Original Article

DOI: 10.7860/JCDR/2016/21092.8451

P3 Fraction: Effect on HbA1c Values by HPLC

Biochemistry Section

MRINAL GUPTA[1], MOHIT THALQUOTRA[2], PRAGNA RAO[3]

ABSTRACT

Introduction: The availability of the haemoglobin A1c test has been a major advance in diabetic care and its measurement has become an integral part for the management of diabetes. When glycated haemoglobin (HbA1c) is estimated by High Performance Liquid Chromatography (HPLC), one of the fractions that are eluted is known as "P3" fraction which is labelled as "degenerated haemoglobin". The P3 fraction is not taken into account while estimating HbA1c or HbA values.

Aim: To study the effect of P3 on the final reported value of HbA1c by HPLC and the significance of this fraction in the final chromatogram. The possibility that HbA1c fraction is under reported due to increase in P3 fraction was also examined.

Materials and Methods: 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% (group I - <6%, group II - 6 – 12%, group III - >12%).

Result: P3% as well as P3 area increases as the HbA1c increases (p-value - <0.001). Statistical significant increase was seen as HbA1c% increases, P3% also increases and correlation (r = 0.6) became stronger with increasing HbA1c levels. HbA1a%, HbA1b% fraction increases with increase in HbA1c% and HbA1a area, HbA1b area also shows the same increase with increasing HbA1c%.

Conclusion: From this study we conclude that 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 haemoglobin in the sample.

Keywords: Glycated haemoglobin, HbA1a%, HbA1b%, Variant haemoglobins

INTRODUCTION

Glycated haemoglobin (HbA1c) most accurately reflects the previous two to three months of glycaemic control [1]. Glycated haemoglobin arises from the irreversible non enzymatic attachment of glucose to one or both N-terminal valines of the haemoglobin β chain [2,3]. High-Performance Liquid Chromatography (HPLC) is currently considered the reference method of the DCCT (Diabetes Control and Complications Trial) and the method of choice of the National Glycohaemoglobin Standardisation Program [4,5]. HPLC provides adequate throughput and improved precision as compared to immune turbidimetric method. HPLC estimates HbA1c by generating the peaks in the chromatogram for different fractions of haemoglobin 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 [6]. P3 denotes the level of degraded haemoglobin and it should ideally be less than 10% for reporting the value of glycated haemoglobin. It is formed due to the post translational modification of adult haemoglobin and the levels rise as the samples gets older or more of the haemoglobin is degraded; the level of post translational modifications further increases with the increase in blood glucose concentration inside the Red Blood Cells (RBC) [7]. While 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 reverse phase HPLC. The aim of this study was to assess the effect of different fractions on the HbA1c and the effect of P3 fraction on the final reported value of HbA1c.

MATERIALS AND METHODS

The retrospective clinical study was conducted in Department of Biochemistry, Kasturba Medical College, Manipal from March 2013-November 2013. All the samples received in the laboratory from March 2013-November 2013 for HbA1c estimaton were included in the study and the HbA1c chromatograms were assessed [Table/Fig-1].

Study was conducted after obtaining the ethical committee clearance. Serum samples analysed for glycated haemoglobin were anonymised and retrospective data was analysed. Data was collected from the graphs obtained on analysis of EDTA blood sample for glycated haemoglobin using BIORAD VARIANT II turbo in 430 subjects. Variant II turbo is fully automated and works on the principle of automated cation exchange HPLC. The final report was presented visually and the area under each peak of chromatogram was integrated to provide percentage of each fraction present in the sample as well as area under each curve. Peaks were differentiated based on retention times of the component in the column. Peak area is the absorption units in μvolts/second of the analyte at 415 nm. Area % is the percent area of the analyte as a fraction of the samples total area.

A total of 430 samples were divided into 3 groups based on HbA1c levels. Patient data was divided into three groups based on HbA1c levels. Group I had patients with glycated haemoglobin <6.5%, group II with glycated haemoglobin 6.6 -12% and group III had glycated haemoglobin >12% [Table/Fig-1].

