

## Comparative study of glycated hemoglobin by ion exchange chromatography and affinity binding nycocard reader in type 2 diabetes mellitus

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### ABSTRACT:

The aim of this study was to compare the level of glycated hemoglobin (HbA1c) in type 2 Diabetes Mellitus (DM) patients by two different methods namely Ion Exchange Chromatography and Affinity Binding Nycocard Reader. This is a cross-sectional study conducted on confirmed type 2 diabetes mellitus patients (n=100) who visited Out Patients Department of the Universal College of Medical Sciences Teaching hospital, Bhairahawa, Nepal from November 2012 to March 2013. The diagnosis of diabetes mellitus was done on the basis of their fasting (164.46±45.33 mg/dl) and random (187.93±78.02 mg/dl) serum glucose level along with clinical history highly suggestive of type 2 DM. The HbA1c values of (7.8±1.9 %) and (8.0±2.2 %) were found in DM patients as estimated by those two different methods respectively. The highest frequency was observed in HbA1c > 8.0% indicating maximum cases were under very poor glycemic control. However, there were no significant differences observed in HbA1c value showing both methods are comparable in nature and can be used in lab for ease of estimation. The significant raised in HbA1c indicates complications associated with DM and monitoring of therapy become hard for those patients. Despite having standard reference method for HbA1c determination, the availability of report at the time of the patient visit can be made easy by using Nycocard Reader and Ion Exchange Chromatography techniques without any delay in communicating glycemic control, clinical decision-making and changes in treatment regimen.

**Keywords:** HbA1c, Ion exchange chromatography, Nycocard reader, Type 2 Diabetes

### INTRODUCTION

The prevalence of Type 2 Diabetes mellitus (T2DM) is increasing at alarming rates both in the developing and the newly industrialized countries of the world.<sup>1</sup> It is highly prevalent in the elderly and associated with various co-morbidities, such as obesity, hypertension, hyperlipidemia and cardiovascular disease. The glycated hemoglobin (HbA1c) is non-enzymatic condensation of glucose to the valine residue of  $\beta$ -hemoglobin to form aldime and then by amadori rearrangement to ketoamine. It has provided clinicians worldwide with a means whereby average blood glucose values can be quantified over the previous 8-10 weeks. The true need for the international standardization of HbA1c measurements became an important objective for scientists and clinicians worldwide only after the results of the Diabetes Control and Complications Trial (DCCT) were published in 1993.<sup>2</sup> Although high performance liquid chromatography is the gold standard method, the disadvantage of it is that results are not available at the time of patient visit, delay in reporting time, communicating health feedback, delay in clinical decision making, changes in the regimen prescription may be missed. There are many commercial

methods available for the routine measurement of HbA1c based on different analytical principles, such as immunoassays, ion-exchange chromatography and affinity chromatography.<sup>3</sup> The choice of diagnostic method will depend on local considerations such as cost, availability of equipment, population characteristics, presence of a national quality assurance system etc.<sup>4</sup>

The aim of this study was to compare the level of Glycated Hemoglobin (HbA1c) in T2DM patients by two different methods viz a viz Ion Exchange Chromatography and Affinity Binding Nycocard Reader. We have designed Diabetes patients into different categories like very good glycemic control, good glycemic control, poor glycemic control and very poor glycemic control to assess association between the HbA1c value with Ion Exchange Chromatography and Affinity Binding Nycocard Reader.

### MATERIALS AND METHODS

This is a comparative, cross-sectional study carried out in Type 2 diabetes mellitus patients (n=100), visiting Universal College of Medical Sciences Teaching hospital (UCMSTH), Bhairahawa, south-western, Nepal, who are confirmed by Fasting Blood Glucose (FBG) based on World Health Organization (WHO) criteria during

period of November 2012 to March 2013. Fasting and Random blood Glucose concentration were determined for the patients on same day. The EDTA- blood samples, stored at 4°C were assayed for HbA1c by using standard protocols by following methods.

