Evaluation of cation exchange HPLC method of Bio-Rad and its correlation to Beckman Coulter Immuno Turbidimetric assay

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ABSTRACT
To evaluate the performance of ion exchange HPLC method using Bio-Rad D10 analyser and compare with Immunoturbidimetric method (IT) of Beckman Coulter (BC) for the measurement of HbA1c in human blood. 100 patients blood samples referred for HbA1c test by the diabetic clinic, both Males and Females with a wide range of age groups were used for this study. Blood samples for HbA1c was collected as per standard protocols. Measurement of HbA1c was carried out using both methods. Clinical Laboratory Standard Institute (CLSI) protocols for the evaluation of a new method was followed in this study. The intra and inter assay precision obtained were 1.0 % and 1.41 % and the intra and inter accuracy were 1.5 % & 1.3 % for HPLC method and the figures for IT method was 1.5 % and 2.0 % for Intra & Inter assay precision and 1.5 % & 0.8 % for intra and inter accuracy. All statistical parameters obtained in this study are well within those recommended by International Federation of Clinical Chemistry (IFCC) & World Health Organization (WHO). The linearity obtained for both the methods are similar (4.2 to 16 %). HPLC method using Bio-Rad D10 analyser is comparable to the method based on IT of Beckman Coulter in terms of precision and accuracy. Hence the laboratory confidently switched over to this method for measuring HbA1c.

Introduction
The past decade has given us a better insight into the clinical uses and problems associated with glycated haemoglobin measurement. HbA1c has been used as a clinical marker for blood glucose control during the preceding 2 – 3 months. Several methods such as colorimetry, electrophoresis, column chromatography and immunoagglutination have been used to measure HbA1c. The HPLC method has been used as a standard method for HbA1c determination. After years of using an immunoassay-based method for HbA1c measurements in our center, we recently changed to a HPLC-based method. The change of method enabled us to identify the presence of clinically silent hemoglobin variants in diabetic patients from the abnormal peaks in the chromatograms, which was not possible when using the immunoassay-based method. The objective of our study is to compare the results obtained for HbA1c using HPLC and Immunoturbidimetric methods.

In the present study we compared analytical performance of D10 Hemoglobin Testing System (BIO RAD Laboratories) which is based on cation exchange HPLC principle with Beckman Immunoturbidimetric method (Performed on Olympus AU 400). The aim of this study was to evaluate a method based on guidelines recommended by CLSI which is extremely accurate, precise, cost – effective and practical that is suitable for routine use in the clinical chemistry laboratory.

Review of literature
The measurement of glycated hemoglobin (A1C) assay has become the gold-standard to monitor glycemia control for over two decades. Anchored in the knowledge that elevated A1C values increase the likelihood of the microvascular complications of diabetes (and perhaps macrovascular complications as well), clinicians have used A1C test results to guide treatment decisions, and the assay has become the cornerstone for the assessment of diabetes care.1

Glycosylated hemoglobin level gives information about the mean average blood glucose level during the previous 2 -3 months and this test shows how well diabetes has been controlled in the last 2 to 3 months and whether diabetes medicine needs to be changed.2

Two main types of Glycosylated hemoglobin viz HbA1 or HbA1C are commonly used in diabetes monitoring. The normal range for HbA1C is 4 – 6 % and values for HbA1 is 2 % higher as HbA1C is a smaller part of HbA1. HbA1c values between 6 - 7.5% indicates moderately control and between 7.5 % - 8.5 % and values from 8.6 % to 10 % indicates poor control of the disease and values over 10% is considered alarmingly high.3

Glycohemoglobin measurement remains the optimal indicator of glycemic control in diabetic patients, but translation of findings from clinical trials to clinical practice worldwide demands consistent values across all assays. To ensure that the important prognostic information still applies to all diabetic patients with the application of the reference method(s), the hemoglobinA(1c) values reported in the major clinical trials will have to be translated into statistically and computationally compatible values based on the new reference system(s).4

Standardisation of HbA1c test between laboratories is difficult to achieve, and most assays are currently calibrated to the values used in the Diabetes Control and Complications Trial (DCCT). With the availability of more specific reference standards it is now proposed that HbA1c is expressed as mmol
HbA1c per mol of non-glycated haemoglobin. An HbA1c of 7% is approximately equal to 53 mmol/mol.(5)