The following criteria were followed for acceptance of HbA1c report:

Total area count should be within 1 million – 3.5 million. HbF should be less than 5 %. HbA1c should be within reportable range (3.5-19.0%).

Groups	HbA1c (%)	n	Males	Females	Mean 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

[Table/Fig-1]: Demography of the subjects.

STATISTICAL ANALYSIS

Statistical analysis was done using SPSS v 16.0. Analysis of result was done by ANOVA and multiple regression analysis. The p-value <0.05 was taken as statistically significant and p value <0.001 was taken as highly significant. Pearson's correlation test was done to assess correlation between different parameters.

RESULTS

As seen from [Table/Fig-2], P3% showed statistically significant increase (p<0.001) in group III compared to group I as well as in group II compared to group I. P3 area increased from group I to group II to group III but the increase was not statistically significant. When glycated haemoglobin levels increased, P3 levels also increase and so did the P3 area. HbA1a% was significantly increased (p<0.001) in group III compared to group I, but the increase was not statistically significant between group II and group I. HbA1a area was significantly increased in group III compared to group I, but the difference was not significant in group II compared to group I. HbA1b% was significantly increased from group I to group II to group III and same was seen with HbA1b area.

On correlating different fractions with P3 fraction we found positive correlation of P3% with HbA1c% in group I (r=0.1), group II (r=0.4) and group III (r=0.6). As the HbA1c% increases, P3% also increases and correlation became stronger with increasing HbA1c levels. P3% showed positive correlation with HbA1a% in group I (r=0.6), group II (r=0.3) and group III (r=0.3). P3% showed positive correlation with HbA1b% also in all the groups; group I (r=0.01), group II (r=0.4) and group III (r=0.1).

On correlating different fractions with P3 area, we found positive correlation of only in group III. P3 area was positively correlated with HbA1c area (r=0.2), P3 area with HbA1a area (r=0.5) and P3 area with HbA1b area (r=0.2) only in group III.

PARAMETER	GROUP I (HbA1c <6.5%)	GROUP II (HbA1c 6.6-12%)	GROUP III (HbA1c >12%)
HbA1c % (mean ± SD)	5.5 ± 0.4 (n = 112)	7.9 ± 1.5* (n = 268)	13.6 ± 1.34* (n = 50)
HbA1c area (mean ± SD)	68671 ± 18274.9 (n = 112)	105010 ± 38438.2* (n = 268)	215970 ± 50848* (n = 50)
P3 % (mean ± SD)	3.7 ± 0.6 (n = 112)	4.4 ± 0.8* (n = 268)	5.6 ± 0.9* (n = 50)
P3 area (mean ± SD)	72029 ± 26559 (n = 112)	73632 ± 20229.4 (n = 268)	74782 ± 25572.1 (n = 50)
HbA1a% (mean ± SEM)	0.5 ± 0.06 (n = 71)	0.4 ± 0.02 (n = 113)	1.3 ± 0.6* (n = 50)
HbA1a area (mean ± SEM)	8239.3 ± 810 (n = 71)	7504.3 ± 418 (n = 113)	22136 ± 14021* (n = 50)
HbA1b% (mean ± SEM)	2.2 ± 0.05 (n = 112)	2.8 ± 0.04* (n = 264)	3.9 ± 0.1* (n = 50)
HbA1b area (mean ± SEM)	37101 ± 1103 (n = 112)	50980 ± 1739.4* (n = 264)	80056 ± 4860.6* (n = 50)

[Table/Fig-2]: Various fractions obtained on HPLC.
*p value is significant (<0.05)

