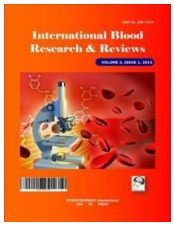


International Blood Research & Reviews
5(2): 1-9, 2016, Article no.IBRR.24410
ISSN: 2321–7219

SCIENCEDOMAIN *international*
www.sciencedomain.org

Glycated Hemoglobin Evaluation by Means of Ion Exchange Chromatography and Immunoassay in Normal and Hemoglobinopathy Patients

Shyamali Pal[1*]

[1]JMD Diagnostics Private Limited, P-336, C.I.T Road Scheme VI M, Kolkata – 700054, India.

Author's contribution

The sole author designed, analyzed and interpreted and prepared the manuscript.

Article Information

DOI: 10.9734/IBRR/2016/24410
Editor(s):
(1) Tadeusz Robak, Medical University of Lodz, Copernicus Memorial Hospital, Poland.
Reviewers:
(1) Alexander Berezin, Medical University of Zaporozhye, Ukraine.
(2) Marco Matteo Ciccone, University of Bari, Italy.
Complete Peer review History: http://sciencedomain.org/review-history/13417

Original Research Article

Received 19th January 2016
Accepted 10th February 2016
Published 24th February 2016

ABSTRACT

Aims and Objectives: High performance liquid chromatography which is basically an Ion Exchange Chromatography, is the most popular as well as expensive method for glycated hemoglobin estimation but biased results have been observed in hemoglobinopathies. Sometimes results cannot be reported also. In such situation the laboratory adopted immunoassay method and successfully reported the results. Therefore, a comparative analysis of glycated hemoglobin results of normal and abnormal hemoglobin were essential as high performance liquid chromatography is the most sought after method.
Study Design: Number of patients with normal adult hemoglobin compared were 120. Patients with Hb-AE thalassemia were 21. Patients with β-thalassemia trait were 36. Hb-E-β thalassemia patients were 6, β-thalassemia homozygous 10 and HbSS patients tested were 4. The glycated hemoglobin values obtained were from euglycemic to severe diabetic range. The instruments used for measurement are D10 BIORAD and Cobas Integra 400plus.
Data Analysis: Deviations calculated from results of same patient obtained from two system. Regression coefficients of each group were calculated. Trend analysis was done and graphically presented.
Results and Discussion: Regression analysis of normal, HbAE&β-variant patients show coefficients are 0.988,0.976 &0.961 respectively proving excellent comparability of two methods.

**Corresponding author: E-mail: Shy23_pal@yahoo.co.in;*

But trend analysis of deviations show right shift of 85%HbAE& 86.1%β-variants .Similar bias was observed in external quality assessment results also. The immunoassay results were comparable with boronate affinity chromatography and enzymatic estimation method. The deviation in HPLC observed only when in external assessment sample a window was seen. Therefore, observations of the trend of patients and EQAS sample indicates low bias of HbA1c estimation by HPLC method in D10.In severe forms of Hemoglobinopathy i.e. HbSS, Hb-E-β and β-thalassemia homozygous 75%,67% & 60% results could not be obtained from D10. The acceptability of immunoassay results checked by comparing glucose fasting and estimated average glucose calculation values.

Conclusion: Immunoassay, the second popular method is reliable, less expensive method and results remain unaffected in hemoglobinopathies.

Keywords: HbA1c; HPLC; immunoassay; HbA0; Tina Quant.

ABBREVIATIONS

HPLC: Ion exchange high performance liquid chromatography; HbA_1c: Glycated Hemoglobin; CI: Cobas Integra 400plus; r: Regression coefficient; Tina Quant immunoturbidimetric immunoassay (TQ).