In the reference method, hemoglobin is cleaved by endoproteinase Glu-C. The resulting glycated and nonglycated N-terminal hexapeptides are separated by HPLC, followed by quantification by electrospray ionization mass spectrometry or capillary electrophoresis. HbA1c is measured as the ratio of glycated to nonglycated N-terminal peptide and is reported as a percentage. Comparison of pooled blood samples revealed a linear relationship between HbA1c results of the International Federation of Clinical Chemistry (IFCC) reference method and the standardization schemes in the United States, Japan, and Sweden. For example, the calculated regression equation for National Glucose Standardization Programme (NGSP) NGSP – HbA1c = 0.955 (IFCC – HbA1c) + 2.15 provides a numeric link between the IFCC and NGSP values. An important observation of the comparison is that HbA1c results obtained by the IFCC method are significantly lower (1.3–1.9% across the relevant HbA1c range) than NGSP results. These findings have generated considerable debate as to how HbA1c should be reported.(6)

The number of methods and laboratories certified by the NGSP as traceable to the DCCT has steadily increased. College of American Pathologists (CAP) GH2-B survey results reported in December 2000 show marked improvement over 1993 data in the comparability of GHB results. In 2000, 90% of surveyed laboratories reported GHB results as hemoglobinAlc (HbA1c) or equivalent, compared with 50% in 1993. Of laboratories reporting HbA1c in 2000, 78% used a NGSP-certified method. For most certified methods in 2000, between-laboratory CVs were <5%. For all certified methods in 2000, the mean percent HbA1c was within 0.8% HbA1c of the NGSP target at all HbA1c concentrations.(7)

Comparative results from seven different GHB methods calibrated by use of hemolysates assayed by a precise ion-exchange high-performance liquid-chromatographic (HPLC) method for hemoglobin Alc (HbA1c). Regardless of the GHB species measured by the seven methods, results were referenced to the HbA1c content of the calibrators. Without this calibration, GHB value for single sample varied, e.g., from 4.0% to 8.1% and from 10% to 14.2% in the normal and high ranges respectively. Calibration decreased between-method variability (single sample ranges of, e.g., 4.8% to 5.4% and 9.4% to 10.2% in the normal and high ranges, respectively) and improved interassay precision. It has been concluded that this approach to calibration of GHB measurements allows direct comparison of results obtained by different methods and improves precision.(8)

Those most frequently used are ion-exchange chromatography for HbA1c and affinity chromatography for total GHB. In one study, a turbidimetric immunoassay for HbA1c was evaluated that was performed on a Hitachi 911 clinical chemistry analyzer (Boehringer Mannheim). Good linearity in the range of 5% to 15% HbA1c, within-run and between-run coefficients of variation ranging from 2.4% to 5.9% were obtained. Results of 179 diabetic and nondiabetic patients showed good correlation to those of a routine HPLC method (r = 0.96). In addition, HbA2, HbS, and HbF in samples from nondiabetic patients were not detected by the immunoturbimetric assay and the "labile" HbA1c fraction (Schiff base) did not interfere with the new test.(9)

An ion-exchange HPLC system equipped with a Pharmacia Mono S® HR 5/5 column. Calibration of analytical runs substantially increased interassay variation (CV), from 1.7% to 4.4% and from 0.9% to 3.2% for control samples with low (6.5%) and high (14%) glyHb percentages, respectively. Standardization of glyHb results, though essential for interlaboratory comparisons, should not be done at the expense of assay precision, as may occur with thoughtless use of lyophilized calibrators. Therefore recommend the use of carefully determined conversion factors for standardization of glyHb results obtained with ion-exchange HPLC systems that are capable of excellent long-term interassay precision.(10)

In a study involving three methods—the Variant HPLC, the Hi-Auto A1c analyzer system, and the Roche immunoassay— with the Diamat HPLC system. All showed good precision and good concordance with the Diamat HPLC. The reference range for HbA1c has to be determined by the laboratory for each assay system. Interference study showed no statistically significant influence of anaemia, polycythemia, rheumatoid factor, or chronic hemodialysis, although individual HbA1c values can be influenced by polycythemia (when measured with the Hi-Auto A1c analyzer) and by chronic hemodialysis (when measured with the Variant HPLC). HPLC was not suitable for measuring HbA1c in the examined cases of hemoglobin variants; assaying fructosamine seems to be better for monitoring these patients.(11)

HPLC-based ion-exchange methods for HbA1c have recently been automated, and interferences by Hb species such as HbF and HbS have been minimized. However, several reports have described artificially high HbA1c results with hemoglobin variants when an automated ion-exchange HPLC method is used.(12)