### ION EXCHANGE CHROMATOGRAPHY

**Principle:** Whole blood is mixed with lysing reagent containing a detergent and borate ions. Elimination of the labile Schiff's base is thus achieved during the hemolysis. The hemolysate is then mixed for 5 minutes with a weakly binding cation exchange resin. During this time, HbA0 binds to the resin. A special resin separator is used to remove the resin from the supernatant fluid which contains the HbA1. The glycohemoglobin percentage of total hemoglobin is determined by measuring the absorbance of the glycohemoglobin and of the total hemoglobin fraction at 405 nm in ELISA reader (Erba LISA scan II) in comparison with a standard glycohemoglobin preparation carried through the test procedure.

**Procedure:** 500 µl Lyse solution was added into cups and 100 µl Sample, Standard & Control were added into separate cups. The solution was mixed and incubated for 5 minutes at 15-25°C. The solution was then pipetted into labeled Reagent (RGT) i.e., Ion Exchange Resin pre-filled in plastic tubes in Imidazole Borate buffer and Separator (SEP) was inserted so that rubber sleeve is 1 cm above surface of resin suspension. The mixture was than mixed in hematology mixer for 5 minutes. SEP was pushed down the resin so that to it firmly packed. The solution was taken of the SEP to microtitre well and measure in ELISA plate reader at 405 nm.

Total Hemoglobin estimation was done by taking 20 µl hemolysate from lyse sample. 5 ml of distil water was added and mixed. The measurement was done in ELISA plate reader at 405 nm and calculation is done by using following formula:

$$\% \text{HbA1c} = \frac{\text{OD of Total Hb (std)} \times \text{HbA1c (std)}}{\text{OD of HbA1c (std)}} \times \frac{\text{OD HbA1c (sample)}}{\text{OD Total Hb (sample)}}$$

### AFFINITY BINDING NYCOCARD READER

**Principle:** HbA1c is a boronate affinity assay. The kit contains test devices with a porous membrane filter, test tubes pre-filled with reagent and washing solution. The reagent contains agents that lyse erythrocytes and precipitate hemoglobin specifically, as well as a blue boronic acid conjugate that binds cis-diols of glycated hemoglobin. When the blood is added to the reagent, the erythrocytes immediately lyses. All hemoglobin precipitate the boronic acid conjugate binds to the cis-

diol configuration of glycated hemoglobin. An aliquot of the reaction mixture is added to the rest device, and all the precipitate hemoglobin, conjugate bound and unbound, remains on top of the filters. Any excess of colored conjugate is removed with washing solution. The precipitate is evaluated by measuring the blue (glycated hemoglobin) and the red (total hemoglobin), color intensity with the NycoCard reader II, the ratio between them being proportional to the percentage of HbA1c in the sample.

**Procedures:** 5µL of whole blood was added to the cup with reagent 1 mixed well and incubated for 3 minutes. It was remixed to obtain a homogenous suspension. Reaction mixtures (25 µL) were applied to a test device by holding the pipette approximately 0.5 cm above the test. The pipette was emptied quickly in the middle of the test. The reaction mixture was allowed to soak completely into the membrane for 10 seconds. Washing solution (25 µL) was added to the test device. The washing solution was allowed to soak completely into the membrane for 10 seconds. The test result was read within 5 minutes using NycoCard reader II.

### STATISTICAL ANALYSIS

Data were processed with the statistical software; SPSS version 16 and diagram were represented by Prism 6. The mean and SD were presented as continuous variables, and categories were expressed as percentages. For comparison of the mean values, the one way ANOVA was used, and for that of categories, the paired t-test and Spearman correlation were selected. Regression analysis of individual values obtained from the two measuring methods was performed.

### RESULTS

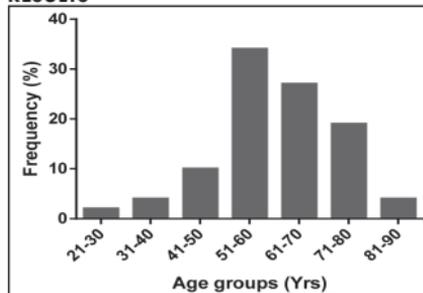


Fig.1: Frequency (%) among different age groups of Type 2 DM patients (n=100)

The figure 1 shows the frequency of patients categorized into different age groups where maximum percentage of patients affected with T2DM fall under age group

