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Selection of an Acceptable Method for the Measurement of Glycosylated Hemoglobin by Evaluating Three Different Methods

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ABSTRACT

The objective of the study is to compare the analytical performance in terms of precision and accuracy of three methods viz., Immunoturbidimetry (IT), Capillary Electrophoresis (CEP) and High Performance Liquid Chromotography (HPLC) for the measurement of glycosylated haemoglobin (HbA1c). A total of 40 routine patients samples were used for the estimation of HbA1c using the three methods and the results obtained were correlated between the three methods. The correlation coefficient 'r' obtained between IT & CEP, IT& HPLC and CEP & HPLC were 0.96, 0.95 & 0.99 respectively, indicating that an 'r' value of 0.99 obtained between CEP & HPLC is in agreement with the CLIA guidelines value of > 0.98,while poor 'r' values of 0.96 and 0.95 were obtained when IT method was compared with both CEP and HPLC methods. As per this study both CEP & HPLC methods gave good precision and accuracy for Bio-Rad controls. Further, CEP and HPLC methods have provisions to identify interference by Haemoglobin variants present in patient sample. The outcome this study recommends the use of one of the two methods viz., CEP and HPLC for routine measurement of HbA1c.

Keywords: HbA1c, Immunoturbidimetry, Capillary Electrophoresis, HPLC.

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INTRODUCTION

Diabetes mellitus (DM), long considered as a disease of minor significance is now taking its place as one of the main threats to human health in the 21st century. Measurement of HbA1c has been recommended for the long-term assessment of glycemic control in diabetic patients. The objective of our study is to compare the results obtained for HbA1c by three commonly used methods, IT, CEP and HPLC. HbA1c is measured by a variety of methods, each of which has a unique normal level. These results obtained are especially significant with respect to the clinically narrow therapeutic window of HbA1c values in type 1 diabetes to avoid rapidly advancing severe hypoglycemia rates and chronic microvascular complication rates, and the glycated hemoglobin threshold for rapidly advancing macrovascular disease in both type 1 and type 2 patients.[1] Determination of HbA1c is one of the most important monitoring procedures for long-term control of diabetes mellitus. Several analytical methods have been developed for the measurement of HbA1c. Those most frequently used methods are ion-exchange chromatography and affinity chromatography. Patients showed good correlation to those of a routine HPLC method. In addition, HbA2, HbS and HbF in samples from nondiabetic patients were not detected by the IT assay and the "labile" HbA1c fraction did not interfere with the new test.[2] The HbA1c values obtained with the new IFCC standardization are significantly lower than the well-known Diabetes Control and Complications Trial (DCCT) values. Due to the specificity of the immunological approach which is very close to the analytes as defined by the *International Federation of Clinical Chemistry* (IFCC) reference method, no further corrections of the obtained % HbA1c values are necessary. A slope/intercept correction formula is derived which allows the transformation of IFCC values into the DCCT numbers if requested[3]. The reference values of the IFCC method (mainly those of the manufacturer) range from 2.85 to 3.81%, being significantly lower than the present DCCT values (4.0-6.1%). Since it may take some time before consumers are ready to accept the new IFCC reference values for general use, it is proposed that the IFCC reference materials and method should be used for calibration of the present methods to the well-known DCCT levels.[4] Total imprecision was less than 5% and the results compared well with those from an established method. The method has a wide analytical range with no carryover between specimens. The HbA1c method on the Variant II gives acceptable analytical performance.[5]. The IFCC Working Group on HbA1c Standardization developed a reference method which uses two steps. In the first step haemoglobin is cleaved into peptides by the enzyme endoproteinase Glu-C, and in a second step the glycated and non-glycated N-terminal hexapeptides of the beta-chain

obtained are separated and quantified by HPLC and electrospray ionisation mass spectrometry or in a two-dimensional approach using HPLC and capillary electrophoresis with UV-detection. HbA1c is measured as ratio between the glycated and non-glycated hexapeptides. Calibrators consisting of mixtures of highly purified HbA1c and HbA0 are used. The analytical performance of the reference method has been evaluated by an international network of reference laboratories comprising laboratories from Europe, Japan and the USA. Possible interferences have been carefully investigated. Due to the higher specificity of the reference method the results are lower than those generated with most of the present commercial methods which currently are calibrated with unspecific designated comparison methods[6]. Exposure of specimens to high temperatures should be avoided regardless of assay methodology. For the ion-exchange methods tested 4°C storage is preferable to -20°C (stability 14-21 days vs. 4-10 days). For studies where long-term stability is required, samples should be stored at -70°C or less [7].

