Original Article

Measurements of HbA$_{1c}$ by High Performance Liquid Chromatography in D-10 analyzer and Immunological Method by Beckman Coulter AU480 System: A Comparative Study

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Abstract

Background: *HbA$_{1c}$ can be measured by different methods. Enzymatic, boronate affinity chromatographic and cation-exchange high performance liquid chromatographic (HPLC) methods are considered as gold standard methods. To make the test cost-effective many laboratories use the immunological method installed in a chemistry analyzer.* **Objective:** *To compare the values of HbA$_{1c}$ measured by high performance liquid chromatographic method and immunological method in two laboratory settings.* **Materials and Methods:** *This observational and comparative study was conducted in Bangladesh Institute of Health Sciences (BIHS) and Enam Medical College, Savar, Dhaka. HbA$_{1c}$ levels measured by HPLC in BIHS were compared with that measured by immunological method in Enam Medical College, Savar, Dhaka. p values <0.05 were considered significant.* **Results:** *Statistically no difference was observed in mean HbA$_{1c}$% between two methods in ⩽7.0% HbA$_{1c}$ group and 7.1-10.0% HbA$_{1c}$ group. Significant statistical difference was found in mean HbA$_{1c}$ >10.0% group, but it was within acceptable limit.* **Conclusion:** *HbA$_{1c}$ can be measured by immunological method installed in an automated chemistry analyzer to make the test cost-effective. But HPLC method is preferable.*
Key words: *High performance liquid chromatographic method, Immunological method, HbA$_{1c}$*

Introduction

Diabetes mellitus is a syndrome of impaired carbohydrate, fat and protein metabolism caused by either lack of insulin secretion or decreased sensitivity of the tissues to insulin.[1] It is characterized by hyperglycemia resulting from the body's inability to utilize glucose for energy. In type 1 diabetes, the pancreas cannot produce insulin and glucose in the blood cannot get entry inside the cell to be metabolized to provide energy. In type 2 diabetes, either the pancreas cannot produce enough insulin or the tissues of the body are insensitive to insulin. The complications of diabetes, involving the eyes, kidneys, nerves and blood vessels of heart, brain, extremities are common to both types of diabetes.[2] Currently more than 5% of the world populations are affected by diabetes and it is getting more and more epidemic.[3]

Management of diabetes requires the long term maintenance of blood glucose level as close as possible to a normal level to minimize the complications of diabetes. Blood glucose measurement is an indication of the patient's immediate past condition (hours). It cannot speak of the true status of blood glucose regulation. An accurate index of the mean blood glucose concentration may be obtained

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only by the measurement of HbA$_{1c}$ every two to three months.[4-6] HbA$_{1c}$ can also be translated to average blood glucose (eAG) to get the approximate plasma glucose level.[7]

HbA$_{1c}$ can be measured by immunological, enzymatic, boronate affinity chromatographic and cation-exchange high performance liquid chromatographic (HPLC) methods.[8] It can also be measured by electrophoresis. Enzymatic and boronate affinity chromatographic and HPLC methods are considered as gold standard methods to measure HbA$_{1c}$. But it is not possible to install these methods in all laboratory settings, especially in the developing countries like ours. Measurement of HbA$_{1c}$ by electrophoresis is also cumbersome and costly. For HbA$_{1c}$ measurement, many laboratories use the immunological method installed in a chemistry analyzer to make the test cost-effective. We designed this study to compare the values of HbA$_{1c}$ measured by HPLC method and immunological method in two laboratory settings.

Materials and Methods

This observational and comparative study was done in the department of Clinical Biochemistry, Bangladesh Institute of Health Sciences (BIHS), Dhaka and the department of Biochemistry, Enam Medical College, Savar, Dhaka during the period of July 2011 to December 2011. Sixty specimens for measurement of HbA$_{1c}$ levels by HPLC method in Bangladesh Institute of Health Sciences were included for this study. Twenty specimens had HbA$_{1c}$ level $\leq 7.0\%$, 20 had 7.1 to 10% and 20 were of >10%. These specimens were also measured for HbA$_{1c}$ levels in Enam Medical College Hospital laboratory by immunoassay method. Results by these two methods were compared by appropriate statistical analyses.

