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# Comparison of Cation Exchange HPLC And Enzymatic Fructosyl Peptide Oxidase (FPOX) Method for Estimation of HBALC

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Abstract: Objective: The objective was to compare and correlate HbA1c values obtained from Tosoh TLC-723 G8 based on cation exchange based HPLC and TBA 12 FR enzymatic fructosyl peptide oxidase (FPOX) method.

Subjects and Methods: The HbA1c values of 610 randomly selected patient samples (310 Female, 300 Male) were estimated by using Tosoh TLC-723 G8 analyser based on cation exchange HPLC and enzymatic fructosyl peptide oxidase (FPOX) (Manufacture by Seikisui medical co. ltd., Japan) programmed on TBA 12 FR biochemistry analyser. The statistical significance was evaluated by student's t test (pair samples) using SPSS software. The Pearson correlation (r) was used for determining strength of linear association between HbA1c measurements by two methods

Results: Intra and Inter assay Coefficient of variance was found to be lower in HPLC method compared to enzymatic FPOX method. The correlation between both methods was good with correlation coefficient (r) of 0.95 and coefficient of determination  $(R^2)$  of 0.9.

Conclusion: Although correlation was found excellent between both methods difference of HbA1c value of few patient was found >1% which may mislead treatment and monitoring of diabetes. The HPLC method proved excellent in terms of faster assay timing and precision of results whereas enzymatic FPOX method with showed excellent correlation with HPLC, cost effectivity, and estimated HbA1c values in presence of hemoglobinopathies.

# I. INTRODUCTION

Since ancient ages, Diabetes mellitus has shown its presence worldwide by various and diverse terminologies. In India, Diabetes mellitus known as "Madhumeha" as early as 2500 BC. Diabetes mellitus is a chronic metabolic disorder marked by hyperglycemia with alteration in normal status of carbohydrate, protein and fat metabolism resulting from defects in insulin secretion, insulin action or both. Diabetes mellitus is associated with 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 disorder, sexual dysfunction and other lethal complications of organ system. (Sarmah et al. 2012) The worldwide prevalence of diabetes in 2000 was approximately 2.8% and is estimated to grow to 4.4% by 2030 which means a projected rise of diabetes from 171 million in 2000 to well over 350 million in 2030. Diabetes prevalence accounts 90-95% of cases in developed countries. By2020, India will be the world capital for diabetes. (Sultanpur et al. 2010)

The results of clinical trials by Diabetes Control and Complication Trials (DCCT) and Nation Glycohemoglobin Standardisation Programme (NGSP) showed that diagnosis and prognosis of diabetes and complication related to it can be control by monitoring accurate and precise glycemic status of patients. Both trials are using International Federation of Clinical Chemistry (IFCC) guidelines for HbA1c estimation. Conventionally, fasting plasma glucose (FPG), 2 hr value in the 75-g oral glucose tolerance test (OGTT), random plasma glucose (RPG) was amongst the battery of test recommended. (WHO report) But these tests have associated limitations which include uncertainty of fasting state, poor reproducibility of 2 h glucose, poor concordance between the fasting plasma glucose and 2 h plasma glucose, and a few days or weeks of change of lifestyle including dieting or increased exercise significantly affect both FPG and OGTT. As compared with both the tests, the use of HbA1c as diagnostic test has advantages including convenience as fasting is not needed for HbA1c assessment, less day-to-day variability, and greater pre-analytical stability with international standardization not inferior to glucose assay. (Rajput et al. 2013) It was year 2009, when an International Expert Committee that included representatives of the American Diabetes Association (ADA), the International Diabetes Federation (IDF), and the European Association for the Study of Diabetes (EASD) recommended the use of the A1C test to diagnose diabetes, with a threshold of  $\geq 6.5\%$  (48 mmol/mol), and ADA adopted this criterion in 2010. The clinical significance



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of HbA1c was supported by Diabetes Control and Complications Trial (DCCT) and the United Kingdom Prospective Diabetes Study (UKPDS). (Sarmah et al. 2012)