1. INTRODUCTION

Diabetes is a worldwide problem and prevalence has been observed to be common in low and middle incoming countries. The majority of 382 million people with diabetes are in the age group 40-59 years. 17% of live births are from pregnant mothers with gestational diabetes [1]. Diabetes is a subtle disease and accounts for an increased risk of cardiovascular risk even if the subject is having no sign of prediabetic/diabetic conditions [2]. As the prediabetic/diabetic conditions are precipitant of impairment of vascular endothelium which may cause atherosclerotic lesion, evaluation of such condition by noninvasive techniques should be considered as subject of prime importance [2]. In the year 2009 American Diabetes Association (ADA) recommended HbA1c evaluation be utilized for the diagnosis of diabetes mellitus and not just for the monitoring of long term glycemic control [3,4]. Such decision is based on the fact that the relative concentration of HbA1c correlates effectively with the average concentration of plasma glucose over an immediate previous period of 3-4 months [5,6]. There are multiple methods for assessment of HbA1c and grossly may be categorized into two groups. In the first category incorporation of glucose to adult hemoglobin (HbA_0) triggers a decrease in the net negative charge of the molecule allowing separation of HbA1c from HbA_0. In HPLC a programmed buffer gradient of increasing ionic strength delivers the sample to cartridge. In the cartridge hemoglobin fraction (HbA1c)/fractions are separated and pass through photometer flow cell where changes in absorbance are measured at 415 nm. In electrophoresis the HbA1c molecules/Hb fractions are separated on the basis of charge by an ionic buffer gradient and separated fractions are scanned [7]. So, the method cannot completely resolve all potential hemoglobin peaks and affected by the presence of specific Hb mutations [7] as well as post translational modifications such as carbamylation or acetylation [8]. A case report on the year 2009 showed detection of HbA1c by immunoturbidimetry (Cobas Integra 400 plus, Tina quant) when the result was undetectable in D10, a HPLC system by BIORAD. Hb analysis identified the presence of Hb-D Punjab [9]. The most common Hb variants are HbS, HbC, HbE and HbD which results from point mutation within the coding region of β- chain. Since this mutations occur at amino acids other than the N-terminal Valine the normal glycation process is not affected. It was stated that such substitution does not affect the charge of Hb variant molecule leading to co-elution/overlapping. The authors found no analytical interference when compared with 7 systems. Only Menarini system showed fluctuations [10]. A case report on the year 2013 has shown interference of HbH on measurement of HPLC and electrophoresis [11]. The guideline for interpretation of results by BIORAD-D10, HbA1c mode has instructed to suspect HbA1c result ≥15%. The reason may be overlapping due to the presence of Hb-variant [12]. Any sample with a combined area of ≥60% in the variant S/C window was stated to be due to homozygous variant/variant β-thalassemia phenotype. Such results should not be reported. Short life span of RBC exhibits decreased HbA1c [12].

Second category are direct enzymatic assay and Tina Quant Immunoturbidimetry. Direct

enzymatic HbA1c Assay is an enzymatic assay in which lysed whole blood samples are subjected to extensive protease digestion with Bacillus sp protease. This process releases amino acids including glycated valines from the hemoglobin beta chains. Glycated valines then serve as substrates for specific recombinant Fructosyl Valine Oxidase (FVO) enzyme, produced in *E. coli*. The recombinant FVO specifically cleaves N-terminal valines and produces hydrogen peroxide. This, in turn, is measured using a horseradish peroxidase (POD) catalyzed reaction and a suitable chromogen. No separate measurement for total hemoglobin (Hb) is needed in this direct enzymatic HbA1c Assay. The HbA1c concentration is expressed directly as %HbA1c by use of a suitable calibration curve in which the calibrators have values for each level in %HbA1c [13]. The method seems to be simple but not readily available in the market. The second popular and readily accessible method after HPLC is Tina Quant competitive inhibition immunoturbidimetry (TQ). The hemolysate prepared from whole blood which forms insoluble complex of polyhapten with excess antiHbA1c antibodies remaining after conjugate formation. In this method total Hb estimation is necessary [14,15]. Two methods, HPLC & TQ were compared and found out to be comparable [12].

The laboratory has observed difficulty in reporting HbA1c when there is clinically silent Hb variant and in homozygous variant could not inform the result by HPLC. As alternative method Tina Quant assay has been used. So, the laboratory wanted to make a comparative analysis of heterozygous and homozygous variants between HPLC and TQ. A comparative study of HbA1c with normal adult hemoglobin was also essential to understand the bias of the methods. Such a full method comparison study including normal and variants were not available. Moreover, the TQ method is basically less expensive, results readily available as the test is being performed in automated system. Such evaluation has helped the laboratory to find out the most suitable method for the laboratory and its users as well as sharing of the inference of the project may help other laboratories also to select the method.