**Table 1:** Age-wise distribution of basic characteristics in Type 2 DM patients (n=100) results are expressed in (Mean  $\pm$  SD)

Age-groups	Age (years)	Ht (m)	Wt (Kg)	BMI (Kg/sq.m.)	BP(Systolic) (mm/Hg)	BP(Diastolic) (mm Hg)
21-30	26.5 $\pm$ 2.12	1.69 $\pm$ 0.02	54.5 $\pm$ 6.36	19.08 $\pm$ 2.71	145.0 $\pm$ 21.21	90.0 $\pm$ 14.14
31-40	37.0 $\pm$ 3.46	1.63 $\pm$ 0.03	60.75 $\pm$ 3.77	22.83 $\pm$ 0.84	147.5 $\pm$ 15.0	97.5 $\pm$ 17.08
41-50	46.6 $\pm$ 3.34	1.62 $\pm$ 0.06	62.7 $\pm$ 9.79	24.18 $\pm$ 4.55	145.5 $\pm$ 19.50	103.5 $\pm$ 19.44
51-60	55.68 $\pm$ 3.54	1.63 $\pm$ 0.05	61.35 $\pm$ 8.64	23.34 $\pm$ 4.28	144.71 $\pm$ 12.61	97.06 $\pm$ 13.38
61-70	66.67 $\pm$ 2.94	1.63 $\pm$ 0.03	61.59 $\pm$ 6.25	23.25 $\pm$ 2.3	154.44 $\pm$ 20.06	105.56 $\pm$ 15.28
71-80	75.08 $\pm$ 3.88	1.65 $\pm$ 0.06	63.74 $\pm$ 4.56	23.45 $\pm$ 2.10	152.11 $\pm$ 24.40	101.58 $\pm$ 14.25
81-90	75.05 $\pm$ 3.88	1.65 $\pm$ 0.06	63.74 $\pm$ 4.56	23.45 $\pm$ 2.1	137.5 $\pm$ 15.0	91.25 $\pm$ 2.5
<b>Total</b>	<b>60.13<math>\pm</math>13.26</b>	<b>1.66<math>\pm</math>0.08</b>	<b>65.5<math>\pm</math>6.66</b>	<b>23.71<math>\pm</math>1.09</b>	<b>147.55<math>\pm</math>16.96</b>	<b>100.20<math>\pm</math>14.23</b>

51-60 years which was 33.0% followed by 61-70 years whose frequency was found to be 26%.

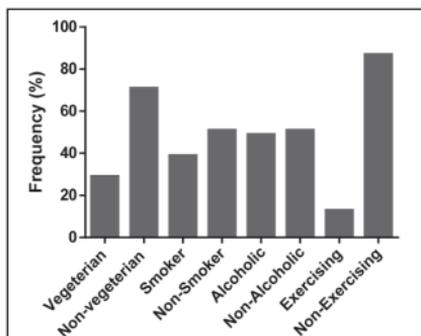
**Fig. 2:** Frequency (%) of General characteristics of Type 2 DM patients (n=100)

Figure 2 shows the frequency of general characteristics of T2DM patients. According to Diet 72% were Non-vegetarian and 28% were Vegetarian, 68% Non smoker and 32% Smoker, 51% were Non-alcoholic and 49% alcoholic, 86% cases were Non-exercising and 14% were exercising.

Table 1 shows the distribution of age-wise distribution of basic characteristics of T2DM patients. The mean age ( $\pm$  SD) of study population was 60.13 $\pm$ 13.26 years, the mean Body Mass Index (BMI) calculated from mean weight (63.74 $\pm$ 4.56 kg) and mean height (1.65 $\pm$ 0.06 m) was (23.45 $\pm$ 2.1 Kg/m<sup>2</sup>), the mean systolic blood pressure was 137.5 $\pm$ 15.0 mm Hg and diastolic blood pressure was 91.25 $\pm$ 2.5 mm Hg respectively. The maximum BMI (24.18 $\pm$ 4.55 kg/sq.m.) was seen in the age group 41-50 years, BP systolic (154.44 $\pm$ 20.06 mm Hg) and BP diastolic (105.56 $\pm$ 15.28 mm Hg) were seen in age group 61-70 years.

