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RESEARCH ARTICLE

COMPARISON OF ANALYTICAL PERFORMANCE OF THREE DIFFERENT METHODS FOR DETERMINATION OF GLYCATED HEMOGLOBIN

[1, *]Dr. Sara Reza, [2]Dr. Asma Shaukat, [3]Maria Mahmud, [4]Dr. Tariq Arain and [5]Umama Reza

[1, 2]Department of Pathology, Quaid-e-Azam Medical College, Bahawalpur, 63100, Pakistan
[3]Department of Advanced Biotechnology, Algonquin College, Ottawa, ON K2G 1V8, Canada
[4]Department of Ophthalmology, Bahawal Victoria Hospital, Bahawalpur, 63100, Pakistan
[5]Biochemistry & Biotechnology Department, Islamia University, Bahawalpur, 63100, Pakistan

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ABSTRACT

Background: The utility of HbA1c for long term assessment of glycemic control requires an accurate, precise and robust measurement system. Currently, immunoassay and HPLC are the most popular methods for HbA1c estimation. Merck Life Sciences has introduced a fully automated direct enzymatic method for quantification of HbA1c from whole blood on MINDRAY chemistry system.
Methods and Findings: HbA1c level of 178 randomly chosen subjects was quantified using three **Methods as follows:** Cation-exchange High Performance Liquid Chromatography (HPLC) [Shimadzu LC-20 AT], Chemiluminescence Microparticle Immunoassay (CMIA) [Abbott architect i2000SR] and enzymatic assay [Mindray BS 400]. HPLC was accepted as comparative method. The analytical performances of the methods were evaluated with imprecision, bias estimation and comparison studies. There was good concordance between the results from CMIA and enzymatic assay when compared with HPLC (r=0.97 and r=0.96, respectively). The Bland Altman plots showed a favorable agreement between the methods, 95% of values were lying within ± 2 SD range from the mean. The average HbA1c measured by HPLC (6.8%) was higher than both the other methods (CMIA 6.4%, enzymatic assay 6.6%).
Conclusion: All methods proved to be sufficiently reliable and the results of these methods showed a strong correlation; though the Direct Enzymatic HbA1c Assay in addition to having all the advantages of both the HPLC and immunoassay methods in areas of accuracy, precision, and applicability to chemistry analyzers, is cost effective, simpler and has less interferences plus it does not require a separate measurement of total hemoglobin content in samples.

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INTRODUCTION

Diabetes mellitus has become a challenging public health problem globally (Ramachandran *et al.,* 2012). The successful treatment of diabetes depends on keeping the blood glucose at a normal level. As blood glucose measurement provides limited value for long term assessment of glycemic control, various other tests have been developed for this purpose. Hemoglobin A1c (HbA1c) has been recommended as the most useful tool in the diagnosis, follow up, and treatment of diabetes (Bonora and Tuomilehto, 2011). Glycated hemoglobin (HbA1c) is a glycoprotein formed by irreversible non-enzymatic binding of D-glucose to the N-terminal amino group valine of hemoglobin β-chain.

Corresponding author: Dr. Sara Reza
Department of Pathology, Quaid-e-Azam Medical College, Bahawalpur, 63100, Pakistan.

The amount of HbA1c in the blood depends on both the life span of red blood cells (average 120 days) and the blood glucose concentration. Because the rate of formation is directly proportional to concentration of glucose in the blood, the HbA1c reflects the integrated values of glucose over the preceding 2 to 3 months. Therefore, HbA1c test is used both as an index of mean glycemia and as a measure of risk for the development of diabetes complications (Syed, 2011; Elshaikh *et al.,* 2014). Accurate HbA1c results are essential for monitoring and appropriate treatment of diabetic patients. Nowadays, the methods for reliable measurement of HbA1c are classified into 3 groups. These methods distinguish hemoglobin from GHb using techniques based on:

- Charge difference (ion-exchange chromatography, HPLC, electrophoresis and isoelectric focusing)

(Elshaikh and Idris, 2014; Weykamp *et al.,* 2009).