2. MATERIALS AND METHODS

2.1 Study Materials

HbA1c of 120 normal adult hemoglobin samples were tested in two instruments Cobas Integra 400 plus (CI) BIORAD D10 (D10). In CI samples were tested by TQ and in D10 by HPLC. The HbA1c results covered the range of 4.5-15.0%. For comparison of normal population, minimum number of patients should be 120 as per CLSI (Clinical and Laboratory Standards Institute) guideline.

The common Hb Variants were tested by these two methods. But getting 120 patients for each Hb variant is a time consuming process and the comparative study needed to be completed within the expiry date of the kits obtained for the project. Number of HbAE thalassemia heterozygous patients tested were 21, β-thalassemia heterozygous were 36,β-thalassemia homozygous were 10, Hb-E-β thalassemia were 6 and HbSS only 4. The patient samples were sent to laboratory for HbA1c testing by HPLC the laboratory compared the data in CI simultaneously. Hence, permission from patients was not necessary for the current study. The homozygous patients results could not be obtained from D10 so plasma glucose were tested and compared with HbA1c. Clinical correlation has also been done. In CI the samples treated within 30 minutes of collection. But in D10 tests were performed at the end of the day to save the expenditure of elution & wash buffer which is a common practice for all D10 users.

2.2 Methods

The D10 HbA1c program is based on chromatographic separation of the analytes by ion exchange high performance liquid chromatography (HPLC). The samples are automatically diluted in the system and injected into the analytical cartridge. The instrument delivers a programmed buffer gradient of increasing ionic strength to the cartridge, where the hemoglobins are separated based on their ionic interactions with the cartridge material. The separated hemoglobins then pass through the flow cell of the filter photometer where changes in the absorbance at 415 nm are measured [16].

In CI HbA1c reacts with anti HbA1c antibody forming soluble antibody-antigen complex. The polyhapten reacts with excess antiHbA1c antibodies to form insoluble antibody polyhapten complex which is measured turbidimetrically. Liberated Hb in the hemolysate is converted to a derivative having a characteristic absorption spectrum measured at pre-incubation phase [15].

Plasma glucose of patients having severe form of variant Hb like HbSS,Hb-E-β andβ thalassemia

homozygous were tested in CI using hexokinase method [17] to assess the acceptability of TQ results.

The laboratory routinely performs internal control check using Diabetic control level 1&2 from BIORAD. One level is being performed in a day, level 1&2 on alternate day. The laboratory is participating in EQAS program also, the EQAS program is ISO 17043:2010 accredited. The parameters HbA1c and glucose are under the scope of accreditation by the national accreditation body.

The trend measurement of HbA1c in normal and hemoglobinopathies were done. The trend in EQAS samples was also observed.

2.3 Statistical Calculation

Inbuilt Quick analysis tool has been used for statistical calculation. Deviation from HPLC was calculated as the objective is to study the deviation from HPLC. Regression coefficient, mean bias and %bias is calculated. Multivariant regression analysis tool has been used to obtain the regression coefficient. From the scattered plot the trend analysis of deviations were done.

2.4 Calculation of Results

In CI calculation of result is as follows:

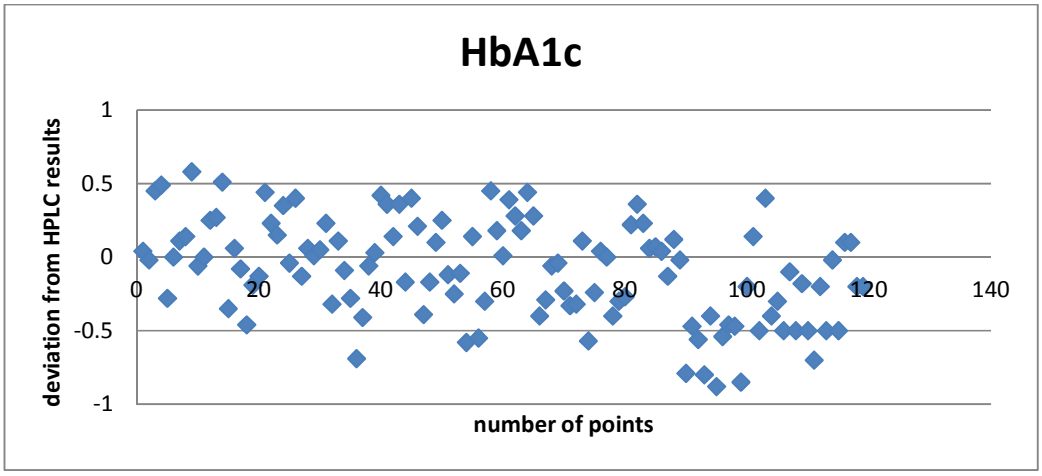
Calculation:
(HbA1c/Total Hb)
x 91.5+2.15=HbA1c (%)[15].