Table 2 shows the age-wise distribution of blood sugar and HbA1c in T2DM. The mean ( $\pm$ SD) fasting blood sugar and random blood sugar were 164.46 $\pm$ 45.33 mg/dl and 187.93 $\pm$ 78.02 mg/dl respectively. The mean ( $\pm$ SD) of HbA1c by Ion Exchange Chromatography was 7.8 $\pm$ 1.9 % and by Nycocard Reader was 8.0 $\pm$ 2.2 %. The maximum FBS and RBS were seen in age group 41-50 years and found to be 156.33 $\pm$ 46.29 mg/dl and 215.0 $\pm$ 113.14 mg/dl respectively. Moreover, the maximum HbA1c % was found in the age group 51-60 years whose value by Ion Exchange Chromatography and Nycocard Reader were found to be 8.2 $\pm$ 2.3 % and 8.6 $\pm$ 2.4 % respectively.

**Table 2:** Age-wise distributions of FBS, RBS and HbA1c in Type 2 DM patients (n=100) results are expressed in (Mean  $\pm$  SD)

Age-groups	FBS (mg/dl)	RBS (mg/dl)	HbA1c Ion Ex (%)	HbA1c Nycocard (%)
21-30	145.22 $\pm$ 21.34	167.33 $\pm$ 65.76	8.0 $\pm$ 0.3	6.3 $\pm$ 0.8
31-40	174.0 $\pm$ 14.14	173.0 $\pm$ 56.80	6.8 $\pm$ 1.6	7.1 $\pm$ 1.7
41-50	156.33 $\pm$ 46.29	215.0 $\pm$ 113.14	7.6 $\pm$ 1.9	7.2 $\pm$ 1.1
51-60	131.67 $\pm$ 2.52	194.33 $\pm$ 24.62	8.2 $\pm$ 2.3	8.6 $\pm$ 2.5
61-70	170.2 $\pm$ 37.76	196.0 $\pm$ 39.7	8.2 $\pm$ 2.1	8.2 $\pm$ 2.4
71-80	163 $\pm$ 25.46	155.2 $\pm$ 62.67	7.4 $\pm$ 1.6	7.2 $\pm$ 1.8
81-90	135 $\pm$ 10.11	177.0 $\pm$ 60.23	7.7 $\pm$ 1.2	8.2 $\pm$ 1.6
<b>Total</b>	<b>164.46<math>\pm</math>45.33</b>	<b>187.93<math>\pm</math>78.02</b>	<b>7.8<math>\pm</math>1.9</b>	<b>8.0 <math>\pm</math> 2.2</b>

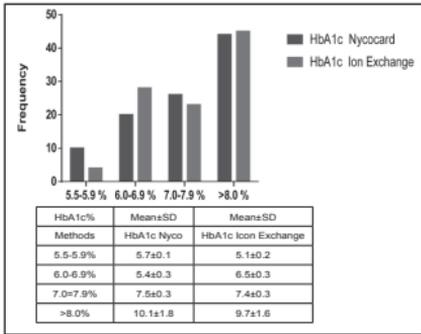


Figure 3: Frequency (%) of General characteristics of Type 2 DM patients (n=100)

Figure 3 shows the frequency and Mean (±SD) of HbA1c % by affinity binding Nycocard Reader and ion exchange chromatography. The highest frequency of T2DM was >8% (very poor glycemic control) by Nycocard Reader and Ion Exchange Chromatography whose mean (±SD) HbA1c % were 10.1±1.8 and 9.7±1.6 respectively. It is followed by HbA1c level 6.0-6.9% (good glycemic control) with 20% and 28% measured by Nycocard reader and Ion Exchange Chromatography whose mean (±SD) HbA1c % were 6.4±0.3 and 6.5±0.3 respectively. Then followed by HbA1c level 7.0-7.9% (poor glycemic control) with 26% and 23% measured by Nycocard reader and Ion Exchange Chromatography whose mean± SD HbA1c % were 7.5±0.3 and 7.4±0.3 respectively. Lastly followed by HbA1c level 5.5-5.9% (very good glycemic control) with 10% and 4.0% measured by Nycocard reader and Ion Exchange Chromatography whose mean± SD HbA1c % were 5.7±0.1 and 5.1±0.2 respectively. The statistically significant difference was observed between groups as compared to very good glycemic control ( $p < 0.0001$ ).

