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Original Research Paper

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GLYCATED HAEMOGLOBIN AND VARIOUS FRACTIONS ON HPLC

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ABSTRACT

Background- Haemoglobin A1c (HbA1c) test has been a major boost in diagnosis & management of diabetes and its estimation has become an important part for the management of diabetes. The term glycated haemoglobin is essentially composed of haemoglobin A1 (HbA1) and HbA1c. Objective of our study was to find out the relationship between sub fractions of glycated haemoglobin and the major fraction,HbA1c and the role of P3 fraction in the final reported value of HbA1c using HPLC.

Method- HbA1c,various fractions of HbA1 and P3 were estimated by HPLC method in 430 subjects. Patient data was divided into 3 groups on the basis of HbA1c%.

Result- HbA1a%, HbA1b% fraction increases with increase in HbA1c% followed by HbA1a area and HbA1b area. Increase in P3% as well as area was also observed with increase in HbA1c.

Conclusion- Sub fractions of glycated haemoglobin and P3 fraction influence the final reported value of HbA1c by HPLC. P3 fraction might indicate the possible presence of variant haemoglobins in the sample.

KEYWORDS : HPLC, HbA1c, Diabetes

INTRODUCTION

Glycated haemoglobin (HbA1c) most accurately reflects the previous 2-3 months of glycaemic control.[1] Glycated haemoglobin is the formed by irreversible non enzymatic glycation at one or both N-terminal valines of the haemoglobin β chain.[2] High-performance liquid chromatography (HPLC) is currently considered the reference method of the DCCT (Diabetes Control and Complications Trial) and the method of the National Glycohaemoglobin Standardisation Program (NGSP).[3,4] HPLC provides adequate accuracy & precision as compared to immune turbidimetric method. BIORAD VARIANT II employs HPLC as the principle for estimating HbA1c and chromatograms are generated showing peaks for different fractions in the sample. Various fractions that are generated in chromatogram by variant II are HbA1a%,HbA1a area,HbA1b%,HbA1b area,HbA0%,HbA0 area, P3% and P3 area.[5] One of the fractions is P3 which denotes the fractions of haemoglobin which have to be eluted out to generate the final value of glycated haemoglobin (HbA1c) in the sample. P3, as shown by some studies, denotes the level of degraded haemoglobin which occurs due to the proteolytic enzymes present inside the erythrocytes and should be >10% for reporting the value of glycated haemoglobin. It is formed due to the post translational modification of adult haemoglobin and the estimated levels rise as the samples gets older or more of the haemoglobin is degraded; the level of post translational modifications further depends on the blood glucose concentration inside the RBC[6]. A combination of increased oxidant production and decreased antioxidant defences in the erythrocytes in diabetes leads to the degradation of haemoglobin by the proteolytic enzymes in the RBC[7]. Most of the studies on glycated haemoglobin have focussed on its synthesis, studies on the degradation of glycated haemoglobin are few. Several glycated haemoglobin samples were analysed using HPLC. The present study was undertaken to study the effect of different fractions on the HbA1c and the effect of P3 fraction on the final reported value of HbA1c.

MATERIAL & METHODS

This study was conducted in department of Biochemistry, Kasturba Medical College, Manipal between March 2012- November 2012.

Data was collected from graphs obtained on analysis of an EDTA blood sample for glycated haemoglobin in 430 subjects. Selection of subjects was solely based on the glycated haemoglobin values. Sample anonymisation was done. Samples were divided into 3 groups based on the HbA1c levels.

Blood sample was collected from each and analysed for glycated haemoglobin without delay using BIO-RAD VARIANT II turbo. Variant II is fully automated and works on the principle of automated cation exchange HPLC, final report is presented visually and the area under each peak of chromatogram is integrated to provide percentage of each fraction present in the sample and the area under the curve.

Criteria for acceptance of HbA1c report (includes limitations)
Total area count should be within 1 million – 3.5 million. Serum triglycerides up to 5680mg/dl, serum total bilirubin up to 20 mg/dl do not interfere with the assay. Heterozygous HbS and HbC do not interfere with A1c assay.

Samples from patients with hemolytic anemia exhibits decreased glycated hemoglobin values due to shortened life span of red cells. Patients with polycythemia or post-splenectomy exhibit increased glycated hemoglobin due to somewhat longer life span of red cells. Ethical clearance was obtained from institutional ethical committee.

STATISTICAL ANALYSIS

Numerical variables were reported in terms of either mean ± SD or mean ± SEM, whichever is significant. Statistical analysis was done by SPSS v16.0.Analysis of result was done by ANOVA and multiple regression analysis. P value < 0.05 and 0.001 was taken as statistically significant and highly significant respectively. Pearson's correlation test was done to assess correlation between different parameters.