Chromatogram is obtained automatically from D10 system [12].

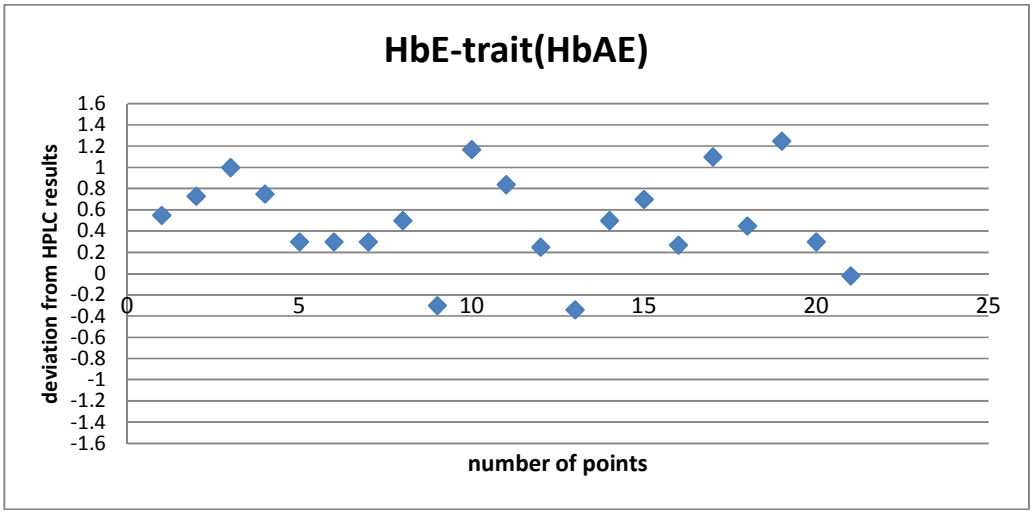
3. RESULTS AND DISCUSSION

Graph 1 presents the HbA1c results of normal adult hemoglobin patients. The graphical presentation and data in Tables 1 & 2 showed both methods are comparable with HbA1c results varying from 4.0%-15.0%. Mean in both methods are almost same, SD's are identical, and bias is negligible resulting excellent regression co efficient(r). No characteristic trend has been observed as number of results showing positive and negative bias are evenly distributed. Graphs 2 & 3 showed Hb variant curves. The results also showed good regression values [Table 2, Table 3, Graphs 2 & 3]. But trends show a different picture. 18 results out of 21 and 31 results out of 36 are showing higher value in CI than D10 showing characteristic right hand shift for TQ estimated HbA1c results.

