Original Article

DOI: 10.7860/NJLM/2022/52355.2584

Biochemistry Section

Unknown Peaks and Spuriously Low Values of Glycated Haemoglobin by High Performance Liquid Chromatography: A Cross-sectional Survey

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ABSTRACT

Introduction: Glycated haemoglobin (HbA1c) has humongous role both in diagnosis as well as in guiding the clinicians in making therapeutic decision in diabetic patients. There are various methods of laboratory estimation of glycated haemoglobin depending on charge and affinity. However, the methods are affected by a number of factors like haemoglobin variants, drugs and erythrocyte survival.

Aim: To identify and evaluate abnormal peaks in chromatogram of High Performance Liquid Chromatography (HPLC) and compare it with boronate affinity chromatography and it also assessed the interference of elution of silent haemoglobin variants or drugs in exactness of HbA1c estimation by HPLC.

Materials and Methods: A cross-sectional survey was conducted in the laboratory of Purwanchal Nagarik Samity, Kolkata, West Bengal, India, from November 2020 to August 2021, where 2500 samples were evaluated for HbA1c estimation. Abnormal variant window, unknown peaks or spuriously low HbA1c were identified in eight samples, where estimation was done by HPLC. These were processed further to investigate for the haemoglobin variants. The glycated haemoglobin in those samples was further estimated by Boronate affinity chromatography.

Results: Out of the eight samples, three patients were Haemoglobin E trait, two were Haemoglobin E homozygous and one had sickel cell trait. However, two samples showed a normal chromatogram in HbA2/HbF mode. The glycated haemoglobin was affected by Haemoglobin E, sickel cell traits as well as other interferences that may cause inappropriate lowering of glycated haemoglobin.

Conclusion: The results of this study and literature review suggests the progression of various micro/macrovascular complications in diabetic individuals can be arrested by maintaining normal HbA1c levels. Therefore, the laboratory consultant should meticulously take into consideration factors like plasma glucose concentration, drug history, biological variation or abnormal haemoglobinopathies. Thus, every laboratory catering to the huge burden of diabetic patients, where the prevalence of haemoglobinopathy is high, should individualise the method of HbA1c estimation.

Keywords: Affinity chromatography, Diabetic patients, Haemoglobin E, Sickle cell trait

INTRODUCTION

Glycated haemoglobin (HbA1c) is produced by the non enzymatic glycation of N-terminal valine residue of β-globin chain of the adult variant of Haemoglobin (HbA) [1]. With the escalation of the number of diabetic patients, the role of HbA1c, both as a diagnostic and prognostic marker for assessment of preceding two to three months glycaemic control as well as a predictor of microvascular complications is humongous [2,3]. Laboratory estimation of glycated haemoglobin is done by utilising the variations in charges (HPLC and capillary electrophoresis), structural characteristics (boronate affinity) and chemical reactivity (immunoturbidimetry and enzymatic methods). The HPLC, though the most commonly used method for glycated haemoglobin estimation, is interfered by the abnormal haemoglobin variants like Haemoglobin E (HbE), Haemoglobin S (HbS) [4]. In recent times, Electronspray Ionization Mass Spectrometry (ESI-MS) has evolved as the reference method for estimation of HbA1c yet the cost per test and technical sophistication are the obstacles faced in routine laboratories of developing countries like India [5].

Though the immunoassay or boronate affinity chromatography is free from meddling effects of abnormal haemoglobin variants but as erythrocyte survival is being altered, the role HbA1c as a glycaemic control indicator remains questionable [6]. Labile HbA1c or pre-HbA1c, the aldimine Schiff base, though eluted as a separate fraction in HPLC may sometimes result in abnormal high fractions due to some abnormal haemoglobin variants. Literature review has suggested that spuriously high or low HbA1c, unknown peaks of haemoglobin variants alters the exactness of HbA1c estimation and resulted in misinterpretation of the chromatogram [7]. There are a list of drugs like dapsone, antiretrovirals that causes low HbA1c values due to drug induced haemolysis [8,9]. There are abnormal variants which co-elutes with glycated haemoglobin, like haemoglobin hope that causes overestimation of HbA1c and spuriously high HbA1c almost in the diabetic range [10]. In cases of HbS, HbE, the net charge of the haemoglobin particularly the glycated N-terminus is affected, resulting in erroneous estimation of HbA1c by HPLC.