The measurement of blood glucose is of limited value for the long term assessment of glycemic control. For the long term assessment of glycaemic status measurement of glycated hemoglobin or HbA1c is now routinely and widely used in clinical practice. HbA1c is a reaction product of glucose and the N terminal value of the beta chain of haemoglobin. The reaction is irreversible and enzyme independent. (Farzana et al) Glucose forms an aldimine linkage (liable fraction) with NH2- of value in the  $\beta$ - chain, undergoing an Amadori rearrangement to form the more stable ketoamine linkage. This process known as glycation of hemoglobin or Amadori shift occurs in a span of 120 days, which is the life span of a normal Red Blood Corpuscles (RBC). The process of glycation depends on the average glucose load during the period of RBC life span, and so the role of HbA1c was rightly accepted for monitoring of plasma glucose concentration over prolonged periods of time. (Bunn et al. 1975)

Diabetes management by measuring HbA1c values is recommended by American Diabetic Association (ADA), American Diabetic Federation (ADF), European Association for the Study of Diabetes (EASD), International Federation of Clinical Chemistry (IFCC) and Singapore MOH Clinical Practice Guidelines. HbA1c 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. (Farzana et al)

At present, more than 20 different assay methods are being used to measure the level of the HbA1c in clinical laboratories. These methods are based on different physical, chemical or immunological characteristic of the Glycated hemoglobin and different analytical principles, such as immuneturbidimetry, cation exchange chromatography, and high-performance liquid chromatography (HPLC). Among all methods, measurement of HbA1c by HPLC became gold standard and has been appointed as the reference method for HbA1c assay by the National Glycohemoglobin Standardization Program (NGSP) in USA. (Sema et.al, 2012).

HbA1c assays are now routinely used in laboratories for patient management, monitoring, education and for patient motivation to control diabetes and related complications. It became necessary to measure HbA1c values accurate and precise, but different methods yielded inconsistent results. Lack of assay standardization, interference by Schiff base and the problems related to its measurement in patients with hemoglobinpathies, fetal haemoglobin, renal failure and haemolytic disease are among the major analytical issues associated with glycated hemoglobin measurement. Also, drugs with strong ionic charges like aspirin may alter HbA1c results. (Farzana et al)

Difference in values obtained for same patient by different method may create confusion and mislead the entire treatment regime, as even 1% of variation in HbA1c values can categorise patient as diabetic or normal. So it is necessary to compare results of various methods used for HbA1c measurement. In this study we correlated, compared and evaluate practical suitability of HbA1c values obtained by using Tosoh TLC-723 G8 analyser based on cation exchange based HPLC and enzymatic fructosyl peptide oxidase (FPOX) (Manufacture by Seikisui medical co. ltd., Japan) method programmed on TBA 12 FR biochemistry analyser.

# II. MATERIALS AND METHODS

This study was performed at Pyramid Grouplab (PGL), New Panvel, Maharashtra. 610 patients were randomly selected from daily work load at PGL, which came as suspected case of Diabetes (1 or 2) and for routine metabolic management of Diabetes. Patients from all age group from 1year to 86 years were included in study.

Venous blood specimens were collected in vacutainer containing EDTA (Ethylene Diamine Tetra acetic Acid) as an anticoagulant (BD vacutainer K2 EDTA 5.4 mg). These specimens are stable for fourteen days stored at 2-8°C or up to eight hours at room temperature before analysis. After collection and prior to analysis, these specimens were mixed gently by inversion to ensure homogeneity. The specimens from different parts of country were collected and packed in specially designed, pre bar-coded specimen transport box containing cool pack for temperature control. After preliminary checks sample information entered in Laboratory Management Software, samples were bar-coded and forward to different section of laboratory. In our study, we have simultaneously processed sample for HbA1c values by Tosoh TLC-723 G8 analyser based on cation exchange based HPLC and enzymatic fructosyl peptide oxidase (FPOX) (Manufacture by Seikisui medical co. ltd., Japan) method programmed on TBA 12 FR biochemistry analyser.

## III. TECHNIQUES

Tosoh TLC-723 G8 HPLC reagent kit and enzymatic fructosyl peptide oxidase (FPOX) kit contains calibrator 1 and 2 and control 1 and 2 provided by manufacturer. Before starting assay both instrument were calibrated with calibrators and controls provided by manufacturer for quality control as per instructions. After successful calibration, samples were processed on Tosoh automated



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Glycohemoglobin analyser HLC-723 G8. The BD vacutainer containing EDTA whole blood is directly processed by inserting sample tube in sample rack and push the start button. The optimum sample volume was 2mL and minimum was 50 µL.