MATERIALS AND METHODS

40 patients attending routine health check-up were enrolled for this study. The age group of the patients were 12 to 75 years Fasting blood samples were collected in Beckon and Dickinson vacutainers containing K- EDTA. All precautions were followed when collecting blood samples, such as the use of sterile needle and syringe and other auxiliary items used for blood collection for routine biochemistry tests. The three methods selected for this study were (1) Immunoturbidimetric principle based DIA LAB kit and fully automatic analyser, Capillary Electrophoresis principle based Sebia Kit and Mini Cap analyzer and High Performance Liquid Chromatography principle based Bio-Rad kit and D-10 analyzer were used for the measurement of HbA1c.

Immuno Turbidimetric (IT) Method

The immunoturbidimetric assay was performed on CS400 discrete selective analyzer using Dialab reagent 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 hemolysis reagent (20µl whole blood + 1000µl hemolysing reagent) for 5 minutes or until complete lysis is evident. This method utilizes the interaction of antigen and antibody to directly determine the HbA1c in whole blood. Total haemoglobin and HbA1c have the same unspecific absorption rate to latex particles. When mouse antibuman HbA1c monoclonal antibody is added, latex-HbA1c mouse antihuman HbA1c antibody complex is formed. Agglutination is formed when goat anti-mouse IgG polyclonal antibody interacts with the monoclonal antibody.

The amount of agglutination is proportional to the amount of HbA1c absorbed onto the surface of lact particles. The amount of agglutination is measured at 660 nm. The Immunoturbidimetric latex method was calculated according to DCCT/NGSP Calculation according to IFCC: HbA1c% = HbA1c (g/dl) x 100 ÷ Hb (g/dl) Calculation according to DCCT/NGSP: HbA1c% = 0.915 x IFCC + 2.15.

Capillary Electrophoresis (CEP) Method

The Capillary HbA1c assay is based on the principle of capillary electrophoresis in free solution. Hemoglobin fractions are separated in silica capillaries, by their electrophoretic mobility and electro osmotic flow at a high voltage in an alkaline buffer. Hemoglobin fractions are directly detected at an absorbance of 415 nm.

High Performance Liquid Chromatography (HPLC) Method

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 pre dilution 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 analyzer delivers a programmed buffer gradient of increasing ionic strength to the cartridge, where the haemoglobins are separated on the basis 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.

RESULTS AND DISCUSSION

The results obtained using the three methods are presented in Tables 1& 2, followed by correlation graphs in figures 1 to 3. The imprecision and inaccuracy for the two levels of Biorad controls and the imprecision for a pooled serum are also presented for all the three methods. It is evident from Table I & Table II while results between CEP & HPLC agrees well as per CLIA guidelines with a correlation coefficient of 0.99, the results between IT and the other two methods does not show good correlation. These observations are further validated as shown in figures 1, 2 & 3 where

R2was found to be good when values are compared between CEP & HPLC methods. Table 3 shows acceptable %bias(accuracy) % CV(precision) obtained for both CEP & HPLC methods. The use of HbA1c levels in the management of diabetes mellitus requires efficient and reliable methods for accurately measuring glycated Hb. There was a good concordance between the results of Capillary 2-FP and HPLC methods. Many methods have been shown to be affected by the Hb variants such as Hb C, D, E, and S. During this study, we found out hemoglobin variants that produced an extra peak on the Variant II chromatogram. The same sample analyzed with G8 showed an underestimated HbA1c level. Only Capillary 2-FP did not show any interference with the variant trait[8]. Earlier studies showed that most of the tested analyzer systems had good correlation with the Diamat HPLC, although the reference intervals varied greatly according to the type of procedure used. Precision was also good on all tested assay systems—all falling within the medically allowable CV (i.e., 5% of the HbA1c value). Diamat HPLC showed significantly greater %HbA1c values in diabetic and nondiabetic persons than did all other methods[9]. When we reviewed most of the earlier studies similar to our study we got the results of following studies: The intra and inter assay precision obtained for IT are 1.5% and 2% which are better than those obtained in a previous study using similar instruments and method (2.4% and 5.9%). 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 [4]. The chromatographic methods for HbA1c gave figures of 0.9% and 1.7% for intra and inter assay precision[6]. HbA1c measurement by automated HPLC using TOSOH analyzer is said to give improved precision[10]but the authors have not given exact figures. In the study involving 23 HbA1c methods, the inter and intra assay % bias obtained were 6% and 9% [11] 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 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 Clinical and Laboratory Standards Institute (CLSI) standard guidelines. All methods available for HbA1c measurement showed generally acceptable precision and accuracy in accordance with the Diamat system. Interference study showed influence of anaemia, polycythemia, rheumatoid factor, and chronic hemodialysis on the values of the DAKO ELISA and of anaemia and polycythemia on the values of the Boehringer-Mannheim Tinaquant assay[12].Interference study showed no statistically significant influence of anemia, polycythemia, rheumatoid factor, or chronic hemodialysis, although individual Hb A1c values can be influenced by polycythemia, when measured with the Hi-Auto A1c analyzer and by chronic hemodialysis when measured with the Variant HPLC. However HPLC was not suitable for