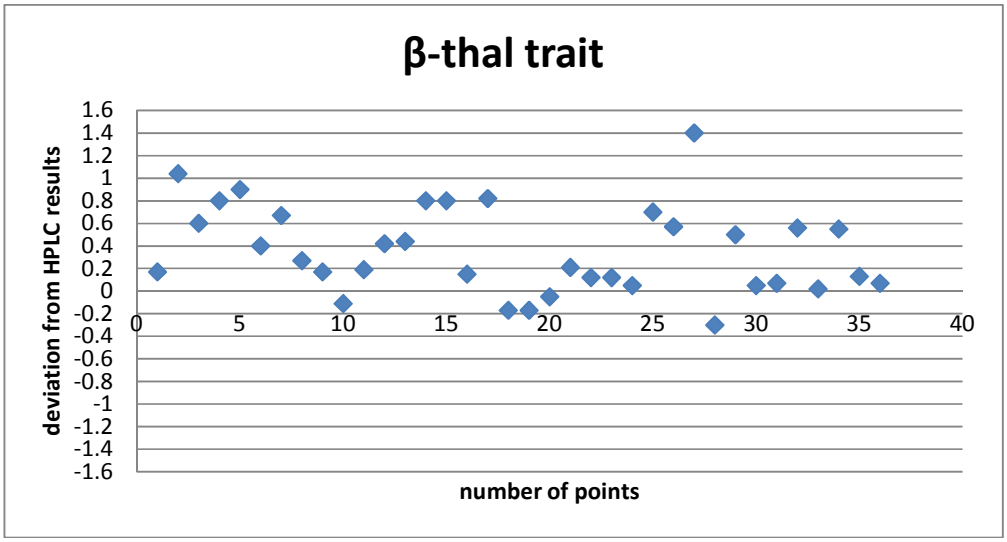
Both the methods are IFCC certified method [19,20]. Previous articles on ion exchange HPLC also proved the efficiency of the method [12,19]. The author opted for the CI method because it is less expensive, can be tested randomly hence may be reported anytime of the day. Disadvantage of HPLC is processing has to be done in a batch to make the process cost effective. Hence urgent reporting is not possible. The laboratory could not report HbA1c of homozygous variant results. The trends in heterozygous patients also prove the low bias of HPLC. Similar results were obtained by other workers [21,22]. In recent article Little et al suggested the same and used Roche TQ method as primary method since the method is least likely to show interference from Hb variants [22].



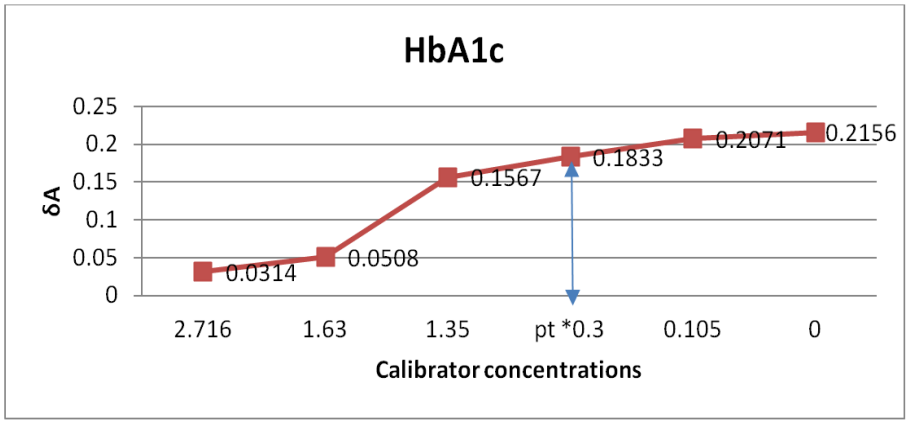
Graph 1. Deviation plot of HbA1c result of CI from HPLC in normal patients



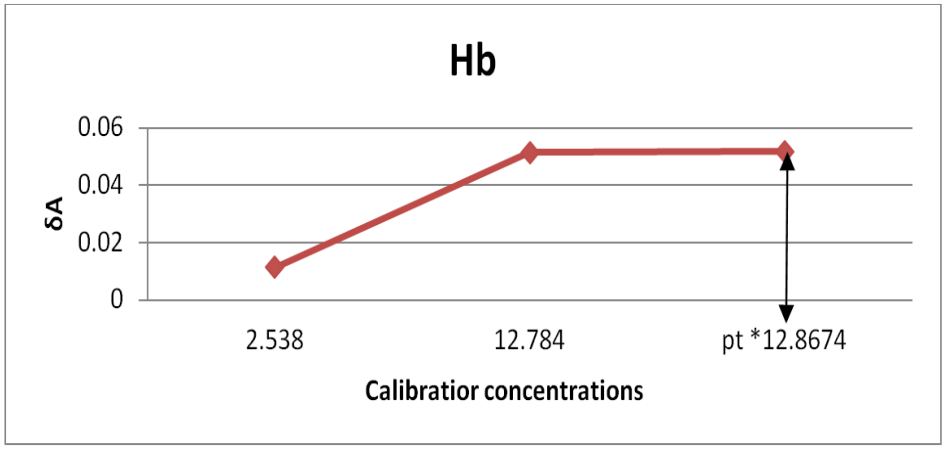
Graph 2. Deviation plot of HbA1c result of CI from HPLC in HbAE patients