- Structural difference (affinity chromatography, immunoassay) (Sacks, 2012; Weykamp, 2013; Weykamp *et al.,* 2009).
- Chemical reactivity (photometry, electrospray mass spectrometry) (Weykamp, 2013; Weykamp *et al.,* 2009).

Many factors interfere with the HbA1c results causing falsely high or low results depending on the assay methods. So there is a need to choose an accurate, easy and practical method which is suitable for routine use in the clinical chemistry laboratory. But it should be kept in mind that any method chosen, must be traceable to high-performance liquid chromatography (HPLC), for it is currently considered the reference method of the National Glycohemoglobin Standardisation Program (NGSP) and the mainstay method for the studies of Diabetes Control and Complications Trial Research Group (DCCT) (Jeppsson *et al.,* 2002; Hoelzel *et al.,* 2004). The HPLC method can detect abnormal Hb variants with favorable precision and CV of less than 1%. However, a large expensive instrument and long run time are required. It takes more than 4 hours to analyze approximately 100 samples. In addition, more technical staff is needed for the maintenance of this equipment (Sakurabayashi *et al.,* 2003; Özçelik *et al.,* 2010; Karami and Baradaran, 2014). The advantage of using immunoassay is that it can be adapted to an automated analyzer so a large number of samples can be measured in a short time. However, this method suffers with the general drawbacks of immunochemistry, i.e. non-linear calibration, which requires multilevel calibration. As stability of the reagent is limited, the calibration curve cannot be held for 24 hours and a relatively frequent recalibration is needed. Also, to quantitate HbA1c, as a ratio, total haemoglobin is measured separately, using a different analytical principle that introduces additional uncertainty to the outcome (Sakurabayashi *et al.,* 2003; Özçelik *et al.,* 2010). Moreover, the reproducibility is less than HPLC i.e. CV: 3–5% (Goodall, 2006). The enzymatic method meets the performance requirements with some additional advantages. There is no extra determination of total Hb (THb) and no calculation of HbA1c content is required. Only one channel and reagent position on analyzer is needed. It is not adversely affected by interferences from common hemoglobin variants in samples. It is a cost effective, user-friendly method and is adaptable to most general chemistry analyzers. However, like immunoassay it has a very low imprecision (Sakurabayashi *et al.,* 2003; Özçelik *et al.,* 2010). In the present study we have made an attempt to compare the analytical performance of a new enzymatic assay with that of an immunoturbidimetry method. The high performance liquid chromatography was used as the comparative method in our study. The aim is to establish an accurate, timely, cost effective and practical method with a high precision that is suitable for Clinical Laboratory of Quaid-e-Azam Medical College, Pakistan, which has a very high workflow.

MATERIALS AND METHODS

Patients and Samples

This study comprised of 178 whole blood samples randomly chosen from the subjects who applied to Clinical Chemistry Laboratory of Quaid-e-Azam Medical College, Pakistan between November to December 2015, for either routine testing or the control of the diabetic status. No further selection criteria were used. Whole blood was collected from all patients in EDTA vials. All samples were kept +4°C until studied. HbA1c levels were measured with three different methods and assays were completed within four hours following blood sampling. This study was approved by the Institutional Ethical Review Committee and informed consent was obtained from each subject prior to the study.

Analytical procedures were conducted according to the following three methods

1. ***High performance liquid chromatography (HPLC) method***

HbA1c level of the patients was measured using an HPLC instrument (Shimadzu LC-20 AT) equipped with SPD-20A detector, which is based on cation exchange chromatographic technique. The manufacturer's instructions were followed for the measurement. This technique requires no predilution of the samples and they are directly introduced in the primary tubes after running two levels of calibrators and controls. The instrument draws sample directly from the EDTA tube and all processing of the sample is performed internally. Samples are automatically mixed, diluted and injected into 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 and changes in the absorbance are measured. A sample report and a chromatogram are generated for each sample. The method and reagents are NGSP certified.

