In normal human erythrocytes, HbA comprises > 90% of the total haemoglobin. Besides HbA, human erythrocytes contain other haemoglobin components that are of considerable interest. Some of these, such as HbA2 and foetal haemoglobin (HbF), like sickle-cell haemoglobin, are the products of alternate globin chain genes and others such as HbA1c are post translational modifications of HbA. The haemoglobin A (Hb A) is converted to HbA1c by interacting with glucose with the amino group of the N-terminal valine of globin chain when glucose enters inside the RBC. This process is a purely intracellular process and is influenced by the concentration of glucose within RBC. Glycation may occur at other sites, producing HbA1 other than HbA1c. Ion exchange chromatography separates specifically HbA1c, whereas weak acid hydrolysis tests detect all the glycation sites of HbA. Therefore, a clinician must search for the method of detection before interpreting the results of Glycated Haemoglobin (GHb) if to be done in a flawless way.1

**Nomenclature**

Glycated Haemoglobin (GHb) is a better term than glycosylated or glycohaemoglobin because, HbA1c production depends on a nonenzymatic amadori chain reaction, but glycosylation means enzymatic reaction between glucose and free amino acids of haemoglobin. Total GHb means HbA1c, HbA1a, HbA1b, and HbS, HbC, HbF, etc. Of all these variants, HbA1c is most sensitive and specific, least affected by different factors and most dependable.1 Before interpreting the data, a clinician must be certain about the component of the glycated Hb.

**Standardisation and New Methods of Reports for GHb**

Current HbA1c assays are aligned to the assay used in the DCCT, so that an individual’s risk of complications can be inferred from the result. The non-diabetic reference range in HbA1c-DCCT is 4.0–6.0%. Until 2009 there was no internationally agreed standard for measuring and reporting HbA1c levels. There is now an international standard for HbA1c, agreed by the International Federation of Clinical Chemistry (IFCC), and from 1st June 2009 all UK laboratories are reporting HbA1c in the new units according to this standard.2

The change was necessary because the new IFCC reference method is more specific for HbA1c than the assay used for standardisation in the DCCT and UKPDS. Now it will be easier to compare the results from different labs throughout the world and interpreting clinical trial results. In future, HbA1c-IFCC results will be reported in mmol of HbA1c/mol of total Hb after standardisation using the IFCC reference method. The non-diabetic reference range in SI units for HbA1c-IFCC using the IFCC reference method will be 20 - 42 mmol/mol, rather than the HbA1c-DCCT-aligned range of 4.0 – 6.0 %. The equivalent of the current HbA1c-DCCT target of < 7.0% is HbA1c-IFCC < 53 mmol/mol in the new units.

The new calibration method, without interferences, gives values approximately 1.5% lower than the DCCT values. Unfortunately the two numbers are still similar enough in appearance to cause confusion. So to avoid confusion IFCC HbA1c will be reported as mmol/mol rather than percentage.1

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HbA1c for the Diagnosis of Diabetes

ADA 2010 recommends the use of HbA1c as a diagnostic marker for diabetes and categories for increased risk of diabetes (formerly known as prediabetes). Persons with HbA1c of 6.5% and above are to be diagnosed as diabetes and HbA1c between 5.7-6.4 are considered to have categories for increased risk of diabetes.

Glycated Hb in Patients with Diabetes

Koeng et al. studied the correlation of HbA1c with diabetic control and observed that glycaemic control as evidenced by glycosuria achieved by 3 weeks, but reduction of HbA1c was lagging behind 3–4 weeks and ended off after 7–8 weeks. Ditzel and Kjaergaard observed a decline in HbA1c after starting the treatment in new onset diabetes seen after 2 weeks and ended off beyond 7 weeks. Chantelau proposed an idea that 1% decrease per 10 days of HbA1c indicates a sustained and sudden reduction in hyperglycaemia. But with the withdrawal of sulphonylurea, HbA1c rose from 12.6% to 14.1% after 1 week in a study by Boden et al.

Although glycation depends on the life span of RBC which is normally 120 days considering the age group among different RBC populations on an average, it is accepted that HbA1c reflects glycaemic status of prior 90 days.

Interpretation of Antecedent Glycaemia from HbA1c

HbA1c values well correlate with glycaemic status in the past, provided the test is done with due care with only a few exceptions. Earlier studies have tried to correlate with values of 7–8 point blood testing at regular interval with the HbA1c results at 1, 2, and 3 months timing and confirmed efficiency in majority of the situations. But actually 7–8 point blood sugar in a day does not cover all the glycaemic excursions throughout the period. It is also noted that recent events alter the HbA1c values more than the distant ones. And 50% of HbA1c result is determined by blood glucose results before 1 month, whereas last 3–4 months’ blood glucose levels only influence 10%.

