COMPARISON OF CATION EXCHANGE HPLC AND IMMUNOTURBIDIMETRIC METHOD FOR DETERMINATION OF HbA\textsubscript{c}

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ABSTRACT
Objective: The objective of this study was to compare and correlate the analytical performance of D10 Hemoglobin Testing System based on Cation Exchange HPLC and Roche Hitachi 902 Immunoturbidimetric method.

Subjects and Methods: A total of 110 patients of Type 2 Diabetes Mellitus were included in the study. HbA\textsubscript{c} was determined using D-10 Hemoglobin Testing system and Roche Hitachi 902 Analyzer.

Results: Both methods showed good correlation with the correlation coefficient (r) of 0.95. Between run Coefficient of variance was found to be lower for HPLC system compared to immunoturbidimetric method. HPLC method also produces a chromatogram that shows the different hemoglobin fractions, allowing identification of different hemoglobin variants.

Conclusion: In the present study both methods showed good correlation but the D10 HPLC system provided adequate throughput and improved precision as compared to immunoturbidimetric method.

Key words: HbA\textsubscript{c}, Diabetes mellitus, Cation exchange HPLC, Immunoturbidimetry.

INTRODUCTION
Diabetes mellitus is a metabolic disorder which is characterized by hyperglycaemia with disturbances of carbohydrate, fat and protein metabolism. It results from defects in insulin secretion, insulin action, or both.\textsuperscript{1-3} Diabetes mellitus has become a major health problem worldwide. The number of cases of diabetes among adults was estimated to be ~ 171 millions in 2000. This number is expected to rise from 171 million to 366 million in 2030.\textsuperscript{4}

Diabetes mellitus is associated with complications of eyes, kidneys, heart, blood vessels and other organ systems. Long – term complications of diabetes include retinopathy with potential loss of vision, nephropathy leading to renal failure, peripheral neuropathy with risk of foot ulcers, amputations and autonomic neuropathy causing gastrointestinal, genitourinary, cardiovascular symptoms and sexual dysfunction.\textsuperscript{5,6} The results of the major clinical trials, The Diabetes Control and Complications Trial (DCCT) and The U.K. Prospective Diabetes Study (UKPDS) showed that the development and progression of diabetic complications can be delayed by monitoring the glycaemic status of patients.\textsuperscript{7,8} The tests most widely used in monitoring the glycaemic status are blood glucose and glycated haemoglobin.\textsuperscript{9,10}

The measurement of blood glucose is of limited value for the long term assessment of glycaemic control. For the long term assessment of glycaemic status measurement of glycated hemoglobin or HbA\textsubscript{c} is now routinely and widely used in clinical practice. HbA\textsubscript{c} is a reaction product of glucose and the N-terminal valine of the beta chain of haemoglobin. The reaction is irreversible and enzyme independent. As red cells are completely permeable to glucose, the quantity of HbA\textsubscript{c} formed is directly proportional to the concentration of glucose. Traditionally glycated haemoglobin reflects mean plasma blood glucose concentration over the previous two – three months.\textsuperscript{11-13}

After the results of diabetes control and complications trial (DCCT) and The U.K. Prospective Diabetes Study the HbA\textsubscript{c} assay has become the gold standard measurement of hyperglycaemia.\textsuperscript{14} Monitoring of HbA\textsubscript{c} is recommended by American Diabetic Association, American Diabetic Federation, European Association for the study of Diabetes, International Federation of Clinical Chemistry and Singapore MOH Clinical Practice Guidelines.\textsuperscript{15} HbA\textsubscript{c} is now generally accepted as the single, most prominent and independent parameter of metabolic control, a risk factor for the development of diabetic complications and is widely used as treatment goal in disease management.\textsuperscript{16-18}

A number of methods for determination of HbA\textsubscript{c} have been developed. These methods are based on different physical, chemical or immunological
characteristics of the Glycated haemoglobin.\textsuperscript{5–19}

As HbA\textsubscript{c} is used for patient management, monitoring, education and for patient motivation to control diabetes so its measurement should be optimally accurate and precise. After the introduction of HbA\textsubscript{c} assays into routine use, it quickly became apparent that different methods produced inconsistent results.\textsuperscript{20,21}

There are numerous analytical problems associated with glycated haemoglobin measurement. Such as the lack of assay standardization, interference by Schiff base and the problems related to its measurement in patients with hemoglobinopathies, fetal haemoglobin, renal failure and haemolytic disease. Some drugs that possess strong ionic charges like aspirin can also alter HbA\textsubscript{c} results.\textsuperscript{22,23}

As different methods for its measurement yield results with undesirable differences, it has become important to compare the results of various methods used by different laboratories. In the present study we compared analytical performance of D10 Haemoglobin Testing System (BIO RAD Laboratories) which is based on cation exchange HPLC with Roche Immunoturbidimetric 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.

