable parameter of glycated-adducts to Hb can be obtained, since the N-terminal values account for the major amount of the glucose bound to Hb. As all method principles were found to correlate well with each other, it seemed reasonable to standardise all GHb assays on the basis of the N-terminal glycation and the IFCC-IUPAC (International Union of Pure and Applied Chemistry) Committee on Nomenclature, Properties and Units (C-NPU) has defined this as ‘Hb β chain (Blood) – N-(1-deoxyfructos-1-yl) Hb β chain’ (23).

Reference method

HPLC methods have been inappropriately used for long time as reference methods for the standardisation of routine tests; they are not totally specific for HbA1c. Indeed, different adducts and amounts of interferants that are not HbA1c can be measured by these techniques depending on the conditions (24). For that reason, the IFCC WG developed a method based on the enzymatic cleavage of the intact Hb molecule with endo-proteinase Glu-C to obtain the β-N-terminal hexapeptides of HbA1c and HbA0. Thus avoiding the heterogeneity created by modifications of other glycation sites of the Hb molecule (25). The peptides can be then separated by reverse-phase HPLC, and quantified by electropray ionisation-mass spectrometry (ESI-MS, option A) or by capillary electrophoresis (CE, option B), as illustrated in Figure 1. Following a ballot of all IFCC member national societies in 2001 the reference measurement procedure was adopted and published as an approved IFCC reference method in 2002 (26).

Primary reference materials

Highly purified materials were prepared from normal blood. The β-N-terminal GHb A and non-glycated Hb A were isolated from whole blood of healthy non-diabetic volunteers; after washing and lysis of cells, pure fractions were prepared using a three step chromatographic procedure (cation exchange SP Sepharose, boronate affinity chromatography and again by cation exchange SP Sepharose). Purity of the preparations was assessed by means of HPLC-ESI-MS and HPLC-CE. The spectrum of HbA1c contains two distinct polypeptides with molecular masses of 15,125, and 15,866 daltons (Da) corresponding to the non-glycated α and β chain, respectively. From the amino acid sequence, the theoretical molecular masses for the non-glycated polypeptide chains are 15,126 and 15,867 Da.

For HbA1c a similar spectrum was obtained: 15,126 Da for the non-glycated a chain but 16,028 Da for the mono-glycated
Correlation with other reference systems

In order to translate the values obtained using the IFCC reference measurement system to values obtained under the previous harmonisation initiatives a method comparison study was performed (28). The aim of the study was to generate equations that reliably prove the relationship between the various reference systems. In order to reduce the confounding effects of biological variation the samples used for the method comparisons were pooled whole blood samples. This reduced effects from factors, such as carboxamylated Hb, varying HbF content and Hb forms that could interfere with the NGSP system in particular. The network results were compared rather than individual laboratory results, each specimen was analysed four times by each laboratory. The process was repeated as four separate studies between 2001 and 2003 in order to adjust for systematic changes, such as calibration, equipment and reagent lots. The mean values from the individual laboratory analyses were used to calculate the overall mean of each of the different standardisation schemes for comparison.

Each of the four method comparison studies were evaluated separately, then ultimately combined, when Kruskal-Wallis testing confirmed there was no statistically significant difference between the studies, to create overall master equations for each of the schemes.

All three DCMs were shown to produce values (when expressed in percentage terms) statistically significantly higher than the IFCC reference method. This was attributable to the lack of specificity of the other methods compared to the IFCC reference method. The NGSP comparison produced the highest percentage HbA1c values due to the method not discriminating against non-HbA1c substances, such as Hb F and carboxamylated Hb. Despite these differences in values each DCM did have a strong linear correlation with the IFCC method allowing reliable linear regression equations – be devised and derived IFCC values from the DCCT and UKPDS studies. Redefining HbA1c reporting units in terms of mmol/mol (SI units) has had the effect of producing numbers that are 10-fold higher than percentage (%) units; the relationship between the IFCC network and NGSP network can be defined using:

$$\text{NGSP} \%= \left[0.0915 \times \text{IFCC (mmol/mol)} \right] + 2.15$$

$$\text{IFCC} \text{ (mmol/mol)} = \left[10.93 \times \text{NGSP (}%\right] - 23.50$$