However, these abnormal variants can easily be identified by abnormal windows/peaks in HPLC. But, interferences of some drugs on these methods may cause underestimation of HbA1c in individual with other healthy erythrocyte survival. The burden of diabetic individual and simultaneously the demand of HbA1c is increasing in India too. But, the prevalence of haemoglobinopathy remains unaltered. However, diabetic experts opine fructosamine to be a better estimate of glycaemic status in patients with abnormal haemoglobin variants and pregnancy [11]. But the cost-efficacy of fructosamine, keeps HbA1c as the gold standard. Thus, the effect of each variant must be examined with each methodology of glycated haemoglobin estimation. The purpose of this study was to evaluate the abnormal peaks in chromatogram of HbA1c and to find any interference on measurement of HbA1c pertinent to a particular methodology. This study may warrant every laboratory catering a huge burden of diabetic patients, where the prevalence of haemoglobinopathy is high, to individualise the method of HbA1c estimation.

MATERIALS AND METHODS

This cross-sectional survey was done in the laboratory of Purwanchal Nagarik Samity, Kolkata, West Bengal, India, after receiving appropriate ethical clearance (Vide Memo.no: PNS/02-11/2020 dated 02/11/2020) from November 2020 to August 2021. The laboratory processes over 2500 samples for HbA1c estimation almost yearly.

Study Procedure

After receiving consent from the patients, 2 mL of venous blood was collected from study participants in a Potassium Ethylenediamine Tetra-acetic Acid (K2EDTA) vial maintaining standard aseptic procedure. However, among the 2500 samples, few were outsourced from other laboratories too. In this instance, there were eight cases out of 2500 where accurate estimation could not be done by HPLC due to unknown peaks or variant window. The HPLC was performed using a BIORAD D-10 analyser. The glycated haemoglobin estimation was done using the Boronate affinity chromatography by the NycoCard™ HbA1c reader (NGSP certified). However, in one sample estimation could not be reported by boronate affinity too.

STATISTICAL ANALYSIS

Descriptive statistics were used to analyse the findings of the study and the values are presented as percentages.

RESULTS

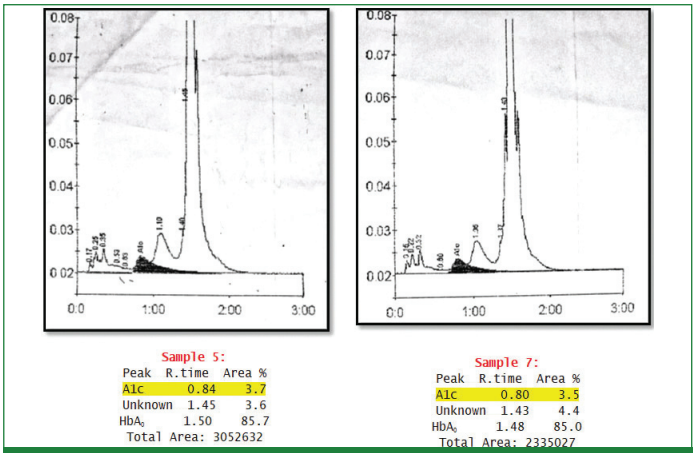
The samples which showed abnormal peaks by BIORAD D10 were subsequently analysed by boronate affinity chromatography (Nyocard) and reportable values were observed [Table/Fig-1]. Moreover, the samples were subsequently analysed by HbA2/HbF to find the abnormal haemoglobin variant. Out of the eight samples, three samples had haemoglobin E trait, two were haemoglobin E homozygous and one demonstrated sickle cell trait. However, interestingly two samples showed a normal chromatogram in HbA2/HbF mode. However, these samples had low HbA1c peak and unknown peaks in the chromatogram [Table/Fig-2]. In addition to this, these samples were further processed by capillary electrophoresis and a normal chromatogram was observed [Table/Fig-3].