MATERIALS AND METHODS
This cross sectional study was conducted at University of Health Sciences, Lahore. We evaluated Type 2 Diabetic patients after approval from institutional ethical review committee and informed consent of the patients. A total of 110 patients of diabetes mellitus were included in the study. These patients were selected from Shalamar Hospital, Lahore and Hamza Foundation, Diabetes Centre, Samanabad, Lahore. The age of the patients was from 25 to 80 years. A sample of 5 ml of blood was collected in EDTA vacutainer tube (BD vacutainer K\textsubscript{3} EDTA 5.4 mg). The samples were kept stored at 2 – 8°C till they were analyzed within a period of two days.

Techniques
HbA\textsubscript{c} was measured by D\textsubscript{10} Haemoglobin testing system (BIO RAD Laboratories) which is based on cation exchange HPLC and Roche Immunoturbidimetric method (Roche Hitachi (902) Cobas System).

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.

The immunoturbidimetric assay was performed on Hitachi 902 analyser under the manufacturer’s instructions. First of all, the sample is diluted with haemolysing agent and kept at room temperature for at least 10 minutes. After that the whole blood processing is performed automatically. The HbA\textsubscript{c} determination is based on turbidimetric inhibition immunoassay. In the first step the Glycohaemoglobin in the sample reacts with anti-HbA\textsubscript{c} antibody (present in the R\textsubscript{1}) to form soluble antigen – antibody complexes. Then R\textsubscript{2} (polyhapten) is added, polyhapten reacts with excess anti-HbA\textsubscript{c} antibodies to form an insoluble antibody – polyhapten complex which can be determined turbidimetrically. The Roche Immunoanalytical method is standardised via IFCC reference system and the results obtained by this method are expressed in mmol/mol. For the conversion of results to % HbA\textsubscript{c} a conversion factor is installed in the analyser by the manufacturers.

Control Material
Total imprecision was determined with commercially available control blood of high and low HbA\textsubscript{c} concentration by repeated analysis. Lyphcocheck Diabetic controls from Bio Rad Laboratories were used.

Data Analysis
The data was entered and analysed by using standard SPSS software version - 16 (SPSS Inc, Chicago) for statistical analysis. Mean (± SD) is given for quantitative variables. Frequency and percentages are given for qualitative variables. Pearson correlation (r) was utilised for determining the strength of linear association between HbA\textsubscript{c} measurements by two methods. The threshold for significance was 0.01 for two – tailed test. Bland and Altman plots were used to calculate mean difference (Bias) and
agreement between two methodologies. It was considered that 95% of all values lying within ±2 SD indicate good agreement.

RESULTS
A total of 110 samples were analysed for HbA1c estimation. The mean age of patients included in the study was 51.48 ± 11.4 years (range 28 – 80 years). There were 34 (30%) males and 76 (69%) females. The descriptive statistics for both the techniques are shown in Table 1. The mean HbA1c was slightly lower for immunoturbidimetric (8.2%) method than HPLC (8.6%). Low and High level controls were tested on consecutive five days in duplicate for the between run precision. Each individual result (N = 15 per level) was taken for the calculation. Lyphocheck Diabetic controls from Bio Rad Laboratories were used (Table 2).

Table 1: Values obtained by D-10 HPLC and Immunoturbidimetric Method.

<table>
<thead>
<tr>
<th>HbA1c (%)</th>
<th>Mean ± (SD)</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-10 HPLC</td>
<td>8.6 ± 2.07</td>
<td>5.3 – 14.6</td>
</tr>
<tr>
<td>Immunoturbidimetry</td>
<td>8.2 ± 3.07</td>
<td>5.2 – 14.2</td>
</tr>
</tbody>
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Table 2: Mean ± SD and between run CVs for HbA1c determined by D-10 HPLC and Immunoturbidimetric method.

<table>
<thead>
<tr>
<th>sControl (Level)</th>
<th>Mean (± SD)</th>
<th>Between run CVs</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-10 HPLC (Level 1)</td>
<td>5.3 ± 0.11</td>
<td>1.8%</td>
</tr>
<tr>
<td>D-10 HPLC (Level 2)</td>
<td>10.1 ± 0.21</td>
<td>1.9%</td>
</tr>
<tr>
<td>Hitachi 902 (Level 1)</td>
<td>5.2 ± 1.12</td>
<td>2.9%</td>
</tr>
<tr>
<td>Hitachi 902 (Level 2)</td>
<td>9.7 ± 1.25</td>
<td>3.1%</td>
</tr>
</tbody>
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Bland and Altman plot was used to calculate mean difference (bias) and agreement between two methodologies. The plot shows the presence of good agreement between the two methods, 95% of values are lying within the ±2 SD range from the mean (Figure 1).