A chromatographic method for HbA1c using a new monodisperse-exchanger has been investigated. HbA1c was separated from other "minor hemoglobins": Hb F, Hb A3 (the glutathione adduct), and the acetaldehyde adduct in alcoholics. The method was fully automated and a single column could be used for more than 1000 runs. The normal reference interval was 4.0–5.2%; the interval for diabetic outpatients was 5.6–12.4%. Within-run and the between-run CVs were less than 0.9% and 1.7%, respectively. Carbamylation in uremic patients who were undergoing hemodialysis increased the proportion of HbA1c to 1%. HbA1c results were compared with results from glucose tolerance tests. In our study, HbA1c less than 5.5% excluded diabetes: subjects with Hb Alc greater than 6.2% showed diabetes. If blood sampled during fasting had been screened with determinations of glucose and HbA1c, only 20% of referred subjects would have needed an oral glucose tolerance test for diagnosis of diabetes.(13)

Various factors may affect the accuracy of HbA1c measurements according to the assay method used, of which hemoglobin variants are one of them. More than 1000 hemoglobin variants have been identified, with many of them being clinically silent. HbA1c deviation of 1% reflects a change of 1.4 – 1.9 mmol/L in average blood glucose concentration. Therefore, a falsely high or low HbA1c value caused by the high prevalence of hemoglobinopathy, in order to avoid mismanagement of diabetic patients. Alternative non-hemoglobin methods of measuring glycemic control such as fructosamine, glycated serum albumin or self-monitoring of
blood glucose may be more appropriate than HbA1c in patients with hemoglobin variants and should be considered.\(^{(15)}\)

Recent improvements in automation combined with new recommendations for precision and accuracy have caused us to re-evaluate our methods for measuring HbA1c. A newly automated high-performance liquid chromatography (HPLC) instrument for measurement of HbA1c (Tosoh Alc 2.2 Plus Glycohemoglobin Analyzer, Tosoh Medics, Foster City, Calif) and compared the results obtained by HPLC to those obtained with an immunoassay (Hitachi 911, Boehringer Mannheim Corporation, Indianapolis, Ind). The Tosoh analyzer was found to be linear in a range of 5.3% to 17% and had a throughput of 20 samples per hour. HbA1c results for 102 patient samples by the 2 techniques showed good correlation, with a slope of 0.87 and an intercept at 1.27%+/- 0.15%. Both the total and within-run coefficients of variation were consistently lower for the HPLC method compared with the immunoassay method. The HPLC method produces a chromatogram that shows the different hemoglobin fractions, allowing identification of abnormal hemoglobin variants. In heterozygous individuals, HbA1c measurements are made with no interference from the hemoglobin variant. In the case of homozygous or doubly heterozygous hemoglobin variants, the Tosoh HPLC identifies the hemoglobin variants as such and correctly does not report a HbA1c value in the presence of a markedly decreased amount of hemoglobin A. The Tosoh HPLC provides adequate throughput and improved precision, and the method is traceable to the Diabetes Control and Complications Trial.\(^{(16)}\)

The similarity in the mean and range of percent HbA1c and in percent total GHb using these different methods can be attributed to two factors: 1) the HbA\(_1c\) ion-exchange method measures only 50–60% of the total GHb present, and 2) 40–50% of the material being measured in the HbA\(_1c\) fraction is not GHb, i.e., offsetting factors fortuitously resulted in values similar to the more specific affinity methods. The greater incremental increase in percent total GHb compared with percent HbA\(_1c\) in people with diabetes can be attributed to the greater amount of GHb being measured with the affinity methods.\(^{(17)}\)

In a study involving 23 HbA1c methods; 9 were immunoassay methods, 10 were ion-exchange HPLC methods, and 4 were capillary electrophoresis, affinity chromatography, or enzymatic methods. An overall test of coincidence of 2 least-squares linear regression lines was performed to determine whether the presence of HbE or HbD traits caused a statistically significant difference from HbAA results relative to the boronate affinity HPLC comparative method. Deming regression analysis was performed to determine whether the presence of these traits produced a clinically significant effect on HbA1c results with the use of +/-10% relative bias at 6% and 9% HbA1c as evaluation limits. Statistically significant differences were found in more than half of the methods tested. Only 22% and 13% showed clinically significant interference for HbE and HbD traits, respectively.\(^{(18)}\)