Graph 3. Deviation plot of HbA1c result of CI from HPLC in β-variant patients



Graph 4A. Absorbance curve of HbA1c



Graph 4B. Absorbance curve of Hb

Calculation: (0.3/12.784)x91.5+ 2.15 = 4.29; HbA1c= 4.29%

Graph 4(A+B). Result format of HbA1c in Tina Quant method

Table 1. Comparison of mean and SD

Patient type	Number of patients	D10(HPLC)		CI(Imm. assay)	
		Mean	SD	Mean	SD
Normal Adult Hb	120	7.65	1.9	7.701	1.9
HbAE	21	6.28	1.8	6.51	1.66
Hb-β-variant	36	5.62	1.4	5.91	1.3

Table 2. Statistical analysis

Patient type	Trend of deviation			Mean deviation	% deviation	Bias	Regression co-efficient
	+ve	-ve	zero				
Normal Adult Hb	52	65	3	0.28	3.66	0.65	0.988
HbAE	18	3	0	0.567	9.03	3.66	0.976
Hb-β-variant	31	5	0	0.404	7.2	5.16	0.961

Table 3. HbA1c (%) results of β-thalassemia homozygous, Hb-E-β-thalassemia & HbSS

β-Thalassemia homozygous				Hb-E-β Thalassemia				HbSS			
D10	CI	Glu (mg/dl)	EAG (mg/dl)	D10	CI	Glu (mg/dl)	EAG (mg/dl)	D10	CI	Glu (mg/dl)	EAG (mg/dl)
3.8	5.24	109	103.7	4.7	5.1	95	99.7	4.1	10.5	286	254.6
3.4	4.9	95	94	4.8	4.91	88	94.2	x	6.1	115	128
3.5	5.1	98	99.7	X	4.62	79	86	X	4.5	77	82.4
3.7	5.13	101	100.5	X	4.91	87	94.2	x	5.75	106	118.3
X	4.6	89	85.3	X	5.47	109	110				
X	4.62	78	86	X	4.41	75	80				
X	4.16	85	73								
X	4.62	92	86								
X	4.0	70	68.1								
x	4.31	81	77								

Glu: Fasting Glucose; EAG: Estimated Average Glucose; Calculation: 28.7xHbA1c-46.7 [18]

Table 4. Comparison of EQAS results of HbA1c in different methods

Months	Boronate affinity chromatography	Enzymatic assay	Ion exchange HPLC(D10)	Immunoturbidimetry (CI)
January 2014	5.8	5.0	5.0	5.2
February 2014	6.1	6.3	5.9	6.4
March 2014	8.7	8.3	8.7	8.4
April 2014	5.8	5.8	5.4	5.8
May 2014	8.8	7.1	8.2	6.7
June 2014	5.8	5.7	5.2	5.5
July 2014	6.8	6.0	5.8	6.8
August 2014	8.5	8.7	8.4	8.9
September 2014	6.2	6.3	6.0	6.5
October 2014	6.5	7.0	5.8	7.0
November 2014	9.0	9.2	7.4	9.5
December 2014	8.5	8.7	6.9	8.5
January 2015	6.9	7.1	5.9	7.2
February 2015	8.6	9.0	7.4	9.0
March 2015	6.8	7.1	5.8	6.9
April 2015	6.4	5.6	5.1	5.6
May 2015	6.9	7.2	6.1	7.1
June 2015	5.6	5.7	5.3	5.6
July 2015	8.7	9.9	8.5	9.4
August 2015	5.8	5.3	5.2	5.2
September 2015	7.1	7.3	6.7	7.1