The correlation analysis was also done between the results obtained by D-10 cation exchange HPLC and immunoturbidimetric method. The immunoassay results are plotted on the x-axis and HPLC results are plotted on the y-axis. The results showed a good positive correlation between both the methods tested. A significant value of < 0.001 was obtained. The correlation coefficient (r) is 0.95 and the coefficient of determination (r²) was 0.90 (Figure 2). Regression analysis between D-10 HPLC and Immunoturbidimetric method yielded a slope of 0.95 ± 0.30, an intercept of 0.95 X + 0.8 and an SDEV of 0.43. A significant value of < 0.001 was achieved.

DISCUSSION
The availability of the hemoglobin A1c test has been a major advance in diabetic care and its measurement has become an integral part for the management of diabetes. A relationship has been established between the glycaemic control and risk of diabetic complications.7,8 However different methods for its measurement tend to yield results with un-
desirable differences. It has become very important to compare the results of various methods used by different laboratories.11

In this study, HbA1c results obtained with D-10 Haemoglobin Testing System (BIO RAD Laboratories) which is based on Cation Exchange HPLC were compared with Roche Immunoturbidimetric method (Performed on Hitachi 902). The motivation for this study was to evaluate a method with a greater practicability and improved precision. The Bio Rad D-10 Hemoglobin Testing System is the newly introduced fully automated analyser based on Cation Exchange HPLC. It consists of a single module that provides an integrated method for sample preparation, separation and determination of specific haemoglobins in the whole blood. Marzullo et al have critically evaluated the analytical performance of D-10 hemoglobin testing system. They concluded that D-10 is an easy to use automated procedure which offers accurate HbA1c quantification.24

Both methods have been compared in terms of precision and their overall correlation was also assessed with the help of correlation analysis. In the present study we found an interassay CV of 1.8% (HPLC) and 2.3% (immunoassay) for Level 1 control and 1.9% (HPLC) and 2.5% (immunoassay) for Level 2 controls. Previously the Hitachi 911 immunoassay was compared with Diamat HPLC and found that the interassay CV for Hitachi 911 immunoassay was 3.4% while it was 3.3% on Diamat HPLC. There was an excellent correlation between Bio Rad D-10 HPLC Hemoglobin testing system and Architect Immunoassay by Abbott Diagnostic. They found an interassay CV of 1.6% for D-10 HPLC and 2.1% on Immunoassay.25

Our 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). While the improved precision which is observed in the study of Beaune et al and in the present study can be attributed to the recent advances in HbA1c assay standardization, lower total CVs make it easier to detect significant trends or shifts in a diabetic patient’s blood glucose control.25

In the present study, comparison between HbA1c by HPLC and HbA1c by immunoassay was done on 110 patients ranging from 5% to 14%. Results showed a correlation coefficient of 0.95. Beaune et al conducted a comparative study on D-10 HPLC and Arcitect immunoassay on 161 samples. They found the correlation coefficient of 0.98.26 Similarly 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.27 Aside from these issues concerning precision there has been much discussion about how the method is standardized and the effects of common interferences from unstable and/or abnormal haemoglobin. The D-10 HPLC system participated in the National Glycohaemoglobin Standardisation Program and is traceable to the DCCT reference method. The National Glycohemoglobin Standardization Program (NGSP) is responsible for the calibration of the HbA1c methods in many parts of the world enabling direct comparison to DCCT targets.26

The Roche Immunoturbidimetric method is standardised via IFCC reference system and the results obtained by 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 equalize the results of immunoassay to HPLC. The recent recommendation by International organizations is 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.

One of the major concerns with various methods is that unstable haemoglobin variants may interfere with the HbA1c measurement. A major disadvantage of these HbA1c immunoassays is that they do not consistently detect the presence of abnormal haemoglobin variants. Since 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 previous studies also mentioned that the immunoassay methods do not identify the haemoglobin variants.21,27 On the other hand 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.

Regarding the pre-treatment of sample, the Roche Immunoassay requires a pre-dilution step while D-10 HPLC method requires no pre-dilution or manual handling of the sample and vacutainer tubes are directly introduced in the analyser. The assay time for HPLC is 3 minute per sample while it is ten minutes per sample for Roche Immunoassay. The Hitachi 902 Immunoassay has higher throughput rate but each sample has to be pre-diluted with haemolysing reagent and kept at room temperature for at least 10 minutes before measurement. The minimum sample requirement for Roche Immunoassay is 10 µl and for D-10 HPLC system it is 5 µl. For D-
10 HPLC finger stick samples can be collected in capillary tube for analysis. No specimen related carries over is present in both methods. The total direct per test cost of the D-10 HPLC is higher than the Hitachi 902 Immunoassay.

In conclusion, although there are a number of different methods for measuring HbA1c, there remains a need for 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 in linear in the HbA1c range of 3 – 18%. In addition the instrument also demonstrates excellent within run and total CVs. In the present study 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 hemoglobin.

REFERENCES