DISCUSSION

According to our results, there was increase in all the fractions including P3 fraction with increase in HbA1c levels. Also, the correlation of P3% increased with increase in the levels of glycated Hb. Glycated haemoglobin is a well characterized Amadori product. Labile intermediate aldimine or Schiff base is formed that undergoes an Amadori rearrangement to form stable ketoamine [7]. Clinical significance of glycated haemoglobin sub fractions is very less investigated. Depending on the order in which they elute from the column in HPLC subfractions were named as haemoglobin A1a, haemoglobin A1b, haemoglobin A1c of haemoglobin [4]. Glucose utilization in the body is by way of entering into the body cells and undergoing glycolysis. This leads to the formation of various products of glycolysis and these products which are formed in the glycolytic pathway of the cells are eluted as different fractions on the HPLC column. Glycated haemoglobin result from the non-enzymatic attachment of glucose (in HbA1c), fructose 1, 6-diphosohate (in hbA1a) or glucose-6-phosphate in (in HbA1b).

From the results, we observed that HbA1a%, HbA1b%, show significant increase with HbA1c% or as the HbA1c increases these fractions also show a significant increase. Numerous studies have shown the increase of minor haemoglobin fractions (HbA1a, HbA1b) with increase in glycated fractions [8,9].

Apart from glycated fractions seen on chromatogram by HPLC, one more that was seen regularly was the P3 fraction. Very less is studied about this fraction of haemoglobin. P3 fraction signifies the degraded haemoglobin in the sample. Erythrocyte contains several proteolytic enzymes which can degrade oxidatively damaged haemoglobin which can be considered as a natural Fenton reaction with its Fe^{2+} and hence prone to oxidative damage [10]. Increased oxidative stress may also be contributed by autoxidative glycosylation and the release of free radicals by glycated proteins. Apart from glycated proteins; free glucose, unattached to proteins, in the presence of transition metals can generate free radicals and hydrogen peroxide further contributing to the oxidative damage of the haemoglobin [7]. Oxidant damage of haemoglobin thus makes it vulnerable to degradation, fragmentation and conformational change by proteolytic enzymes inside the erythrocytes [11]. Simultaneously, antioxidants such as reduced forms of glutathione, Vit E are lowered [12] and the activity of erythrocyte Cu-Zn SOD is decreased with increased blood glucose as seen in diabetes [13]. A combination of increased oxidant production and decreased anti-oxidant defences in the RBCs in diabetes leads to subsequent degradation of haemoglobin. Hence, there is every possibility that P3 fraction which denotes degraded fraction of haemoglobin in the sample may well be due to the oxidative damage of haemoglobin and subsequent proteolytic degradation of haemoglobin which depends on the glucose level of the patient. Normal acceptable cut off value of P3 fraction in the sample to report the value of HbA1c is <10%. Abnormal haemoglobin can also lead to increased P3 levels. Abnormal haemoglobin or variants are the haemoglobin with point mutation of one of the amino acids. Some of these variants are found to elute in the P3 window of chromatogram. These variants can be Hb Camden, Hb Hope, Hb J Oxford, Hb Austin, Hb N-Baltimore to name a few [14,15]. These are silent variants and do not present clinically but lead to spurious elevation of P3 fraction and hence the spuriously increased final HbA1c values.

This study emphasizes the role of erythrocytic proteolytic degradation of haemoglobin while focusing that it increases with increase in glycated haemoglobin levels and thus the glycaemic status. Furthermore, abnormal haemoglobin should always be kept in mind while interpreting the final values of HbA1c by HPLC.

LIMITATION

Our study uses the samples from a particular geographical region. Further studies with larger sample size and with samples from different geographical regions should be taken up to substantiate the findings of our study.

CONCLUSION

P3 fraction affects the final reported value of HbA1c by HPLC. Presence of P3 fraction may indicate the presence of variant haemoglobin in the sample which can lead to misinterpretation of the HbA1c results and compromise in patient care.

REFERENCES

[1] Shanthi B, Revathy C, Manjula Devi AJ, Subhashree. Effect of iron deficiency on glycation of haemoglobin in nondiabetics. *J Clin Diagn Res*. 2013;7(1):15-17.
[2] Lahousen T, Roller RE, Lipp RW, Schnedl WJ. Silent haemoglobin variants and determination of HbA1c with the HPLC Bio-Rad Variant II. *Journal of Clinical Pathology*. 2002;55(9):699-703.