HPLC method[3]

We used the Bio-Rad D-10^{TM} Hemoglobin A$_{1c}$ program which is intended for the percent determination of HbA$_{1c}$ in human whole blood. The D-10 Hemoglobin A$_{1c}$ Program is based on chromatographic separation of HbA$_{1c}$ on a cation exchange cartridge. Separation is optimized to minimize interferences from hemoglobin variants, labile A$_{1c}$ and carbamylated hemoglobin. The D-10^{TM} Hemoglobin A$_{1c}$ program also offers automatic

sampling from a primary whole blood tube, followed by sample dilution and an analysis time of three minutes per sample.

The samples are automatically diluted on the D-10 and injected into the analytical cartridge. The D-10 delivers a programmed buffer gradient of increasing ionic strength to the cartridge where the hemoglobin is separated based on their ionic interactions with the cartridge material. The separated hemoglobin then passes through the flow cell of the filter photometer where changes in the absorbance are measured at 415 nanometer wavelength. The D-10 software performs reduction of raw data collected from each analysis. Two-level calibration is used for quantification of the HbA$_{1c}$ values. A sample report and a chromatogram are generated for each sample. The A$_{1c}$ area is calculated using an exponentially modified Gaussian (EMG) algorithm that excludes the labile HbA$_{1c}$ and carbamylated peak area from the A$_{1c}$ peak area.
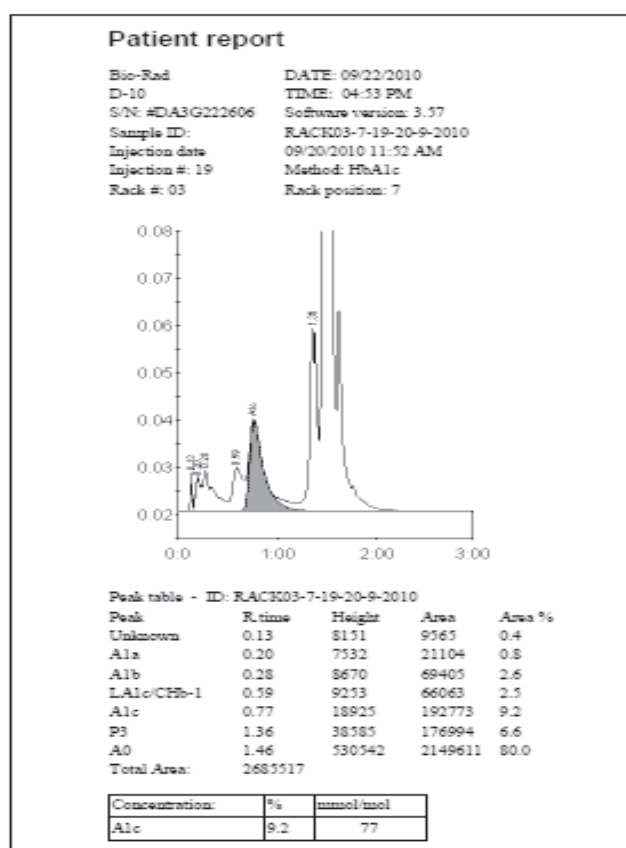

Fig 1. Representative chromatogram of ion-exchange HPLC (shaded area indicates the area of HbA$_{1c}$ eluent)

Immunoassay method[9]

We used the Beckman Coulter AU480 analyzer. The absolute HbA_{1c} and total hemoglobin (THb) values generated as part of the HbA_{1c} assay are intended for the quantitative determination of hemoglobin A_{1c} concentration as a percentage of total hemoglobin in human whole blood.

The assay for percent HbA_{1c} involves the use of four reagents: Total hemoglobin R1, HbA_{1c} R1 antibody reagent, HbA_{1c} R2 agglutinator and hemoglobin denaturant. In the pre-treatment step the whole blood is mixed with hemoglobin denaturant in a 1:41 dilution and incubated for a minimum of five minutes at room temperature. The red blood cells are lysed and the hemoglobin chain is hydrolyzed by the protease present in the reagent. Total hemoglobin is measured via the conversion of all hemoglobin derivatives into alkaline hematin in the alkaline solution of a non-ionic detergent. Addition of the pre-treated blood sample to the total hemoglobin reagent results in a green solution which is measured at 600 nm wavelength. HbA_{1c} is measured in a latex agglutination inhibition assay. An agglutinator, consisting of a synthetic polymer containing multiple copies of the immunoreactive portion of HbA_{1c}, causes agglutination of latex coated with HbA_{1c} specific mouse monoclonal antibodies. In the absence of HbA_{1c} in the sample, the antibody-coated microparticles in the HbA_{1c} R1 and the agglutinator in the HbA_{1c} R2 will agglutinate. Agglutination leads to an increase in the absorbance of the suspension. The presence of HbA_{1c} in the sample results in a decrease in the rate of agglutination of the HbA_{1c} R1 and the agglutinator in the HbA_{1c} reagent R2. The increase in absorbance is therefore inversely proportional to the concentration of HbA_{1c} in the sample. The increase in absorbance due to agglutination is measured at 700 nm wavelength.