The analyzer dilutes the whole blood specimen (3 uL) with Hemolysis & Wash Solution, and then injects a small volume of this specimen onto the TSKgel G8 Variant HSi column. Separation is achieved by utilizing differences in ionic interactions between the cation exchange group on the column resin surface and the hemoglobin components. The hemoglobin fractions (designated as A1a, A1b, F, LA1c+, SA1c, A0, and H-V0, H-V1, HV2) are subsequently removed from the column by performing a step-wise elution using the different salt concentrations in the Variant Elution Buffers HSi 1, 2, and 3. The separated hemoglobin components pass through the LED photometer flow cell where the analyzer measures changes in absorbance at 415 nm. The run time is approximately 1.6 minutes per sample. A sample report and a chromatogram are generated for each sample. For samples, having less quantity as well as samples showing "area high" flag was diluted (e.g. 1:100, 1:200 as per requirement) manually with G8 variant Dilution solution for further analysis. The values are obtained as SA1c (Stable A1c) in % and were recorded on software as well as printout generated by instrument on thermal paper.

Once samples are processed on Tosoh automated Glycohemoglobin analyser HLC-723 G8 same samples are further processed for HbA1c detection by Enzymatic FPOX method from Norudia N HbA1c reagents (Manufacture by Seikisui medical co. ltd., Japan) on fully automated TBA 120 FR instrument from Toshiba, Japan. EDTA whole blood sample was centrifuged for 5 minutes at 800g and after that from lower layer of blood cells,  $25 \,\mu$ L of Red blood cells are taken in 1.5mL vial. 500  $\mu$ L of Norudia N HbA1c pre-treatment solution is added in the vial and mixed well. Pre-diluted calibrators, controls and pre-barcoded samples were loaded on TBA 120 FR analyser sequentially. From pre-treated samples,  $12\mu$ L sample was aspirated by instrument, mixed with 180  $\mu$ L of Reagent 1 of Norudia N HbA1c kit, and incubated at 370C for 5 min before taking absorbance. After measurement of absorbance Reagent 2 of Norudia N HbA1c kit were mixed and incubated at incubated at  $37^{0}$ C for 5 min and absorbance was measured. The absorbance ranges from 480nm to 800nm were for difference calculation. Instrument estimated HbA1c value (in  $\mu$ mol/L), Hemoglobin (Hb) value (in  $\mu$ mol/L) and estimated average glucose (in mmol/L and mg/dL) which was displayed on software and printed on normal dot matrix printer paper. Estimated HbA1c values in  $\mu$ mol/L were converted HbA1c values in % by formula: HbA1c (MGSP %) = 98.2 x HbA1c ( $\mu$ mol/L)/Hb ( $\mu$ mol/L) + 1.97. The HbA1c results are expressed in % as well as  $\mu$ mol/L as per IFCC/NGSP guidelines. The results are recorded by TBA 120 FR software and printed on paper by dot matrix printer connected to instrument. In both the analytical technologies, erroneous results were repeated and if error remains consistent then patient history and fresh sample was requested.

The HbA1c values obtained from both the techniques were converted in to estimated average glucose (eAG) (in mmol/L and mg/dL) by Nanthan's linear regression equation;  $eAG(mg/dl)=(28.7 \times HbA1c) - 46.7$  as per recommendation from NGSP. Conversion factor is installed in the analyser by the manufacturer in Tosoh HLC-723 G8 analyser and manually programmed in TBA 120 FR biochemistry analyser.

#### **IV. CONTROL MATERIAL**

The intra and inter assay precision of both methods was determined with commercially available control blood of low and high HbA1c concentrations by repeated analysis. Lyphocheck Diabetics Control Level 1 and Level 2 from Bio Rad Laboratories were used as external controls.

## IV. DATA ANALYSIS

The generated data was analysed by using standard SPSS software for statistical analysis and expressed as mean ( $\pm$  SD). The statistical significance was evaluated by student's t test (pair samples) using SPSS software. It was considered that 95% of all values lying within  $\pm 2$  SD indicate good agreement. The Pearson correlation (r) was used for determining strength of linear association between HbA1c measurements by two methods.