2. ***Immunoturbidimetric Method***

The HbA1c values were measured by ARCHITECT HbA1c 2-step pretreatment immunoassay following the kit manufacturer's instructions. In this method sample is incubated with pre-treatment reagent to lyse the red blood cells. Pre-treated sample is then incubated with magnetic microparticles with a silica surface. Hb and HbA1c in the sample bind to the silica surface of the microparticles. Following a wash cycle, anti-HbA1c acridinium-labeled conjugate is added to create a reaction mixture. Following another wash cycle, pre-trigger and trigger solutions are added to the reaction mixture. The resulting chemiluminescent reaction is measured. The traceability of Architect Immunoturbidimetric method is given to IFCC as well as NGSP/DCCT reference method ensuring good comparability to other tests.

3. Enzymatic Assay Method

The HbA1c values of hemolysed samples were measured by Mindray kit according to the kit manufacturer's instructions. In the first reaction, the concentration of Hb is measured at absorbance of fixed wavelength, and simultaneously the fructosyl dipeptides are generated from the N-terminus amino groups of the beta-chain of HbA1c by the reaction of protease. In the second reaction, the reaction of fructosyl peptide oxidase (FPOX) with fructosyl dipeptides, generated hydroperoxide

allows 10-(carboxymethylaminocarbonyl)-3,7-bis(dimethyla mino) phenothiazine sodium salt to develop a color in the presence of peroxidase. The change in absorbance is measured by spectrophotometer. This method has been standardized against JCCLS standard CRM-004a. The experimental protocol followed EP-9 CLSI guidelines for method comparison and bias estimation.

Control Material

Repeated analysis of commercially available control materials of high and low HbA1c concentration were performed for determining total imprecision after testing their commutability and stability. Lyphocheck Diabetic controls from Bio Rad Laboratories were used. All results were expressed as % HbA1c. Determination by each method was performed in different areas of the laboratory by independent analysts in three replicates and single run. The analysts performing the tests knew neither the experimental design nor the result of the other method.

Statistical Analysis

The data was entered and analyzed by using standard SPSS software version - 20 (SPSS Inc, Chicago) for statistical analysis. Mean (± SD) and frequency/ percentages were used to present the variables. Pearson correlation (r) was utilized for determining the strength of linear association between HbA1c measurements by the above mentioned laboratory methods. Bland and Altman plots were constructed using GraphPad Prism 6 software and were used to calculate mean difference (Bias) and agreement between the methodologies. The measurements were compared using paired sample t-test and a p-value of less than 0.05 was considered statistically significant.

RESULTS

A total of 178 samples were analyzed for HbA1c estimation. The mean age of patients included in the study was 48.5 ± 10.2 years (range 23 – 74 years). There were 72 (41%) males and 106 (59%) females. The descriptive statistics for both techniques are shown in Table 1. The mean HbA1c was slightly lower for CMIA (6.4%) and enzymatic (6.6%) methods than HPLC (6.8%). The correlation analysis was also done between the results obtained by HPLC and CMIA method as well as for HPLC and enzymatic assay (Figure 1-A and 2-A). The HPLC results were plotted on the x-axis while those from CMIA and enzymatic methods were plotted on the y-axis. The results showed a good positive correlation between both the methods tested. Results depicted that there was no significant difference between these two mean numbers ($p > 0.05$). The initial comparison results of these methods using Spearman test, a correlation coefficient (r) of 0.97 and 0.96 for CMIA and enzymatic assays, respectively (Figure 1-A and 2-A) was obtained.

The Bland Altman plots of differences between the HPLC and other methods are shown in (Figure 1-B and 2-B). These plots were used to calculate mean difference (bias) and agreement between these methodologies. The plot showed the presence of good agreement between the methods, 95% of values were lying within the ± 2 SD range from the mean (Figure 1-B and 2-B). 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 (Table 2). Data of method comparisons obtained by a set of whole blood samples (n=178) are summarized in Table 3. The desirable analytical goals for bias and imprecision are 1.5% and 0.9%, respectively (Ricos *et al.,* 1999).