Table 5. Hb fractions of discrepant EQAS HbA1c

Month	A1a (%)	A1b (%)	Unknown (%)	LA1c/ CHb1 (%)	A1c (%)	P3 (%)	A0 (%)	Variant window (%)
May.14	3.3	1.3	3.3	1.5	8.6	14.4	70.2	
Jul. 14	4.3	-	3.0	2.3	6.3	17.3	54.7	14.5 at 1.63 min
Oct.14	1.6	1.3	2.0	2.7	5.9	13	74.4	---
Nov.14	1.9	1.5	2.4	3.3	7.3	11.6	72.7	---
Dec.14	1.9	1.5	1.6	3.7	6.8	13.3	70.9	---
Jan.15	1.2	2.9	2.9	7.2	5.8	10.5	77	---
Feb.15	1.6	1.2	1.9	2.5	7.2	9.1	77.7	---
Mar.15	1.6	1.8	2.1	2.1	5.5	9.4	79.4	---
May.15	8.0	-	2.0	2.4	4.6	7.8	82.6	19.5 at 1.65 min
Jul.15	3.1	1.6	3.2	2.4	9.1	12.5	71.2	---

In homozygous patients, the concentration of adult Hb is low and overlapping affects the test. The HbSS with diabetes is a rare phenomenon. But the author obtained a few such case [Table 3]. The results are either very low or could not be reported. As in TQ method the ratio of total Hb & HbA1c in the hemolysate is being estimated the results were bias free. To check the validity of the results fasting glucose values were measured and found out to be well correlated [Table 3].

The question is when TQ is IFCC certified, less expensive, bias free for heterozygous Hb variant method and accepted as primary method for estimation of HbA1c why HPLC is the most popular method all over India. The presentation of chromatogram in HPLC adds value to the results. The laboratory created the result format [Graphs 4A, 4B] and requested Roche Diagnostics for software to obtain automated graphical presentation from the instrument. The advantage of TQ method is when the result is below the measuring range (<4.3%) the absorbance may be obtained from the system and by manual calculation test result may be reported. In homozygous Hb variant patients such option helps the laboratory and HbA1c may

be crosschecked estimating blood glucose and comparison with estimated average glucose [17]. The data of present project supports such conclusion as per the observations of Table [3].

The laboratory tested the possibility of positive trend of HbA1c results by TQ. So, the laboratory checked the EQAS results. The results of almost 2 years have been tabulated in Table [4]. Except May 2014 result there appeared to be a good correlation of Boronate Affinity Chromatography, Enzyme assay and TQ method. One variation in two years may be attributed as random error. But HPLC method showed low bias against three methods in 10 out of 21 months. The chromatogram of those months obtained from the laboratory and Hb fractions were studied. The Hb fractions presented in Table [5] showed variant window at retention time of HbA2 in July 2014 and May 2015 & others showing high P3 level. Diagnosis should not be done for EQAS sample but it proved that the chromatograms showing variant pattern affected HbA1c results in HPLC.

4. CONCLUSION

Tina Quant is reliable, cost effective method and HbA1c results do not get affected due to the presence of common Hb variants. The method is patient friendly also as results may be reported within 30 minutes after collection of sample. The presentability factor should not be important and the effectiveness to serve the patients are expected to be major concern. However, the automated graphical presentation can make the determination process presentable also.

CONSENT

It is not applicable.

ETHICAL ISSUES

- The study design has been done to find out alternative method when Ion Exchange HPLC results cannot be obtained. This is purely a scientific study not for any commercial interest.
- The blood samples were received by the laboratory for diagnostic testing. So, method comparison does not require permission of the patients.
- As the study does not involve any medication permission from ethical committee is not applicable.

ACKNOWLEDGEMENT

[1] JMD Diagnostics Private Limited. [2] Roche Diagnostics India Limited. [3] Dr. Siddhartha Gupta, HOD, Kolkata Port Trust Hospital.

COMPETING INTERESTS

Author has declared that no competing interests exist.