## V. RESULTS

610 samples were analysed for HbA1c estimation, out of which 31 samples (15female, 16male) has not given any value by HPLC method. These samples were sent for further analysis by other methods. In this study, for further statistical analysis these 31 samples were excluded (i.e., out 610 samples 579 samples subjected to statistical analysis). The mean age of patients included in the study was  $42.97 \pm 19.3$  years (range 1 - 86). There were 295 (51%) females and 284 (49%) males.

In this study, calibration was done daily before assessing samples and controls. After calibration validity of calibration was checked by running control level 1 and level 2 for both methods. On the successful validation of calibration, samples were processed for



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HbA1c estimation. The inter and intra assay precision with calculated by repeating Lyphocheck Diabetics Control (Bio Rad) Level 1 and Level 2, 10 times in a run (for intra assay precision) and in duplicate in 10 run in 10 days (for inter assay precision) by both the techniques. This control considered to be external assay control and values are referred from data sheet given by manufacturer.

Level 1		Level 2	
ean	Range	Mean	Range
4.83	3.86 - 5.79	9.36	7.49 - 11.2
5.20	4.70 - 5.70	9.90	8.90 - 10.80
	e <b>an</b> 1.83 5.20	an Range   4.83 3.86 - 5.79   5.20 4.70 - 5.70	anRangeMean4.833.86 - 5.799.365.204.70 - 5.709.90

(Note: All values are expressed in "%")

(Table 1: Lyphocheck Diabetes Control 1 & 2 values with mean and range) (Source: Value sheet lot no. 33860 Bio Rad Laboratories)

	Intra Ass	ay	Inter Assay		
Control Level	Mean (± SD)	<b>CV</b> %	Mean (± SD)	<b>CV</b> %	
SEIKISUI NORUDIA HbA1c (NGSP) Level 1	5.16 ± 0.143	2.8	5.24 ± 0.184	3.5	
SEIKISUI NORUDIA HbA1c (NGSP) Level 2	9.93 ± 0.316	3.2	9.99 ± 0.256	2.6	
TOSOH HLC-723 G8 (NGSP) Level 1	5.45 ± 0.053	1.0	5.45 ± 0.071	1.3	
TOSOH HLC-723 G8 (NGSP) Level 1	9.72 ± 0.042	0.4	9.71 ± 0.057	0.6	

(Table 1 : Mean ± SD and between run CVs for HbA1c values determined by Tosoh HLC-723 G8 HPLC and Seikisui Norudia HbA1c enzymatic FPOX method.)

In present study we found an intra assay CV of 2.8% (FPOX) and 1.0% (HPLC) for Lyphocheck Diabetes Control level 1 and CV of 3.4% (FPOX) and 0.4% (HPLC) for Lyphocheck Diabetes Control level 2. Whereas, an inter assay CV of 3.5% (FPOX) and 1.3% (HPLC) for Lyphocheck Diabetes Control level 1 and CV of 2.6% (FPOX) and 0.6% (HPLC) for Lyphocheck Diabetes Control level 2. However, value observed (Table 2) has given closer values to expected values (Table 1) and CV % very low by both methods, the standard deviation (SD) of FPOX is slightly higher than HPLC method due to variation in observed values. The correlation analysis of HbA1c values obtained from Tosoh TLC-723 G8 based on cation exchange based HPLC and TBA 12

FR enzymatic fructosyl peptide oxidase (FPOX) method was done. The HbA1c values obtained from HPLC and TBA 12 FR enzymatic fructosyl peptide oxidase (FPOX) method was done. The HbA1c values obtained from HPLC method are plotted on the x-axis and FPOX are plotted on the y-axis. The result showed a good positive correlation between both the methods tested. A significant value of p<0.005 was obtained, indicative of significant difference. The correlation coefficient (r) is 0.95 and the coefficient of determination (r<sup>2</sup>) is 0.9.