Table 1. Basic statistical parameters obtained by three methods: HPLC, CMIA and enzymatic assay

Method	Mean HbA1c (%),n=178	Standard Deviation (SD)	Range HbA1c (%)	Coefficient of Variation (CV)
HPLC	6.8	1.8	4.0-10.6	0.27
CMIA	6.4	1.7	4.0-10.3	0.27
Enzymatic assay	6.6	1.8	4.1-10.2	0.26

Table 2. Mean ± SD and between run CVs for HbA1c determined by LC-20 AT HPLC, Architect i2000SR (CMIA) and Mindray BS 400 (enzymatic method)

Control (Level)	Mean (± SD)	Between run CVs
LC-20 HPLC (Level 1)	5.0 ± 0.12	1.2%
LC-20 HPLC (Level 2)	10.2 ± 0.20	1.3%
Architect i2000 (Level 1)	5.1 ± 1.03	2.3%
Architect i2000 (Level 2)	9.8 ± 1.15	2.5%
Mindray BS400 (Level 1)	5.2 ± 1.01	2.1%
Mindray BS400 (Level 2)	9.7 ± 0.98	2.3%

Table 3. Comparison of HPLC with CMIA and enzymatic assay: Individual results for method-comparison studies

Method	Correlation coefficient (r)	Coefficient of determination (r^2)	Bias	Slope	Intercept	95%	p-value
HPLC and CMIA	0.97	0.94	0.40±0.46	0.91±0.02	0.19±0.13	-0.51 –1.32	0.0372 (NS)
HPLC and Enzymatic	0.96	0.93	0.16±0.50	0.92±0.02	0.37±0.14	-0.83 –1.15	0.4109 (NS)

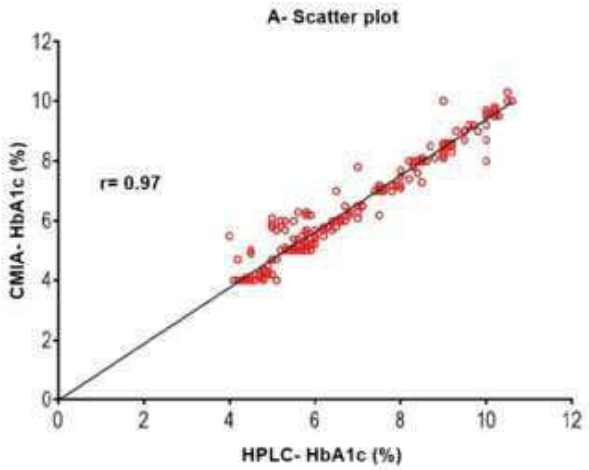

Figure 1A.

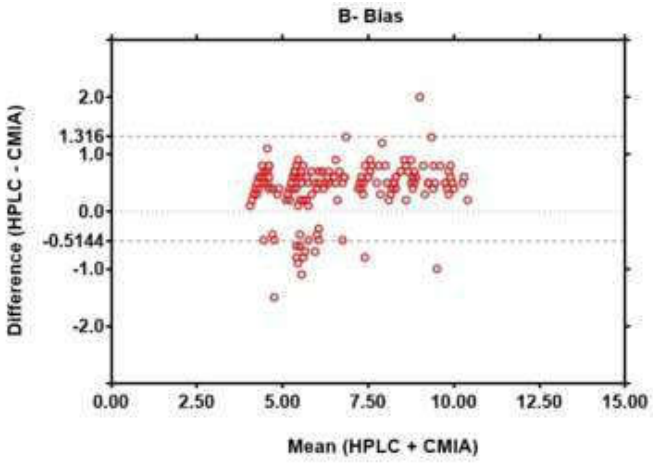

Figure 1B.