Reference range data

The high specificity of the reference method results in lower values for %HbA1c in patient samples since the nonspecific components measured as ‘HbA1c’ in routine methods are not measured by the reference method. Consequently, a reference range utilising the IFCC reference method for non-diabetic individuals has been evaluated. EDTA-washed red cells collected during the period from April to May 2000 from 120 subjects (60 women and 60 men), the participants of a Danish population study (DiaRisk, Steno Diabetes Centre, Copenhagen, Denmark) were analysed. The subjects were normo-glycaemic according to the current WHO-ADA criteria. The mean age of
the group was 43.5 years (range 32–60 years). Laboratories of
the international network running both the HPLC-CE method
and the HPLC-ESI-MS method analysed samples in duplicate.
Two quality control samples were analysed in quadruplicate.
There were no statistically significant differences between
men and women so the data for the two groups could be com-
bined. Statistical evaluation of the frequency distribution of the
results showed that the distribution was unimodal and could be
fitted by a Gaussian curve. The mean value was 33 mmol/mol
HbA₅₀ and the standard deviation was 2.4 mmol/mol. From
these data a 95% reference range of 29–38 mmol/mol (4.8%–
5.6% in NGSP units) was established (26).

International consensus

In 2007 an International HbA₁c Consensus Committee was
formed from members of the ADA, the European Association
for the Study of Diabetes (EASD), the IDF and the IFCC (29).
After the development of the IFCC reference method system
the consensus committee agreed the following:

1. HbA₁c test results should be standardised worldwide, in-
cluding the reference system and results reporting.
2. The new IFCC reference system for HbA₁c represents
the only valid anchor to implement standardisation of the
measurement.
3. HbA₁c results are to be reported worldwide in IFCC units
(mmol/mol) and derived NGSP units (%), using the IFCC-
NGSP master equation.

This was further updated in 2010 (30):

1. HbA₁c test results should be standardised worldwide, in-
cluding the reference system and results reporting.
2. The new IFCC reference system for HbA₁c represents
the only valid anchor to implement standardisation of the
measurement.
3. HbA₁c results are to be reported by clinical laboratories
worldwide in SI (Système International) units (mmol/mol,
no decimals) and derived NGSP units (%: one decimal),
using the IFCC-NGSP master equation (DCCT units).
4. HbA₁c conversion tables including both SI (IFCC) and
NGSP/DCCT units should be easily accessible to the dia-
betes community.
5. Editors of journals and other printed material are strongly
recommended to require that submitted manuscripts report
HbA₁c in both SI (IFCC) and NGSP/DCCT units.
6. The reportable term for GHb is HbA₁c, although other
abbreviations may be used in guidelines and educational
material (A1C).

Future activities

As can be seen, the objectives of the IFCC WG project have
been achieved. It is now the objective of the newly formed
IFCC Integrated Project Task Force to assist in the implemen-
tation of standardised HbA₁c methods globally.

Conclusions

The International IFCC WG on HbA₁c standardisation worked
for a number of years to develop a complete reference measure-
ment system based on the concepts of metrological traceabil-
ity. In addition to reference methods and materials, essential
elements of a comprehensive reference measurement system
include the definition (including the unit) of the measurand and
establishment of reference laboratories that will collaborate in
a network to ensure stability and accuracy of standardisation.
The process that has been developed fulfills the concept of a
metrological Reference Measurement Procedure; thus provid-
ing clinical laboratories with calibration traceable to primary
reference material with known uncertainty, and that uncertainty
kept to a minimum due to the processes involved. Although
much of Europe has adopted mmol/mol as the reporting unit,
the use of SI units has not yet been accepted globally.

The provision of HbA₁c values that are standardised and
globally accepted will enable clinical targets to be set that are
applicable to all countries, and using this enhanced moni-
toring tool should result in improving glycaemic control in
patients with diabetes.

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