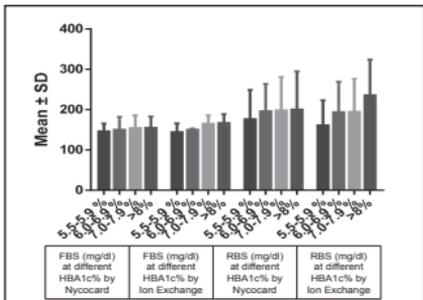


Figure 4: FBS & RBS (Mean±SD) Different level of HbA1c% by Affinity Binding Nycocard Reader & Ion Exchange Chromatography

Figure 4 shows mean ± SD of blood sugar level at different level of HbA1c% by two different methods. The maximum FBS level (155.0±28.27 mg/dl) and RBS (200.40±94.72 mg/dl) were seen in HbA1c % level >8 % followed by 7.0-7.9 %, 6.0-6.9 % and 5.5-5.9 % respectively.

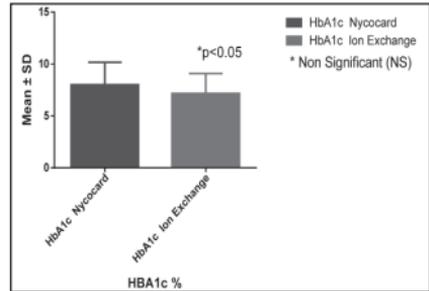


Figure 5: Comparison of HbAc % (Mean SD) by Nycocard Reader & Ion Exchange Chromatography

Figure 5 shows the comparison between mean± SD of HbA1c% by two methods. There was statistical non-significant difference of HbA1c% by Nycocard Reader and Ion Exchange Chromatography ( $p > 0.05$ )

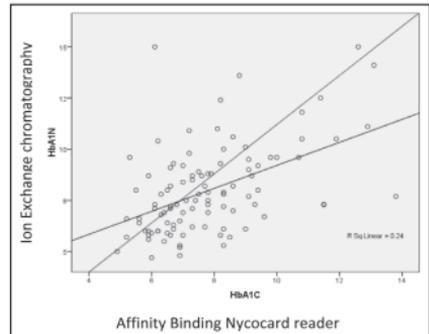


Figure 6: Regression analysis between Nycocard Reader (IFCC Standardized method) and Ion exchange chromatography

The Regression analysis between Nycocard Reader (IFCC Standardized method) and Ion exchange chromatography yielded a coefficient of determination  $r^2 = 0.24$  ( $P < 0.0001$ ) as shown in Figure 6. We also found positive correlation between HbA1c% Nycocard Reader and Ion Exchange chromatography ( $p < 0.0001$ ), between HbA1c% Nycocard Reader & FBS, RBS ( $p < 0.001$ ), between HbA1c% Ion Exchange Chromatography & FBS, RBS ( $p < 0.001$ ) shown in table 3.

**Table 3:** Correlation between Glucose concentration and HbA1c

Variables	HbA1c (Nyc)	HbA1c (Ion Exchange)
HbA1c (Nyc)	1	0.49**
HbA1c (Ion Exchange)	0.49**	1
FBS	0.16*	0.11*
RBS	0.23*	0.18*

\*\*p&lt;0.0001,\*p&lt;0.001

## DISCUSSION

The significant rise in HbA1c level in the present study indicates the complication associated with Diabetes mellitus and monitoring of therapy become harder for those patients. Moreover, there were significant rise in systolic and diastolic BP in study population indicating risk of hypertension and associated cardiovascular diseases. Hypertension is common in patients with type 2 diabetes, with prevalence rate of 40-60% over the age range of 45-75 years.<sup>5</sup> Hypertension multiplies the risk of cardiovascular and renal disease already present in diabetic patients. The major reasons for the increasing number of people with T2DM are population growth, aging, urbanization and increasing prevalence of obesity and physical inactivity. Long term complications of T2DM include retinopathy, nephropathy, peripheral and autonomic neuropathy and cardiovascular diseases cause huge medical and socioeconomic burden on the society and impose enormous strains on health care systems.<sup>6</sup> Medical burden of diabetic patients increases long before actual diagnosis for the disease is established.<sup>7</sup>