Figure 1A. Scatter plot and 1B: Bland Altman plot between HPLC (LC-20 AT) and CMIA (Abbott Architect i2000SR) showing a good agreement

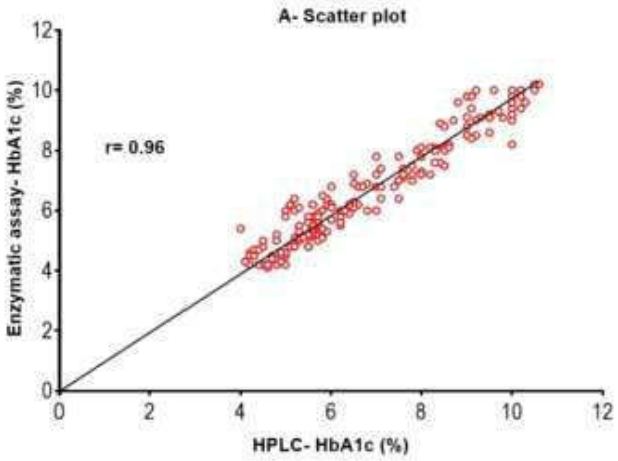

Figure 2A.

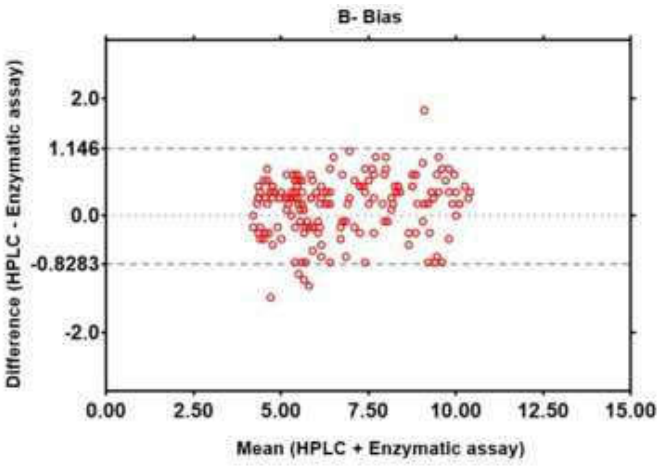

Figure 2B.

Figure 2A. Scatter plot and 2B: Bland Altman plot between HPLC (LC-20 AT) and enzymatic assay (Mindray BS 400) showing a good agreement

DISCUSSION

Several studies have been conducted to report a relationship between HbA1c methods based on different principles. However different methods for its measurement tend to yield results with unacceptable differences. It is therefore, very important to compare the results of various methods used by different laboratories. There are some steps that are necessary for the method-comparison studies in order to maintain the quality of clinical trials. They include the overall precision and bias of the methods, the correlation with the reference method and agreement of the results (Genc *et al.,* 2014). In our study, the analytical performance of Chemiluminiscent Micropaticle Immunoassay (CMIA) and enzymatic HbA1c methods was evaluated in terms of precision and overall correlation was also assessed in comparison to cation-exchange HPLC, the reference method.

The motivation for this study was to evaluate a method with a greater practicability and improved precision. Both the methods indicated a good precision and accuracy.

They also showed good agreement with HPLC by showing a narrow dispersion around the regression lines. These results are similar to those obtained in other previous method-comparison studies (Karami and Baradaran, 2014; Yasmeen *et al.,* 2011; García-Alcalá *et al.,* 2009; Vucheva *et al.,* 2012). A previous study evaluating the analytical performances of HPLC reported intra- and inter-assay CVs were 1.8% and 1.9% (Yasmeen *et al.,* 2011), and these findings are in accordance to our findings. Previous precision studies revealed CVs for the two candidate methods as: immunoassay, 1.94% (Karami and Baradaran, 2014) and direct enzymatic assay, 0.5% (Teodoro-Morrison *et al.,* 2014). Our study revealed an excellent precision with <1% error in patients results which not only indicated a close harmony with these previous studies but also showed a good agreement with the goals of NGSP (<2.0%) and IFCC (<2.8%) (Clinical and Laboratory Standards Institute, 2004) and those available on Westgard homepage (Ricos *et al.,* 1999). This minimal imprecision may be due to the detection principle of enzymatic assay which depends on the color production by POD and H2O2. So this method provides a rapid and uniform reaction in the same way as