Sl. No.	Sample Id	Age/Gender	HbA1c by HPLC (%)	HbA1c by boronate affinity (%)	HbA2/HbF mode
1.	Sample no. 1	54 y/Male	3.5	5.7%	HbE carrier/trait
2.	Sample no. 2	39 y/Male	Not detected	6.5%	HbE Homozygous
3.	Sample no. 3	47 y/Female	3.6	5.5%	HbE carrier/trait
4.	Sample no. 4	59 y/Male	Not detected	6.9%	HbE Homozygous
5.	Sample no. 5	58 y/Female	3.7	6.5	Normal Chromatogram
6.	Sample no. 6	34 y/Female	5.8	6.1	HbS carrier/trait
7.	Sample no. 7	28 y/Female	3.5	5.8	Normal Chromatogram
8.	Sample no. 8	61 y/Male	4.2	6.1%	HbE carrier/trait

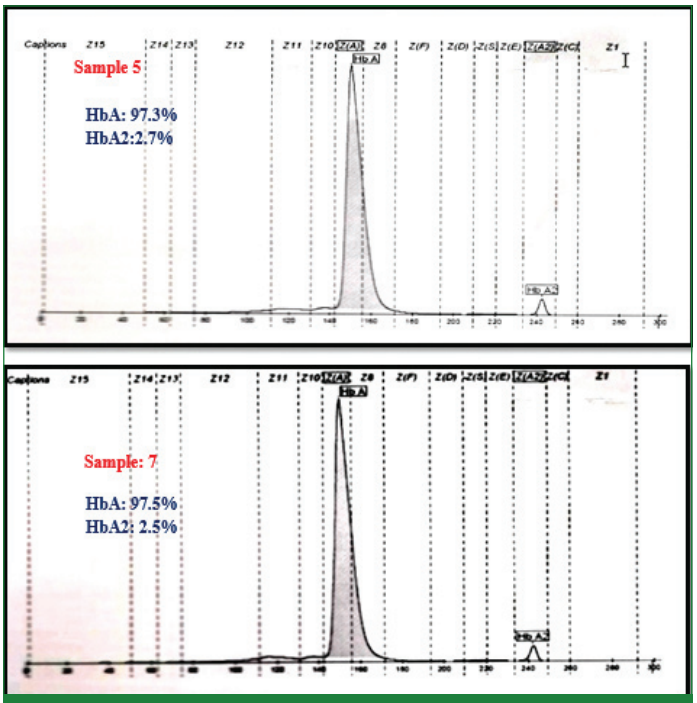
[Table/Fig-1]: Showing the age, gender and glycated haemoglobin values of different samples as measured by different methods.

DISCUSSION

In this study, Sample no. 1,3,8 were all diabetic patients and came for routine testing. Their HPLC suggested chromatogram which was perfectly normal except all of them had a variant window after HbA with retention time between 1.52-1.64 seconds. The variant window of all the four patients was less than 30% area count. These samples were further analysed with the BIORAD10 analyser and HbA2 in all the patients were less than 30%. In the cases number 2 and 4, HbA1c was estimated for the first time for evaluation of



[Table/Fig-2]: Showing the chromatogram of HbA1c of spuriously low haemoglobin.



[Table/Fig-3]: Showing the normal chromatogram observed after processing samples by capillary electrophoresis.

hyperglycaemia. In both the patients, HbA1c could not be estimated by HPLC and had a variant window >90% at retention time of 1.56 seconds. These two cases were diagnosed as HbE homozygous by HPLC evaluation on HbA2/HbF mode.

HbE variant, the second most prevalent haemoglobinopathy, is mostly found in North eastern India, arises due to replacement of glutamic acid residue at 26th postion of β globin chain by lysine. A research by Little RR et al., suggests that this mutation results in an underestimation of HbA1c by HPLC in comparison to boronate affinity chromatography and Immunoturbidimetry [12]. The sample number six had a variant S-window and was diagnosed as sickle cell trait. However, the rationale and results of HbA1c estimation by HPLC and immunoassay is debatable. The raised HbF fraction and the mutation causes erroneously over estimation of HbA1c in sickle cell heterozygous individuals. The chromatogram of spuriously low HbA1c patients (case five and case seven) were meticulously investigated and compared with an absolutely normal chromatogram. The area count of both the abnormal chromatogram was within the acceptable limits (1 million to 5 million). The baseline of the chromatogram was within acceptable limits.