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## VI. DISCUSSION

Both methods have been compared in terms of precision and their overall correlation was also assessed with the help of correlation analysis. It is recommended that HbA1c methods used should fulfil the standards of NGSP and DCCT as reference by keeping CV % less than 5%. (Sacks et al. 2002) This criterion has been successfully achieved by both methods used in this study where HPLC has shown lowest CV%. The lower or improved CV% helpful in HbA1c standardization, and can be used to detect significant trends or shifts in a diabetic patient's blood glucose control. The precise HbA1c measurement is important in diabetes diagnosis and prognosis. However, when same samples were processed on different methods, tend to yield results with difference in values. Inspite of significant data, we have also observed that 75 samples (13% of sample analysed) have difference in HbA1c values more than 1%. As per WHO study published in 2011 there are different percentages of HbA1c value are involved in different complications related to diabetes. People with diabetes who reduced their HbA1c by less than 1% can cut their risk of dying within 5 years by 50%, according to Swedish research presented at the annual meeting of the European Association for the Study of Diabetes, Sept. 2012 (EASD). This clearly indicates that even a single percentage of difference in normal or abnormal HbA1c may mislead treatment and management of diabetes patient. It is strongly recommended that results should be correlated with clinical history of patient.

In view of this, it is necessary to compare the results of various methods used by different laboratories. In this study HbA1c values obtained by both methods were compared and correlated to find out method with ease in operation and excellent in precision. Tosoh HLC-723 G8 is fully automated Glycohemoglobin analyser based on cation exchange HPLC with unique non-porous column system. Whereas, Norudia N HbA1c kit (Manufacture by Seikisui medical co. ltd., Japan) based on FPOX method can be easily programmed on automated or semi automated biochemistry analyser (In this study we used TBA 120 FR automated biochemistry analyser, from Toshiba, Japan). Both the methods are NGSP certified.

Tosoh HLC-723 G8 HPLC system participated in National Glycohaemoglobin Standardisation Program and is traceable to the DCCT reference method. Norudia N HbA1c by FPOX method is standardised via IFCC reference system and the values obtained from this method shown good correlation and significant difference with values obtained from HPLC system. As per guidelines for diabetes diagnosis given by international organisation like WHO, NGSP, ADA, etc. that the methods for measurement of HbA1c should be standardised according to NGSP and method should be traceable to DCCT method. This criterion is matched by Tosoh HLC-723 G8 HPLC method.

The presence of unstable hemoglobin variant may shorten the life span for , this may interfere with the HbA1c measurement. Tosoh HLC-723 G8 HPLC method recognises these hemoglobin variant and mark them as separate peak (i.e., H-V0, H-V1, H-V2) on chromatogram, but due to shorten life span of RBC HbA1c values may not reflected or reflected values are non-reliable. FPOX method lacks this feature, but it reflects value of HbA1c in presence of hemoglobin variants. In this study, 31 samples have not given any value on HPLC but have shown values on FPOX method. So FPOX method may be useful for samples with hemoglobin variants.

Norudia N HbA1c by FPOX method requires a pre-treatment of every sample before analysis for HbA1c values while Tosoh HLC-723 G8 HPLC method requires no pre-treatment or manual handling of the sample. EDTA whole blood vacutainer tubes can be directly introduced in analyser with sample loader for 90 samples or 270 samples and assay timing is just 1.6 min per sample. For high throughput Tosoh HLC-723 G8 HPLC method is strongly recommended due to this feature. Although, Norudia N HbA1c by FPOX method can be programmed on fully automated biochemistry analyser, the pre-treatment of each and every sample still remains the manual procedure which adds more time for final result assessment. Norudia N HbA1c by FPOX method require 25 µL of sample while Tosoh HLC-723 G8 HPLC method even a 5 µL sample can be manually diluted to 1:200 by hemolysis wash and dilution solution and can be used of analysis. The overall cost per test of Tosoh HLC-723 G8 HPLC method is higher than Norudia N HbA1c by FPOX method. Because Tosoh HLC-723 G8 HPLC method is analyser for analysis of Glycohemoglobin and hemoglobin variants and Norudia N HbA1c by FPOX method can be easily done manually or can be easily programmed on semi automated or automated biochemistry analysers.

In view of above all points we can conclude that there is a need for an extremely accurate, precise and practical method that is suitable for routine use in the clinical chemistry laboratory. This study indicates Tosoh HLC-723 G8 HPLC method match the criteria. Still current study is endeavour towards assessing this criteria by analysis two commonly used methods with smaller sample load i.e., 610 patient samples. We recommend that similar or improvised study has to be done on the larger scale with large samples load from different populations in India as well as across the world. Although correlation was found excellent between both methods difference of HbA1c value of few patient was found >1% which may mislead treatment and monitoring of diabetes. The HPLC method proved excellent in terms of faster assay timing (1.6mins per sample) and precision of results whereas enzymatic



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