One hundred eleven laboratories, using 21 different methods based on five different principles, determined glycohemoglobin (GHb) percentages in two identical series of six lyophilized hemolysates and three similarly processed calibrators, distributed 3 months apart. To assign GHb percentages to calibrators, we used HbA1c results from nine participants who used the Bio-Rad Diamat high-performance liquid chromatographic method. Three-point calibration with assigned values improved mean intralaboratory variation (CV) from 6.6% to 3.5%. For samples with low (5.5%) and high (14.1%) GHb percentages, respectively, calibration decreased interlaboratory variation per method (from 10% to 4% and from 6% to 3%), inter-method variation (from 18% to 4% and from 16% to 3%), and overall interlaboratory variation (from 25% to 7% and from 15% to 4%). Without calibration, 71% of the laboratories did not meet the clinically desirable intralaboratory CV of 3.5%; calibration reduced this proportion to 39%. We conclude that, irrespective of the analytical method used, calibration greatly reduces all sources of GHb variation.\(^{(19)}\)

The essential role of glycohemoglobin measurements in diabetes care and the currently poor comparability of results between different methods used in clinical laboratories makes an international standardization absolutely necessary. The IFCC working group on HbA1c standardization is going to develop a reference system for the international standardization which is based on HbA1c as the biochemically well-defined major glycohemoglobin component, and consisting of primary and secondary reference materials, a reference method and adjusted reference and target values. The system will serve for the standardization of all glycohemoglobin assays.\(^{(20)}\)

**Material And Methods**

A total of 100 patients attending the diabetic clinic in SRM Hospitals, Kattankulathur comprising both male and female in the age group of 26 to 80 years were selected for the study. 5 ml of blood was collected in EDTA vacutainer tube (BD vacutainer K2 EDTA 5.4 mg). Analysis for HbA1c was done on the same day by both methods. This study was carried out over a period of one month. The samples were analyzed one the same day. As the department is equipped with state of art discrete selective analyser viz olympus AU400 and fully automated D10 Haemoglobin testing system (BIO RAD Laboratories), HbA1c measurements were carried out using the above two instruments. Appropriate assayed accuracy controls from Bio- rad laboratory USA were included in each batch of measurement to validate the accuracy of the results obtained in the study. The department participates in proficiency testing survey to validate the interlab precision and accuracy on national level.

As this study was to compare HbA1c results for two methods, patients selection with respect to inclusion or exclusion criteria were not followed.

**Assay protocol for D10 Bio-rad analyser**

The Bio Rad D-10 Haemoglobin Testing System is the newly introduced fully automated analyser based on Cation Exchange HPLC. The dual kit reorder pack contains whole blood primer, Calibrator 1 and 2, calibrator diluent, wash reagent, elusion buffer 1 and 2 and analytical cartridge. The manufacturer’s instructions were followed for the quality control and calibration. This technique requires no predilution or manual handling of patient’s samples. The samples are directly introduced in their primary tubes following calibrators and control samples. The instrument draws sample directly from the EDTA vacutainer and all processing of the sample is performed internally. Samples are automatically mixed, diluted and injected into the cartridge. The analyser delivers a programmed buffer gradient of increasing ionic strength to the cartridge, where the haemoglobins are separated on the bases of their ionic interactions with the cartridge material. The separated haemoglobins are then passed through the flow cell of the filter photometer, where changes in the absorbance at 415 nm are measured. The run time is approximately 3 minutes per sample.
with a throughput of 20 samples per hour. A sample report and a chromatogram are generated for each sample.

**Assay protocol for Beckman Coulter (BC) Immuno Turbidimetric (IT)**

The immunoturbidimetric assay was performed on Olympus AU 400 discrete selective analyser as per the manufacturer’s instructions given in the kit leaflet. Before performing automated analysis on samples, the test requires manual preparation of a sample hemolysate. Samples were mixed with HbA1c denaturant containing porcine pepsin, buffer pH 2.4 (10µl whole blood + 400µl denaturant reagent) for 5 minutes in accordance with the testing method. The HbA1c is measured in a latex agglutination inhibition assay. An agglutinatin, consisting of a synthetic polymer containing multiple copies of the immunoreactive portion of HbA1c, causes agglutination of latex coated with HbA1c specific mouse monoclonal antibodies. In the absence of HbA1c in the sample, the antibody-coated microparticles in the HbA1c APT R1 and the agglutinator in the HbA1c APT R2 will agglutinate. Agglutination leads to an increase in the absorbance of the suspension. The presence of HbA1c in the sample results in a decrease in the rate of agglutination of the HbA1c APT R1 and the agglutinator in the HbA1c APT R2. The increase in the absorbance is therefore inversely proportional to the concentration of HbA1c in the sample. The increase in absorbance due to agglutination is measured at 700 nm. The Immunoturbidimetric method was calculated according to DCCT/NGSP Calculation according to International Federation of Clinical Chemistry (IFCC): HbA1c% = HbA1c (g/dl) x 100 ÷ Hb (g/dl) Calculation according to DCCT/NGSP: HbA1c% = 0.915 x IFCC + 2.15.