measuring HbA1c in the examined cases of hemoglobin variants; assaying fructosamine seems to be better for monitoring these patients[13]. TINIA (Turbidimetric inhibition immunoassay and boronate affinity) chromatography methods showed good agreement and correlation with the comparative method, whereas a significant difference was obtained between the mean levels of HPLC and CEP[14]. Considerable differences in the probability of reporting unreliable HbA1c results between different NGSP (National Glycohemoglobin Standardization Program) certified platforms were observed. Risk estimates for individual laboratories' HbA1c methods can be used to assess QC practices and residual risk of an unreliable HbA1c result[15]. Diazyme had a better performance and showed a greater concordance with HPLC among others, although it was not an ideal alternative for HPLC[16]. Since the HbA1c test is now recommended for diagnosing diabetes, and minimal variation of the concentration affects the clinical therapy, it is very important that the results are reliable and interference-free. Capillarys 2-FP analyzer is suitable for this purpose and sometimes it showed some advantages with respect to the HPLC analyzers tested, especially when Hb variants are present[17]. The difference in glycation percentage between the PolyCAT A and Diamat methods is 2-3% over the whole concentration range. These results point to the limitations of Diamat as a reference method to be used to calibrate other methods for determining HbA1c. Further, a switch from one method to another is likely to cause considerable problems in the clinical follow-up of certain patients[18]. There was good concordance between results of particle-enhanced immune turbidimetric assay (PEITT) and HPLC methods ($r = 0.9401$, $p<0.0001$ and by Deming Regression; $y = 0.9978x + 0.24$). The average HbA1c (7.52±1.40 %) measured by HPLC was higher than the other methods (TINIA: 7.12±1.66 % and PEITT: 7.26±1.39 %, $p<0.0001$). The measured total time spent on 240 samples was 81 min. for TINIA, 54min. for PEITT and 540 min. for HPLC. It has been found that, the PEITT method, which is reliable, faster, and easier to perform, can be used as an alternative to TINIA and HPLC measuring system within the known imprecision limits" [12]. Earlier we had performed a study to evaluate the performance of ion exchange HPLC method using Bio-Rad D10 analyzer and compared with IT method of Beckman Coulter (BC) for the measurement of HbA1c in human blood. 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[19]. 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 %. Our

present study shows that the results between CEP &HPLC agrees as per correlation coefficient of 0.99,whereas the results obtained for IT method does not show good correlation with both CEP & HPLC methods. Good precision and accuracy were obtained for both CEP and HPLC methods, but not for IT method.

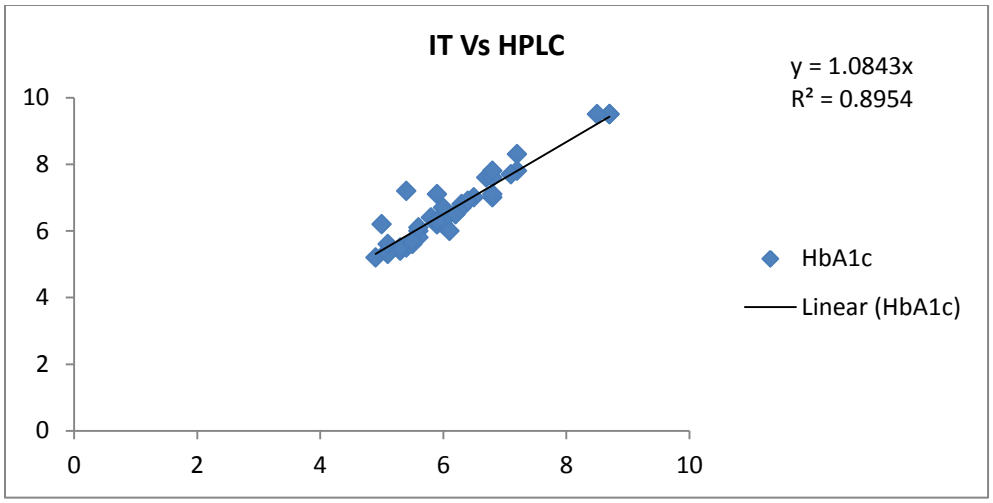

Figure 1: Correlation between IT and HPLC Methods for All Patients (N=40)