The maximum value of HbA1c was observed for age groups 51-60 years are  $8.6 \pm 2.5$  % and  $8.2 \pm 2.1$  % by Ion Exchange Chromatography and Affinity Binding Nycocard Reader respectively. Amongst the DM patients, the frequency was found to be highest for the HbA1c value >8.0% (very poor glycemic control) by these two methods. This indicates the maximum patients are at risk for developing complication related with Diabetes mellitus. This also reflects poor management of diabetic patients in this region because of ignorance, poverty, poor health education, unawareness about Diabetes control programme etc. A positive correlation between fasting glucose, random glucose and glycated hemoglobin in cases indicates the exposure of glucose correlate with the glycation which corroborate with the study done by Baral *et al*.<sup>8</sup>

Ion Exchange Chromatography HbA1c has lower isoelectric point and migrates faster than other Hemoglobin (Hb) components. The current ion exchange assays correct for HbF and carbamylated Hb and does not interfere by them. In a Boronate Affinity HbA1c,

glucose binds to m-aminophenylboronic acid and has minimal interference from haemoglobinopathies, HbF and carbamylated Hb. It measures not only glycation of N-terminal valine on  $\beta$  chain, but also  $\beta$  chains glycosylated at other sites and glycosylated  $\alpha$  chains.<sup>5</sup> The coefficient of variation (CV) <5% is usually obtained. There is no biochemical interference from hemoglobin variant for the affinity and immunochemical methods but liable to interference with RBC turnover in blood is high.<sup>2</sup> Adopting the new IFCC standardization procedure will result in HbA1c percentage values being lowered because of the higher specificity on the reference method.<sup>3</sup> The IFCC reference is unaffected by interfering substances that result in the non-specificity in certain methodologies. The conversion formulae of IFCC into DCCT/NGSP unit (%) is equal to  $0.09148 \times \text{IFCC units (mmol/mol)} + 2.152$  and vice versa  $\text{DCCT/NGSP unit (\%)} = 10.93 \times \text{DCCT/NGSP unit (\%)} - 23.50$ .<sup>4</sup>

Although HPLC is the standard method for assay of HbA1c the disadvantage is that the results are not available at the time of the patient visit. In present study, the average time consumption by those two methods are less (5 min by Nycocard reader and 15 min by Ion exchange chromatography), blood glucose concentration correlate with HbA1c % as well as statistical non-significant difference show the comparability nature of these two methods with ease of performance. In one of the randomized study comprising 201 patients (both type 1 and type 2 diabetic patients with insulin treatment), one group received immediate feedback, while the other group received delayed feedback via phone call or letter. Patients were followed for 1 year. Results indicated that immediate A1C feedback helped to improve subsequent glycemic control at 6 and 12 months.<sup>9</sup> The availability of report at the time of the patient visit can be made easy with the use of Nycocard Reader and Ion Exchange Chromatography techniques without any delay in communicating glycemic control, opportunities for clinical decision-making and changes in treatment regimen. Implementing inexpensive, easy-to-use interventions can reduce the huge economic burden of diabetes. Many of these interventions are cost effective and/or cost saving; even in developing countries.<sup>10</sup> The measurement of glycated hemoglobin is central to good-quality diabetes care. This is a measure by which healthcare providers can relate blood glucose control to the risk of complications. The working group by International Diabetes Federation (IDF) was established to develop a standard and harmonise HbA1c reporting.<sup>11</sup>

The conventional methods, reference ranges, diagnostic criteria, risk assessment pattern are updated regularly for increasing its quality and reliability for the diagnosis and

management of patients with Diabetes mellitus. Better management plans based on the current evidence can help significantly to reduce the long-term complications associated with type 2 diabetes.

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