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Research Paper

Interference by Various Fractions of HbA1 While Estimating Total HbA1c% by High Performance Liquid Chromatography in Diabetics, if Any?

Medical Science

KEYWORDS : Glycated Hb, Fasting plasma glucose, HbA1 fractions, HbF, carbamylatedHb, HbA0, High performance liquid chromatography

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ABSTRACT

Glycated Hb, measured as HbA1c is accepted as the gold standard marker for long-term glycemic control, for the preceding six to eight weeks. Aim of study was to measure total HbA1c% and fractions of HbA1 in diabetics and healthy controls, correlate total HbA1c% with fractions of HbA1 and fasting plasma glucose (FPG) levels in diabetics and healthy controls,to measure each fractions of HbA1 which might cause fluctuations in the value of HbA1c .The study was conducted in department of Biochemistry, Kasturba Medical College, Manipal.(n= 467).Total HbA1c and various fractions of HbA1 were estimated by high performance liquid chromatography method. Plasma glucose was estimated by hexokinase method.Total HbA1c%, HbF%,HbA1b% were significantly increased in diabetic patients compared to healthy controls . HbA1a% was not significantly increased in diabetic patients, compared to healthy controls. Weak positive correlation was found between total HbA1c% and FPG.HbF% was significantly increased in diabetic patients. Elevated HbF% in diabetic patients might be due to an intereference or cross reactivity of HbA1c% with HbF% during estimation, so HbF, which is actually a part of HbA1c is eluted out separately and which might lead to underestimation of HbA1c , it could result in sub¬optimal therapy and, thereby, increased risk of diabetic com-plications. FPG levels correlated less well with HbA1C%, which showed that HbA1c might be reliable on estimated average glucose (eAG) rather than FPG.

Introduction

Glycated haemoglobin, measured as HbA1c is the result of irreversible posttranslational attachment of glucose to the N-terminal amino acid valine of the Hb A0 (major Hb) β chain . It is accepted as an important indicator of glycemic control for the preceding six to eight weeks[1,2] and risks for diabetic complications in patients with diabetes mellitus.

The term glycated haemoglobin includes both haemoglobin A1(HbA1) and haemoglobin A1c (HbA1c). HbA1 refers to the non-enzymatic binding of several species of carbohydrate to haemoglobin, whereas in HbA1c the carbohydrate is specifically glucose.[4] HbA1c is formed by the stable amadori rearrangement of a precursor known as labile A1C (LA1C, aldimine fraction).[5] LA1c is characterized by the reversible binding of glucose to Hb as a Schiff base. HbA1 can be further separated into HbA1a1%(glycation with F1,6 diphosphate) HbA1a2% (glycation with glucose 6 phosphate), HbA1b% (glycation with unknown carbohydrate), HbA1c%(glycation with D- glucose). Carbamylated hemoglobin is formed by the reaction of cyanate, derived from urea, with the N-terminal valine of the Hb β chain . Fetal Hb ($\alpha_2\gamma_2$) peak is also seen in HPLC chromatogram.

Biorad variant II turbo HbA1c autoanalyzer utilizes principles of reverse phase high performance liquid chromatography(HPLC) [11].

HPLC allows separation of minor and major HbA fractions such as Labile A1c%, HbA1a1%, HbA1a2% , HbA1b% , HbA1c%, HbF%, HbA0%, CHB % .

The purpose of this study was to evaluate the measurement of minor and major fractions of HbA1 by using HPLC method, and to observe and identify the fractions of HbA1 which might cause fluctuations in the levels of HbA1c.

We also focused on estimating the correlation between each fractions of HbA1 and total HbA1c% in non- diabetics, patients with impaired glucose tolerance and diabetics.

Materials & methods

This study was conducted in Department of Biochemistry, Kasturba Medical College, Manipal for 9 months duration (March 2012- November 2012). Patient's informed consent and Institutional Ethical Committee Clearance was obtained. Total 467 patients were included in the study.

Samples (n=467) were collected in EDTA vacutainers for HbA1c% and in fluoride vacutainers for plasma glucose analysis. HbA1c was estimated by HPLC method, using (Bio-rad) variant II turbo HbA1c analyzer. In the HPLC method, the samples were automatically diluted on variant II turbo sampling station (VSS) and injected into the analytical cartridge. The variant II turbo chromatographic station (VCS) dual pumps delivered a programmed buffer gradient of increasing ionic strength to the cartridge, where the hemoglobins were separated based on their ionic interactions with the cartridge material. It separated HbA1c from other hemoglobin fractions based on differences in ionic charge. The absorbance at 415nm was measured. A sample report including retention times of detected peaks and a chromatogram were generated by clinical data management (CDM) for each sample. From the graph, percentage of minor and major glycoHb fractions were taken into consideration. Biorad quality control low level and high level were run daily once. [12]

FPG was estimated using Hexokinase method in Cobas 6000 autoanalyzer[13].

Statistical analysis was done using SPSS V16.0. Student's t test was performed, to compare the mean between different groups. ANOVA test was applied for comparison of mean between more than two groups. Pearson's correlation coefficient was used to

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correlate between various parameters.