HbA1c estimation is also useful to differentiate between stress hyperglycaemia and pre-existing undetected diabetes in emergency situations when insulin therapy is to be advocated in the later one. HbA1c estimation is also useful to plan therapy in newly detected T2DM as per American Association of Clinical Endocrinologists (AACE) recommendation 2007. If HbA1c is above 10%, insulin should be started from the beginning, and if between 8% and 10% combinations of oral antidiabetic should be of choice. Below the value of 8%, treatment should be started with a single drug.

Glycated Hb and Chronic Complications in Diabetes

Persistent hyperglycaemia produces not only glycation of haemoglobin but also different tissue proteins and advanced glycation end products, which are one of the culprits in the development of organ complications in diabetes. Thus, persistent raised GHB values for long term (usually more than 2 years) are associated with organ damage. Klein et al. found 1.9 times higher risk of retinopathy with HbA1c above the highest quintile in T1DM with diabetes for more than 4 years. The risk increased with age and longer duration. Chase et al. in a population of

<table>
<thead>
<tr>
<th>Table 1 : Factors affecting HbA1c results</th>
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<tr>
<td>Increase HbA1c level</td>
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<td>----------------------</td>
</tr>
<tr>
<td>HbF and HbG</td>
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<tr>
<td>Uraemia</td>
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<td>Lead poisoning</td>
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<td>Alcoholism</td>
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<td>Iron deficiency state</td>
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<td>Hyperbilirubinaemia</td>
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<table>
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<th>Table 2 : Assay methods of glycated Hb</th>
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<tr>
<td>Ion exchange chromatography</td>
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<tr>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>Isoelectric focusing</td>
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<td>Agar gel electrophoresis</td>
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3–4 months’ blood glucose levels only influence 10%. Thus, in newly diagnosed diabetes with effective therapy, sharp fall in HbA1c is noted in the first 2 months followed by less gradual drop.

Because of wide scale and unpredictable fluctuation of glucose metabolism, HbA1c results which translate these changes over 24 hours a day, is a better marker for planning and modulating therapy in T1DM. Of course HbA1c cannot guide a physician regarding the dose of insulin but compel him to change the doses.

In cases of T2DM, it was thought that HbA1c estimation is not that necessary as fluctuations in fasting glucose (which affect the result most) are not that marked. But it is accepted that HbA1c together with blood sugar values is a powerful platform to moderate the therapy.

In cases of pregnancy (though estimated level is affected on lower side), HbA1c is very valuable and should be done every month with a target to keep below 6%, as we require aggressive control to avoid foeto-maternal outcome. HbA1c done at first trimester is also a valuable tool to differentiate between gestational and pregestational diabetes showing higher values in the later situation.

HbA1c estimation is also useful to differentiate between stress hyperglycaemia and pre-existing undetected diabetes in emergency situations when insulin therapy is to be advocated in the later one. HbA1c estimation is also useful to plan therapy in newly detected T2DM as per American Association of Clinical Endocrinologists (AACE) recommendation 2007. If HbA1c is above 10%, insulin should be started from the beginning, and if between 8% and 10% combinations of oral antidiabetic should be of choice. Below the value of 8%, treatment should be started with a single drug.
T1DM observed that microalbuminuria was 3.6 times and retinopathy 2.5 times higher in patients of mean HbA1c over 12.3% than with less than 9.0% (normal value = 6.3 – 8.2%).

The strongest evidence is provided by DCCT study that showed reduction of retinopathy (34%), microalbuminuria (35%), and neuropathy (60%) in intensive control group with HbA1c below 7% than conventional group above 9%. This establishes that improvement in HbA1c value by 2% significantly prevents organ damage.

**Frequency of Testing HbA1c**

Usually, it should be done every 3 months and when controlled after every 6 months. In pregnancy for tight control, monthly determination is necessary.

**Limitations of HbA1c Measurement**

HbA1c results (DCCT or IFCC) will be misleading in certain situations e.g. a variety of haematological conditions where there is abnormal red cell turnover also different other disease conditions.

The results are also altered by medications of comorbid illness and are as follows (Table 1). Physician should interpret the results after considering the above variables. The results can also vary depending on the HbA1c method used by a particular laboratory. Some methods for HbA1c can give more reliable results in some haemoglobinopathies, but if this or any other condition leads to a change in red cell survival, then HbA1c measurement by any means can, at best, be used to track changing trends in glycaemia. Other measures of glycaemia may then be required, such as more reliance on self monitored blood glucose values or the use of a serum fructosamine assay, if available.

**Assay Methods of GHb**

Physician should be acquainted with different assay methods before interpreting the results. Till now, high-performance liquid chromatography method with paper electrophoresis is the best and dependable. DCCT adopted a standardisation of the assay method and that should be followed everywhere. The different methods are shown in Table 2.