Calibration and traceabilty[10,11]

Calibrator 1 is used for calibration of the total hemoglobin assay. Calibrators 1 to 6 are used for calibration of HbA_{1c} assay. The calibrator HbA_{1c} values are traceable to the International Federation of Clinical Chemistry (IFCC) HbA_{1c} reference method via IFCC HbA_{1c} reference material. Total hemoglobin values assigned to the total hemoglobin calibrator are traceable to the Institute for Reference Materials and Measurements (IRMM) hemoglobin cyanide standard (BCR-522). The relationship between results from the National Glycohemoglobin Standardization Program (NGSP) network (Diabetes Control and Complications Trial, DCCT aligned) and the IFCC network has been evaluated, and a Master Equation, as mentioned below, has been developed for interconversion of results from IFCC to DCCT/NGSP units.

$$DCCT/NGSP = (0.915 \times IFCC) + 2.15$$

Definition of the relationship between the two networks links the IFCC traceable results to clinically meaningful HbA_{1c} results from the DCCT and the United Kingdom Prospective Diabetes Study (UKPDS). The Master Equation also provides these DCCT results with traceabilty to a higher order reference method.

Percent HbA_{1c} results generated during this assay are automatically recalculated to DCCT aligned units by the instrument, using the IFCC approved Master Equation. Results are therefore expressed in DCCT aligned units as recommended by the IFCC.

Statistical analysis

Statistical analyses were performed by Paired t test, Bland-Altman plot and Pearson's test using MedCalc® version 11.4 for Windows. p values <0.05 were considered significant.

Results

Table I shows the mean HbA_{1c}% and comparison in different HbA_{1c} levels by two methods. In ≤7.0% HbA_{1c} group it was 6.27 ± 0.48% by HPLC method in D-10 analyzer and 6.11 ± 0.62% by immunological method in Beckman Coulter AU480 system. In 7.1–10% HbA_{1c} group it was 8.24 ± 0.83% by HPLC method in D-10 analyzer and 8.16 ± 1.07% by immunological method in Beckman Coulter AU480 system. In >10.0% HbA_{1c} group it was 12.66 ± 2.06% by HPLC method in D-10 analyzer and 11.98 ± 2.29% by immunological method in Beckman Coulter AU480 system.

Table I: Mean HbA$_{1c}$% in different HbA$_{1c}$ levels by two methods

HbA$_{1c}$ level	Methods		p values
	HPLC	Immunological	
≤7.0% (n=20)	6.27 ± 0.48%	6.11 ± 0.62%	>0.05
7.1–10% (n=20)	8.24 ± 0.83%	8.16 ± 1.07%	>0.05
>10.0% (n=20)	12.66 ± 2.06%	11.98 ± 2.29%	<0.05

for HbA$_{1c}$>10.0%. Casewise performance of the two methods in different HbA$_{1c}$ ranges is presented in Fig 3.