**Validation of Reliability of results**

Both precision and accuracy were determined using commercially available control blood of low and high HbA1c concentrations by repeated analysis. Lyphochek Diabetic controls from Bio Rad Laboratories were used.

**Statistical Analysis**

A software [www.vassarstats.net](http://www.vassarstats.net) was used to calculate correlation coefficient and arriving at extrapolation equation. Seven guidelines recommended by CLSI for evaluation of a new method was followed and the statistical results obtained are presented in a series of tables.

**Results**

**Table I** shows the mean and correlation coefficient (r) obtained (HPLC Vs IT of BC) for the two methods compared. While the mean values between the two methods agrees well for all patients and females, for males the mean result is 3.6% higher than that of IT method. However, the r value for all the 3 groups are the same 0.95.

**Table II** presents the intra and inter assay precision for the two methods. The intra and inter assay precision obtained in this study are well within the WHO guidelines values of 4% and 8%.

**Table III** gives the intra and inter assay accuracy (% bias) for the two methods. All % bias for both intra and inter assay are < 2% indicating good performance.

**Table IV** shows the % carry over in results when a low value sample is loaded immediately after a sample with high HbA1c value. The mean carry over out of 3 samples tested, two samples carry over is negative for HPLC and two shows positive for IT. However, the % carry over is less than 2% and hence negligible.

**Figure I** Illustrate the correlation graph for all patients for the two methods compared ‘Y’ and ‘R’ values are found to be 0.9788 and 0.8903.

**Figure II** Illustrate the correlation graph for all patients for the two methods compared ‘Y’ and ‘R’ values are found to be 0.9605 and 0.8861.
Table IV Sample carry over

<table>
<thead>
<tr>
<th>Sample No</th>
<th>HPLC (Biorad D10)</th>
<th>IT (Beckman)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High sample value</td>
<td>Low sample value</td>
</tr>
<tr>
<td>1</td>
<td>13.9</td>
<td>5.5</td>
</tr>
<tr>
<td>2</td>
<td>12.4</td>
<td>5.1</td>
</tr>
<tr>
<td>3</td>
<td>9.2</td>
<td>7.7</td>
</tr>
</tbody>
</table>

Discussion

The method selected and evaluated in this study viz cation exchange HPLC has been accepted as a reference method by the author’s laboratory evaluated its performance in comparison with the method already used in the laboratory. This approach is to fulfill the guidelines of CLSI for the purpose of accreditation. The intra and inter assay precision obtained for HPLC is 1.5% and 2% which are better than those obtained in a previous study using similar instruments and method (2.4% and 5.9%)\(^{(19)}\). An ion exchange HPLC system of pharmacia gave figures of 1.7% and 4.4% and these figures too are higher than the results obtained by this study\(^{(10)}\). The new method evaluated is said to minimize the interference by Hb F and Hb S variant and hence the method selected by us is considered good\(^{(22)}\). The chromatographic method for HbA1c gave figures of 0.9% and 1.7% for intra and inter assay precision and these figures are more or less in agreement with those obtained by us namely 1.0% and 1.4%\(^{(13)}\). HbA1c measurement by automated HPLC using TOSSOH analyser is said to give improved precision\(^{(16)}\) but the authors have not given figures. In the study involving 23 HbA1c methods, the intra and inter assay % bias obtained were 6% and 9%\(^{(18)}\) which are much higher than our figures of 1.0% and 1.3%.

On the whole the method evaluated by us in comparison with IT of BC are much lower for both intra and inter assay precision and accuracy, suggesting that this method may be accepted for routine use, since the comparison undertaken are in agreement with CLSI standard guidelines.

Conclusion

HPLC method evaluation in comparison with IT of BC was carried out as per CLSI guidelines. The intra and inter assay precision obtained were 1.0% and 1.41% and the intra and inter accuracy were 1.5% & 1.3% for HPLC method and the figures for IT of BC method was 1.5% and 2.0% for Intra & Inter assay precision and 1.5% & 0.8% for intra and inter accuracy. All statistical parameters obtained in this study are well within those recommended by IFCC & WHO. The correlation coefficient obtained for all patients, males and females separately are 0.95 and the regression equation obtained for all the three groups are similar. The methods compared are found to be linear form 4.2 to 16.0 %. Hence the laboratory confidently switched over to this method for measuring HbA1c.

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