clinical biochemistry reagents (e.g., glucose or glutamic oxaloacetic transaminase). When comparing between run CVs of control materials, it was found that CMIA and enzymatic assays have higher CVs than HPLC. Previously an interassay CV of 1.6% for D-10 HPLC and 2.1% on immunoassay was found. Our results are concordant with these previous studies that immunoassay has higher variation (CV) than HPLC. The precision of these methods is within the medically allowable CV (< 5% recommended by National Academy of Clinical Biochemistry and International Federation of Clinical Chemistry) (Beaune *et al.,* 2003). The lower CVs in enzymatic assay make it easier to detect significant trends or shifts in a patient's sample. In our study, estimated mean difference of the methods from HPLC were found very small; 0.40 (-0.51 – 1.32) for CMIA and 0.16 (-0.83 – 1.15) for enzymatic assay. Allowable bias for HbA1c was suggested by Rohlfing as ≤ ±1.5% desirable and ≤ ± 0.8 % optimal (Rohlfing *et al.,* 2008). The values obtained in our study were at optimal level. In a previous study immunoassay was well associated with the HPLC showing a mean bias of 0.19% (Genc *et al.,* 2014) while that of enzymatic assay was found to be 2.0-2.2% (Rohlfing *et al.,* 2008). The good relationship and concordance between the enzymatic and HPLC methods, as indicated in other studies (Teodoro-Morrison *et al.,* 2014; Penttilä *et al.,* 2011; Liu *et al.,* 2008), support the reliability of properly standardized enzymatic method. Results showed a correlation coefficient of 0.97 and 0.96 from CMIA and enzymatic assay, respectively when compared with HPLC. 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 (Beaune *et al.,* 2009). Similarly Hawkins RC, found a correlation coefficient of 0.98 (Hawkins, 2003). They compared the HbA1c results of 110 patients performed on Bio Rad Diastat HPLC and Bayer DCA 2000 immunoassay. Liu et al during their method validation for Diazyme Direct Enzymatic HbA1c Assay on the Hitachi 917 auto-analyzer, showed a correlation coefficient of 0.98 (Liu *et al.,* 2008). while Teodoro-Morrison et al found a correlation coefficients ranging from 0.984 to 0.996 for ARCHITECT HbA1c enzymatic assay when compared to Menarini Adams HA-8160, Bio-Rad Variant II and Variant II Turbo instruments (Teodoro-Morrison *et al.,* 2014). The results obtained by both CMIA and enzymatic assay methods were lower than the HPLC. Our results are concordant with the previous studies which showed similar results (Hoelzel *et al.,* 2004; Karami and Baradaran, 2014; Goodall, 2005; Groche *et al.,* 2003). Perhaps this difference in HPLC and other two methods reflects the possibility that the HbA1c peak was affected by other substances and by abnormal Hb variants (Tsai *et al.,* 2001). One of the major concerns with various methods is that unstable haemoglobin variants may interfere with the HbA1c measurement. The size and characteristics of our study population were not suitable for an investigation of possible interference of hemoglobin variants and various metabolites on HbA1c levels. Further studies by the interference analysis are needed to examine the effect of such factors on the glycated hemoglobin measurements.

Conclusion

Most of the studies till now have not proved any superiority between the available methods of HbA1c estimation. In the present method-comparison study, we found that results produced by enzymatic assay did not show any significant difference between the bias and correlation with gold standard method HPLC as compared to conventional methods like immunoassay. But the enzymatic assay was faster than the other two methods because it does not require measurement of THb. So it can be concluded that the enzymatic method although not an ideal substitute but is reliable, faster, cost-effective, easier to perform and hence; can be used as an alternative to immunoassay and HPLC measuring system within the known imprecision limits.

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