RESULTS

Patient data was divided into three groups on the basis of glycated haemoglobin levels and mean and standard deviation / SEM was calculated for each fraction obtained as shown in the tables below:

Table 1 : Demographic details of the patients

Groups	HbA1c (%)	n	Males	Females	Mean ± SD age(years)
I	<6%	112	74	38	51.5±13.6
II	6-12%	291	185	106	57.9±11.4
III	>12%	27	17	10	52.3±12.9

*p value < 0.001

Table 2 : Mean and SEM of the HbA1a % and HbA1a area in each group

PARAMETER	Group I (n =71)	Group II (n =158)	Group III (n =5)
HbA1a% (Mean ± SEM)	0.5 ± 0.06	0.4 ± 0.02	1.3 ± 0.6*
HbA1a area (Mean ± SEM)	8239.3 ± 810	7504.3 ± 418	22136 ± 14021*

Comparing HbA1a% in group I vs group II , we did not find any statistically significant difference but while comparing group I vs group III we found p value < 0.001 which is statistically significant. On comparing HbA1a area in group I vs group II , we did not find any statistically significant value but while comparing group I vs group III we found p value < 0.001 which is significant.

Table 3 : Mean and SEM of the HbA1b % and HbA1b area in each group

PARAMETER	Group I (n=112)	Group II (n =289)	Group III (n =25)
HbA1b%	2.2 ± 0.05	2.8 ± 0.04*	3.9 ± 0.1*
HbA1b area	37101 ± 1103	50980 ± 1739.4*	80056 ± 4860.6*

*p value <0.001

Comparing HbA1b% in group I vs group II & group I vs group III we find statistically significant p value. On comparing HbA1b area in group I vs group II & group I vs group III we find p value < 0.001 which is significant.

Table 4 : Mean and SD of the HbA1c % and HbA1c area in each group

Parameter	Group I (n =112)	Group II (n =291)	Group III (n=26)
HbA1c %	5.5 ± 0.4	7.9 ± 1.5*	13.6 ± 1.34*
HbA1c area	68671 ± 18274.9	105010 ±38438.2*	215970 ±50848

*p value <0.001

Comparing HbA1c% between group I vs group II and group I vs group III shows an increase which is statistically significant. On comparing HbA1c area between group I and group II as well as between group I and III there is a statistically significant increase.

On correlating different fractions with P3 fraction we found that with Hba1c% , P3% is positively correlated and it becomes stronger as we go from group I to group III. In case of HbA1a% with P3%, positive correlation is seen which is strongest in group I and it goes on decreasing as we go from group I to group III. In HbA1b, positive correlation is found with P3% but it is strongest in group II.HbA0% is negatively correlated with P3. In HbA1b, positive correlation is found with P3% but it is strongest in group II, though the correlation is not very strong.

DISCUSSION

Glycated haemoglobin A1c(HbA1c) is the well established amadori product and the role of HbA1c in the management of diabetes mellitus (DM) has been of great significance. Clinical significance of glycated minor haemoglobin sub-fractions is less studied. Detailed studies have showed sub fractions, which were named haemoglobin A1a, haemoglobin A1b, haemoglobin A1c (HbA1c), haemoglobin A0 of haemoglobin depending on the order in which they eluted from the column in HPLC. The heterogeneity of Haemoglobin arises from posttranslational modifications, mostly glycation. Glycated Haemoglobins, which are referred to in order of their elution as HbA1a1, HbA1a2, HbA1b and HbA1c result from the no enzymatic attachment of glucose (in HbA1c), fructose 1,6-diphosphate (in HbA1a1) or glucose 6-phosphate (in HbA1a2).

In this study we studied the different sub fractions of glycated haemoglobin and their relation with the glycated haemoglobin (HbA1c).From the results, we observe that HbA1a%, HbA1b%, show significant increase with HbA1c% whereas HbA0% shows significant decrease with HbA1c%.Also, HbA1a area,HbA1b area show significant increase while HbA0 area with HbA1c area . Numerous studies[8,9] have shown the increase of minor (HbA1a, HbA1b) fractions with increase in glycated fractions and decrease in HbA0 with HbA1c .That might be because as the glycation fraction increases, haemoglobin fraction being almost constant, the non glycated fraction must decrease.

Apart from glycated and nonglycated fractions seen on chromatogram for glycated haemoglobin by HPLC, one more fraction that is seen regularly is the P3 fraction. Very less is studied about this fraction. Haemoglobin A1c makes up 60% of all glycated haemoglobins (total glycated haemoglobin). Point mutation of one of the amino acids in the protein chains of the haemoglobin molecule occurs and are called haemoglobin variants. Variants can be present in the population without any significant clinical features. Hb variants are known to interfere with the estimation of glycated haemoglobin by HPLC. Elution of a particular haemoglobin from the column depends on its retention time so if retention time of the variant matches with that of the fraction, it will lead to the spurious increase in the final peak in the chromatogram According to our results, whenever there is increase in the HbA1c ,there is a corresponding increase in the P3 fraction and the P3 area and the correlation is constant as well as strong with the HbA1c area. Variants like Hb S, and Hb C, have always been known to interfere with HbA1c results but there are some silent variants that are present in the population without any clinical implications. Some variants can be Hb Camden, Hb Hope and Hb JOxford, Hb Austin, Hb N-Baltimore, Hb Fukuyama,to name a few.[10,11] We hypothesize that these silent variants which get eluted in less studied fraction windows like P3 window of the chromatogram which might lead to overestimation or underestimation of final HbA1c value. More so, studies should be taken up to study the influence of other fractions of haemoglobin on the final value of HbA1c for the efficient assessment of glycaemic status of the patient.

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