REFERENCES

1. Van Dieren S, Beulens JW, Vander Schouw YT, Grobbe DE, Neal B: The global burden of diabetes and its complications: An emerging pandemic, Eur. J. Cardiovasc. Prev. Rehabil, 1S, 3-8; 2010.
   DOI: 10.1097/01
2. Ciccone MM, Scicchituno P, Cameli M, Cecere A, Cortese F, Dentamaro I, et al. Endothelial function in prediabetes, diabetes and diabetic cardiomyopathy: A review: Journal of Diabetes and Metabolism. 2155-6156,1-10; 2014.
3. American Diabetes Association. Standards of medical care in diabetes: Diabetes Care: S1, 33, 11-61; 2010.
4. Gillett MJ: International expert committee report on the role of the A1C assay in the diagnostics of Diabetes: Diabetes Care 2009:327, 1327-1344; 2009.
5. Sacks DB: A1c versus glucose testing; A comparison: Diabetes Care. 2011;34,518-523.
6. Sacks DB: Measurements of hemoglobin A1c: A new twist on the path to harmony: Diabetes Care: 2012;35:2674-2680.
7. Bry L, Chen PC, Sacks DB: Effects of hemoglobin variants and chemically modified derivatives on assays for glycohemoglobin: Clin Chem: 2001;47:153-163.
8. Weykamp CW, Pendens TJ, Siebelden CW, Muskiet FA, Van Der Slik W: Interference of carbamylated and acetylated hemoglobin in assays of glycohemoglobin by HPLC, electrophoresis, affinity chromatography and enzyme immunoassay. Clin Chem. 1993;39:138-142.
9. Thevarajah M, Nadzimah MN, Chew YY. Interference of hemoglobin A1c detection using ion exchange high performance liquid chromatography method by clinically

silent hemoglobin variant in University Malaya Medical Centre (UMMC)- A case report. Clin Biochem. 2009;42(4-5):430-434.

10. Little RR, Rohlfing CL, Hanson SE, Schmidt RL, Lin CN, Madsen RW, Roberts WL. The effect of increased fetal hemoglobin in 7 common HbA1c assay methods. Clin Chem. 2013;58(5):945-947.
11. Mehmet Agill, Halil Yaman, Aydin FN, Canir E, Cayci T, Kurt YG, Akgul EO, Aydin Ebrahim. HbH interference on measurement of HbA1c with Ion exchange HPLC. A case report. Acta Inform Medica. 2013;21(3):216-18.
12. Bio RAD D10 ^{TN} hemoglobin A1c program. Instruction Manual. 13-14. Ref. 220-0101.
13. Liu L, Hood S, Wang Y, Bezverkov R, Dou C, Datta A, Yuan C. Direct enzymatic assay for HbA1c in human whole blood sample. Clin Biochem. 2008;(7-8):576-580.
14. Fleming JK. Evaluation of HbA1c on the Roche Cobas Integra 800 closed tube system. Clin Biochem. 2007;40(11):822-827.
15. Tina Quant ®HbA1c Gen. 3Turbidimetric inhibition immunoassay (TINIA) for the in vitro determination of hemoglobin A1c in whole blood or hemolysate. Instruction Manual. Roche Diagnostics; 2013.
16. Kunst A, Draeger B, Ziegenhorn J. In: Bergmeyer. Method of enzymatic analysis, 3rd ed. Metabolites 1. Carbohydrates. 1984;6:163-172.
17. Nathan DM, Kuenen J, Borg R, Zheng H, Schoenfeld D, Heine RJ: Translating the A1cassay into estimated average glucose values. Diabetes Care. 2008;31(1):1-6.
18. Finke A, Kobold U, Hoelzel W, Weycamp C, Jeppson JO, Miedema K. Harmonizing Hemoglobin A1c testing. Clin Chem Lab Med. 1998;36:299-308.
19. Jeppson JO, Kobold U, Barr J, Finke A, Hoelzel W, Hoshino T, Miedema K, Mauri P, Mosca A, Paroni R, Thiempont L, Umemoto M, Weycamp CW: Approved IFCC reference method for the measurement of HbA1c in human blood. Clin Chem Lab Med. 2002;40:78-89.
20. Bhat VS, Dewan DK, Krishnaswamy PR: Diagnostic dilemma of HbA1c detection in presence of hemoglobinopathy: A case report. Indian J Clin Biochem. 2011;26(1):91-95.
21. Karami A, Baradarun A: Comparative evaluation of three different methods for HbA1c measurement with high performance liquid chromatography in diabetic patients. Advanced Biomedical Research. 2014;3:1-7.
22. Little RR, La'ulu S, Hanson SE, Rohlfing CL, Schmidt RL: Effect of 49 different Hb variants on HbA1c measurement in eight methods. Journal of Diabetes Science and Technology. 2015;9(4):446-451.

Peer-review history:
The peer review history for this paper can be accessed here:
http://sciencedomain.org/review-history/13417