[3] Hoelzel W, Weykamp C, Jeppsson JO, Miedema K, et al . IFCC reference system for measurement of haemoglobin A1c in human blood and the national standardization schemes in the United States, Japan, and Sweden: a method-comparison study. *Clin Chem*. 2004;50(1):166-74.
[4] Weykamp C, Garry John W, Mosca A. A review of the challenge in measuring haemoglobin A1c. *Journal of diabetes science and technology* (Online). 2009;3(3):439-45.
[5] Chandalia HB, Krishnaswamy PR. Glycated haemoglobin. *Current Science*. 2002;83(12):1522-32.
[6] Little RR, Rohlfing CL, Tennill AL, Connolly S, et al. Effects of sample storage conditions on glycated haemoglobin measurement: Evaluation of five different high performance liquid chromatography methods. *Diabetes Technology & Therapeutics*. 2007;9:36–42.
[7] Raghothama C, Rao P. Degradation of glycated haemoglobin: Role of erythrocytic proteolytic enzymes and oxidant damage. *Clin Chim Acta.* 1997;264(1):13-25.
[8] Imagawa S, Abe T, Kimura S, Sugita Y, Yamashita K. Studies on subfractions of haemoglobin A1b & haemoglobin A1c in diabetic subjects. *Endocrinol Japon*. 1984;31(6):725-31.
[9] Saiedullah M, Hayat S, Kamaluddin SM, Begum S. Association of glycated minor haemoglobin fraction of A1 with microalbuminuria and glycaemic status in diabetic subjects. D*iab Endocr* J. 2012;40(1):14-16.
[10] Watkins JA, Kawanishi S, Caughey WS. Autoxidation reactions of haemoglobin A free from other red cell components: a minimal mechanism. *Biochem Biophys Res Commun.*1985;132:742-48.
[11] Ragothama C, Rao P. Degradation of glycated haemoglobin by erythrocytic proteolytic enzymes. *Clin Chem Acta*. 1996;245:201-08.
[12] Jain SK, Levine SN, Duett J, Hollier B. Reduced vitamin E and increased lipofuscin products in erythrocytes of diabetic rats. *Diabetes*. 1991;40:1241-44.
[13] Taniguchi N. Clinical significance of SOD changes in ageing, diabetes, ischaemia and cancer. *Adv Clin Chem.* 1992;29:1-59.
[14] Joutovsky A, Hadzi-Nesic J, Nardi AM. HPLC retention time as a diagnostic tool for haemoglobin variants & haemoglobinopathies: A study of 60,000 samples in a clinical diagnostic laboratory. *Clinical Chemistry.* 2004;50(10):1736-47.
[15] Schnedl WJ, Lahousen T, Wallner SJ, Krause R, Lipp RW. Silent haemoglobin variants and determination of HbA1c with the high resolution program of the HPLC HA-8160 haemoglobin analyser. *Clinical Biochemistry.* 2005;38(1):88-91.

PARTICULARS OF CONTRIBUTORS:
1. Senior Resident, Department of Biochemistry, National Institute of Tuberculosis and Respiratory Diseases, New Delhi, India.
2. Demonstrator, Department of Biochemistry, Govt. Medical College, Jammu, Jammu and Kashmir, India.
3. Associate Dean and Professor, Department of Biochemistry, Kasturba Medical College, Manipal University, Manipal, Karnataka, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:
Dr. Pragna Rao,
Associate Dean and Professor, Department of Biochemistry, Kasturba Medical College,
Manipal University, Manipal, Karnataka, India.
E-mail: drpragnarao@gmail.com

FINANCIAL OR OTHER COMPETING INTERESTS: None.

Date of Submission: **May 03, 2016**
Date of Peer Review: **Jul 01, 2016**
Date of Acceptance: **Aug 04, 2016**
Date of Publishing: **Sep 01, 2016**