Moreover, the P3 was less than 8%, meaning that the sample quantity did not deteriorate. The samples were analysed by HbA2/HbF method to exclude any structural haemoglobinopathy. A succinct analysis of pros-cons of various methods used for estimation of glycated

haemoglobin has suggested that HbA1c values may be spuriously elevated or reduced, depending of the type of methodology and presence of associated haemoglobinopathy. In scenarios, where there is concomitant elution of abnormal haemoglobin fractions with haemoglobin A variant may produce false low values [8]. Haemoglobin J-Baltimore, is one such variant of haemoglobin, where there is minimal functional alteration of HbA and this altered variant does not yield a separate peak in chromatogram [9,13]. However, to confirm this both the patients were investigated by capillary electrophoresis. The capillary electrophoresis was normal. Both the patients were investigated, the sample number five was of female patient of 58 years. This patient was suffering from Type 2 Diabetes Mellitus (DM) and the value of 6.5% corroborated with her glycaemic control. However, the patient was on low dose aspirin for last five months.

A few studies have shown that chronic use of Aspirin reduces the rate of glycation and causes low HbA1c, however, on the contrary, some studies have suggested that Aspirin has no interference on HbA1c estimation by cation exchange HPLC [14-17]. Low dose aspirin is recommended to arrest primary and secondary cardiovascular complication in diabetics. However, this aspirin induces an acetylation of haemoglobin thereby increasing the net negative charge. However a randomised control trial by Camargo JL et.al suggested that Aspirin has no interference on HbA1c estimation by cation exchange HPLC [18]. This warrants a study with a large sample size. The sample number of 7 was that of a pregnant patient and was a part of routine antenatal protocol. A physiological rise of HbF is not so exubarent to effects the chromatogram. No significant drug history was found in this patient. However, there was a tailing of the peak of HbA1c followed by an unknown peak. This may be due to some unknown interferences.

This study creates a scope for investigation of interferences of drugs like aspirin in glycated haemoglobin estimation by various methods as well as warrants further studies on effect of pregnancy on various methods of HbA1c estimation. Hence, after focusing on all methods we found that the abnormally low level of haemoglobin A1c fraction may be due to some interference that results in misinterpretation of results.

Limitation(s)

Literature review suggests that drug has a strong interference in HbA1c estimation. However, due to the limitations imposed by small sample size the findings of the study cannot be generalised.

CONCLUSION(S)

In conclusion, not a single method of glycated haemoglobin estimation is free from interferences. In reporting of HbA1c by HPLC, the pathologist/biochemist should be meticulous to consider the factors like plasma glucose concentration, drug history or abnormal haemoglobinopathies. However, some Haemoglobin variants are silent, they may lead to underestimation and over estimation of HbA1c misguiding the physician in treatment of diabetes. Moreover, in a country like India where the burden of diabetic individuals is escalating and haemoglobinopathy is also common, the method of HbA1c should be individualised. Thus, every laboratory catering diabetic patients should make a data bank of concomitant haemoglobinathy in the diabetic patients, if any. This will prevent the misinterpretation of test results and guide the clinician in appropriate decision making.

Acknowledgement

The authors is grateful and sincerely acknowledge the inspiration to authors/editors/publishers of all those articles/journals/books from where literatures for the discussion have been collected and cited. The author of this article is immensely indebted to Dr. Indranil Basu, HOD Clinical Chemistry and Quality Manager, Unipath Speciality Laboratory Ltd., Kolkata for arrangement of Capillary Electrophoresis in SebiaCapillays2.

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PLAGIARISM CHECKING METHODS: [Jain H et al.]
- Plagiarism X-checker: Sep 11, 2021
- Manual Googling: Nov 20, 2021
- iThenticate Software: Dec 31, 2021 (2%)

ETYMOLOGY: Author Origin

AUTHOR DECLARATION:
- Financial or Other Competing Interests: None
- Was Ethics Committee Approval obtained for this study? Yes
- Was informed consent obtained from the subjects involved in the study? Yes
- For any images presented appropriate consent has been obtained from the subjects. NA

Date of Submission: **Sep 10, 2021**
Date of Peer Review: **Oct 26, 2021**
Date of Acceptance: **Nov 21, 2021**
Date of Publishing: **Jan 01, 2022**