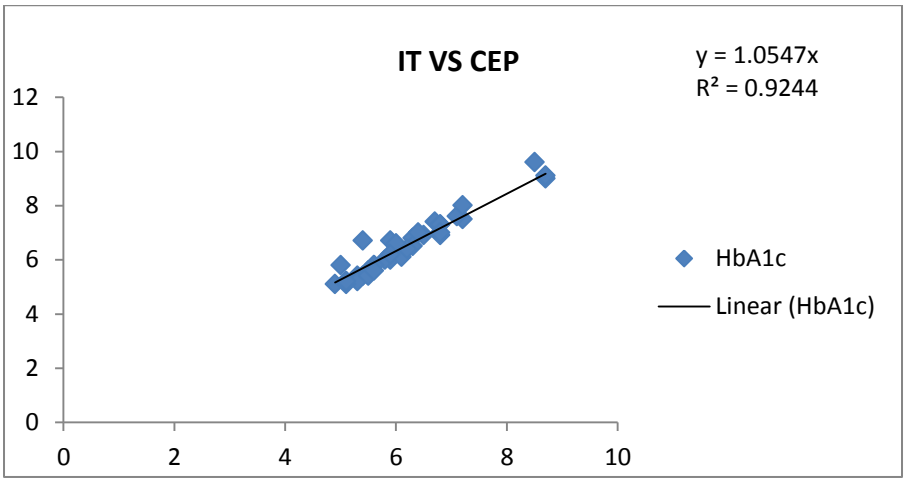

Figure 2: Correlation between IT and CEP Method for All Patietns (N=40)

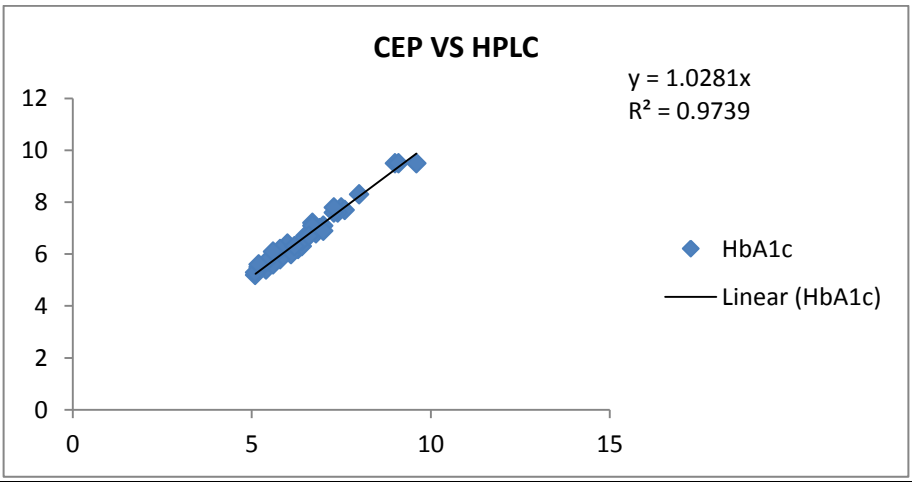

Figure 3: Correlation between CEP and HPLC Method for All Patients (N=40)

Table 1: Mean and SD for HBA1C (All Three Methods)

		IT	CEP	HPLC
All patients n=40	MEAN	6.16	6.49	6.67
	SD	0.95	1.11	1.14
Male patients n=25	MEAN	6.45	6.73	6.93
	SD	0.71	0.96	0.98
Female Patients n=15	MEAN	6.1	6.4	6.6
	SD	1	1.1	1.2

Table 2: Accuracy and Precision for Bio-Rad Controls(All Three Methods)

	Biorad Level- 1		Biorad Level – 2		Pooled serum
	Accuracy (%bias)	Precision (%CV)	Accuracy (%bias)	Precision (%CV)	Precision (%CV)
IT	-3.7	0.95	-2.8	0.86	1.36
CEP	2	0.79	2.6	0	3.48
HPLC	0.85	0.96	1	0.43	1.08

Table 3: Correlations between IT, CEP and HPLC Methods

Methods	R	Y	R2
IT VS CEP	0.96	1.0547	0.9244
IT VS HPLC	0.95	1.0843	0.8954
CEP VS HPLC	0.99	1.0281	0.9739

CONCLUSION

As per the present study, good correlation was obtained between CEP & HPLC methods (r=0.99) and the % bias obtained for the two levels of Biorad accuracy controls were also good with a mean % bias of + 2.3 and + 0.9 for CEP & HPLC methods, while it was -3.25 for IT method. Further, the precision obtained for the pooled serum also was good for both CEP & HPLC but not for IT method.

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