Discussion

In this study we found no significant difference in HbA$_{1c}$ values between the two methods (HPLC in D-10 analyzer and immunological method in Beckman Coulter AU480 system) in ≤7.0% and 7.1–10% ranges of HbA$_{1c}$ levels. But there was significant difference in HbA$_{1c}$ values between the
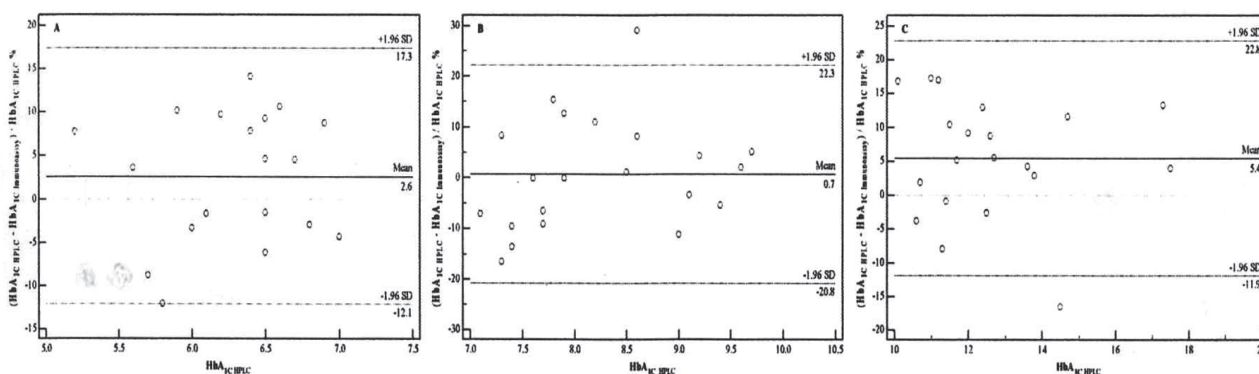

Fig 2. Bias of HbA$_{1c}$ measured by immunological method against HPLC method in ≤7.0% (A), 7.1–10% (B) and >10.0% (C) ranges of HbA$_{1c}$ levels
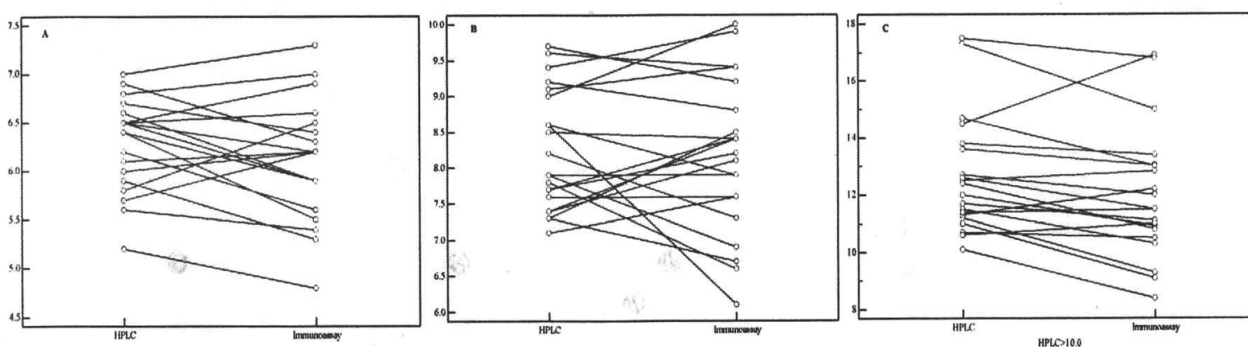

Fig 3. Casewise performance of HPLC and immunological methods to measure HbA$_{1c}$ in ≤7.0% (A), 7.1–10% (B) and >10.0% (C) ranges of HbA$_{1c}$ levels

The bias of HbA$_{1c}$ measured by immunological method against the HbA$_{1c}$ measured by HPLC method was 2.6% for HbA$_{1c}$ ≤7.0%, 0.7% for HbA$_{1c}$ range of 7.1–10% and 5.4% for HbA$_{1c}$ >10% (Fig 2). The correlation coefficients of HbA$_{1c}$ measured by HPLC and immunoassay were 0.671 (p=0.0017) for HbA$_{1c}$ ≤7.0%, 0.588 (p=0.0050) for HbA$_{1c}$ range of 7.1 – 10.0% and 0.870 (p<0.0001)

two methods (HPLC in D-10 analyzer and immunological method in Beckman Coulter AU480 system) in >10.0% range. But in these cases the percentage of difference against HPLC method was 5.4% which was very close to acceptable limit of 5%. Moreover the correlation coefficients between these two methods were strong in different HbA$_{1c}$ ranges. It was also evident from the casewise

performance of the two methods that there was no systemic underestimation or overestimation, values of HbA_{1c} were randomly scattered around the HbA_{1c} values measured by HPLC methods. So, it can be concluded that by having proper and regular calibration and by undertaking strict quality control measures in accordance with good laboratory practices HbA_{1c} can also be measured by immunological method installed in a chemistry analyzer to make the test cost-effective. However, more studies in different laboratory settings are recommended.

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