Results

Patient samples were divided into three groups based on the FPG values. Group I included patients with normal fasting plasma glucose levels (≤ 100 mg/dL), group II included patients with impaired glucose tolerance (101-125 mg/dL), group III included diabetics(>126 mg/dL).

Table-1 shows mean and SD of HbA1c% and various fractions of HbA1 in different groups. HbA1c% and HbF% were significantly increased in diabetic patients compared to normal and patients with impaired glucose tolerance ($p < 0.05$) hence usefulness of HbA1c% in diagnosing diabetes seems proved. It shows that, there is some interferences in HbF estimation, when plasma glucose level increases. HbA1b% was significantly increased in latter two groups, compared to normal patients ($p<0.05$). There was no statistically significant difference for HbA0% , LA1c% and HbA1a% in any of the three groups ($p > 0.05$).

Table-2 shows correlation of total HbA1c% with various measured fractions. Weak positive correlation was found between total HbA1c% and FPG (r=0.595), Significant positive correlation was found between total HbA1c% and HbA1b% (r = 0.561), total HbA1c % and HbF% (r = 0.551),total HbA1c% and Labile A1c% (r = 0.419) and significant negative correlation was found between total HbA1c% and HbA0%(r = - 0.627).

Table-3 shows correlation of HbA1c% with various measured fractions in different groups. Significant positive correlation was found between total HbA1c% and HbA1b% in group II (impaired glucose tolerance) and group III (diabetics). Significant positive correlation was found between HbA1c% and HbF%, HbA1c% and HbA0%, HbA1c% and LA1c% in group III (diabetics).Significant negative correlation was found between total HbA1c% and HbA0% in group II (impaired glucose tolerance) .

Table-1: Mean & SD of various HbA1 fractions in group I , II and III

Parameter	Group-I	Group-II	Group-III
HbA1c%	6.21±1.345	6.39±1.162	9.00±2.917 *
HbA1b%	2.37±0.92	2.48±0.60**	3.00±0.70***
HbF%	0.85±0.45	1.19±0.77	1.66±0.90****
HbA0%	87.6±3.72	86.87±1.87	83.00±4.07
LA1c%	1.25±0.29	1.42±0.82	2.01±0.55
HbA1a%	0.480±0.283	0.479±0.509	0.575±0.935

*** p< 0.05 when compared group I & III**
****p< 0.05when compared group II & III**
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Table-2: Correlation of total HbA1c% with various measured HbA1 fractions and FPG

Parameter	Total HbA1c%
FPG	r = 0.595*
LA1c%	r = 0.419*
HbA1b%	r = 0.561*
HbF%	r = 0.551*
HbA0%	r = - 0.627*

***Correlation is significant at p < 0.01 level (2 tailed)**

Table 3: Correlation of measured fractions of HbA1 with HbA1C% in group I, II & III

Parameter	HbA1c% in Group-I	HbA1c% in Group-II	HbA1c% in Group- III
HbA1b%	r = 0.067	r = 0.347*	r = 0.579*
HbF%	r = - 0.006	r = 0.203	r = 0.579*
HbA0%	r = -0.090	r = -0.594*	r = 0.570*
HbA1a%	r= - 0.19	r= - 0.27	r=0.045
LA1c%	r = - 0.22	r= - 0.078	r= 0.434 *

***Correlation is significant at p < 0.01 level (2 tailed)**

Discussion & conclusion

In this study, HbF% was significantly increased in diabetic patients. This could be due to an intereference or cross reactivity of HbA1c% with HbF% during estimation, so HbF , which is actually a part of HbA1c is eluted out separately and which might lead to underestimation of HbA1c , it could result in suboptimal therapy and, thereby, increased risk of diabetic complications. This finding did not coincide with the study conducted by Curt L Rohfing et al, they showed minimal evidence of interference on HbA1c levels from elevated HbF levels by HPLC method using Bio-Rad Variant II.

In this study, total HbA1a% was not significantly increased in diabetic patients. Total HbA1a% [HbA1a1% (glycation with F1,6 diphosphate) HbA1a2% (glycation with glucose 6 phosphate)] did not correlate significantly with total HbA1c % in diabetic patients. Theoretically, diabetic red cells would be expected to have normal levels of glucose-6-P since even in normal red cells, hexokinase is operating at its Vmax and thus the rate of formation of glucose-6-P should be no higher in hyperglycaemic individuals. whereas the concentration of intracellular glucose is about 200-fold that of glucose-6-P.

In this study, HbA1b% was significantly increased in diabetic patients. Significant correlation was found between HbA1b% and fasting plasma glucose levels in diabetic patients. It was due to the effects of hyperglycemia. This finding coincide with previous study conducted by, Shigehiko Imagawa et al.

In previous studies, fasting glucose correlated less well with HbA1c and results showed that with increasing HbA1c[2], fasting glucose progressively underestimated the level of HbA1c. In this study also, FPG levels correlated less well with HbA1C%, which shows HbA1c might be reliable on estimated average glucose (eAG) rather than FPG.

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