The estimation of HbA1c is used for patient management, monitoring, education and for patient motivation to control diabetes. As such its way of measurement should be optimally accurate and precise. But after the routine use of HbA1c assays, it quickly showed that different methods produced inconsistent results.

There are multiple analytical problems affecting the glycated haemoglobin measurement. They are the lack of assay standardisation, interference by Schiff base and the problems related to its measurement in patients with haemoglobinopathies, foetal haemoglobin, renal failure and haemolytic disease and use of drugs like aspirin that possess strong ionic charges.

As different methods for its measurement reflect results with undesirable differences, it was necessary to compare the results of various methods used by different laboratories. In a recent study Yasmeen et al compared analytical performance of D10 Haemoglobin Testing System (BIO RAD Laboratories) which is based on cation exchange HPLC with Roche Immunoturbidometric method (performed on Hitachi 902). The aim of this study was to evaluate a method which is extremely accurate, precise, cost – effective and practical that is suitable for routine use in the clinical chemistry laboratory. Their results are concordant with the previous studies that Immunoassay has higher variation (CV) than HPLC. The precision is good on both methods within the medically allowable CV (< 5% recommended by National Academy of Clinical Biochemistry and International Federation of Clinical Chemistry). A lower total CVs make it easier to detect significant trends or shifts in a diabetic patient’s blood glucose control.

Hawkins RC, found a correlation coefficient of 0.98. They compared the HbA1c results of 110 patients performed on Bio Rad Diastat HPLC and Bayer DCA 2000 immunoassay. Beaune et al performed a comparative study on D-10 HPLC and Arcitect immunoassay on 161 blood samples. They found the correlation coefficient of 0.98.

Beside the issues related to precision, much has been discussed about how the method is standardised and the effects of common interferences from unstable and/or abnormal haemoglobin. The D-10 HPLC system participated in the National Glycohaemoglobin Standardisation Programme and is traceable to the DCCT reference method. The National Glycohaemoglobin Standardisation Programme (NGSP) is responsible for the calibration of the HbA1c methods in many parts of the world enabling direct comparison to DCCT targets.

The Roche Immunoturbidometric method is standardised via IFCC reference system and the results of this method are lower than the HPLC and are expressed in mmol / mol. Therefore a conversion factor is incorporated into the software of the analyser to convert the results to % unit and equalise the results of immunoassay to HPLC. Recently the International organisations has recommended that the methods for measurement of HbA1c should be standardised according to NGSP and method should be traceable to DCCT method. So the D-10 cation exchange HPLC method meets this criterion.
Before advising for HbA1c, clinicians should also remember that unstable haemoglobin variants may interfere with the HbA1c measurement. A major limitation of these HbA1c immunoassays is that they do not definitely detect the presence of abnormal haemoglobin variants. As the red blood cells with abnormal haemoglobin variants have shortened life spans, the reported HbA1c value may not reflect the preceding 2 – 3 months blood glucose control. The different studies also mentioned that the immunoassay methods do not identify the haemoglobin variants.\textsuperscript{20,21} But the D-10 cation exchange HPLC method produces a chromatogram for each patient sample in which the presence of haemoglobin variants can be easily detected by careful examination of chromatogram. The D-10 HPLC not only correctly identifies the haemoglobin variants but also withholds reporting the results in the presence of markedly decreased amount of haemoglobin A. The total direct per test cost of the D-10 HPLC is higher than the Hitachi 902 Immunoassay.

Though, there are various methods for measuring HbA1c, we urgently need an extremely accurate, precise and practical method that is suitable for routine use in the clinical chemistry laboratory. The D-10 HPLC method appears to satisfy this need. It is fully automated system that requires no sample preparation and has a run time of 3 minutes. It is found to be linear in the HbA1c range of 3 – 18%. In addition the instrument also demonstrates excellent within run and total CVs. In the study of Yasmeen et al, both methods correlated very well, however the HPLC method had significantly improved precision compared with the Immunoassay and has the additional advantage of indicating presence of abnormal haemoglobins.\textsuperscript{16}

In this issue of JAPI, Pranesh G. T. and Faith M. have evaluated the assessment of standardisation of HbA1c testing across clinical laboratories in India and its impact on diabetes management. They analysed the data available from 148 NABL accredited laboratories from all over the India regarding the standardisation of testing of blood glucose and HbA1c. HbA1c testing was only available with 87.1% of the laboratories and gross differences were noted in the test in terms of nomenclature, methodology and analytical performance. On comparison of coefficient of variation, the HbA1c assay demonstrated higher variability than blood glucose estimation between different laboratories. Ion exchange chromatography method appeared to bear less coefficient of variation than spectrophotometry and immune turbidometry.

The results of this study is an eye opener for the clinicians. They should always check the method of testing of HbA1c before accepting the results. Regulatory authorities like NABL should immediately take serious steps to homogenise and standardise the estimation of HbA1c all over the India.

References

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