Original Article

Assay Interferences in HbA1c Measurements : Effect of Hemoglobinopathies and Elevated Fetal Hemoglobin in the BioRad D10

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Introduction : HbA1c or Glycated Hemoglobin, is an invaluable marker in diabetes management and of late in its diagnosis as well. Of the many modalities of testing HbA1c, the High Pressure Liquid Chromatography (HPLC) method maintains wide acceptance and popularity amongst clinicians. We aim to specifically elucidate the interferences caused by Hemoglobinopathies in the BioRad D10 HPLC system.

Methods : HPLC chromatograms of a predominantly adult population visiting a tertiary care hospital and tested between September, 2016 and December, 2017 on the BioRad D10 (Short Program) were retrospectively studied. A few representative chromatograms of each variant type were analyzed separately and reported to illustrate the laboratory's experience in dealing with known interferences on the HPLC system.

Results : HPLC chromatograms for HbA1c quantification (n=6015) yielded 151 abnormal or suspected 'abnormal' peaks (2.5%). Abnormal peaks encountered were high P3 peak (n=2), borderline high HbF (n=1), HbS trait (n=5), Unknown/Variant peak (unidentified Hemoglobinopathy; n=140) and no HbA1c (homozygous/double heterozygous condition with none or little HbA; n=3).

Conclusion : The laboratory may encounter several hemoglobin variants depending on the patient population it serves. Careful scrutiny of chromatograms may help identify the presence of aberrant peaks produced by variants. The HbE, HbD, HbS and HbC traits do not interfere with HbA1c results on the BioRad D10 short program. Others can interfere. Patients with a Homozygous Hemoglobinopathy will show no HbA1c result and alternate methods are required to ascertain glycemic control. Laboratorians need to be aware of the limitations and strengths of their methods of reporting HbA1c especially with an increasing workflow of the biomarker. A laboratory – clinician interaction becomes essential for solving cases that do not align to the apparent clinical picture.

[*J Indian Med Assoc* 2021; **119(12):** 15-8]

***Key words* : HbA1c, BioRad D10, Interference, Hemoglobin variants.**

Six percent of the total adult Hemoglobin (HbA) is HbA1, which comprises of HbA1a1, HbA1a2, HbA1b and HbA1c fractions, each attached to a different chemical moiety and each defined by their electrophoretic traits. The HbA1c fraction is the most abundant of these (in health it makes up about 5% of the total HbA), wherein the circulating glucose molecule gets attached to the beta globin chain of hemoglobin. Thereby, analysis of glycated hemoglobin or HbA1c in blood provides information about an individual's weighted average of blood glucose of the past 100 days - roughly the predicted half life of the red blood cells.

The HbA1c test has been included in 2009 by the American Diabetes Association as one of the first line diagnostic criterion of Diabetes Mellitus, as a possible substitute of the fasting blood glucose test. This inclusion, along with its established reliability in indicating long term glycemic control and prediction of diabetic complications, has catapulted the single HbA1c test as an invaluable tool to the clinicians, for the care of the general population at large.

Editor's Comment :

- Hemoglobinopathies other than HbE, HbD, HbS and HbC traits can interfere in the HbA1c assay on BioRad D10 short program
- Patients with a Homozygous Hemoglobinopathy will show no HbA1c result and alternate methods are required to ascertain glycemic control.
- A laboratory – clinician interaction becomes essential for solving cases that do not align to the apparent clinical picture.

HbA1c can be measured by at least 30 different laboratory methods. Each method has its pros and cons. The HPLC system maintains wide popularity and acceptance among-st clinicians owing to its sole use in the Diabetes Control and Complications Trial (DCCT) and UKPDS trials, wherein changes in HbA1c results were equated to a large alteration in the risk of diabetes complications in patients with type 1 or type 2 diabetes. Moreover, all HbA1c methods now need to be National Glycohemoglobin Standardization Program

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Received on : 12/09/2019
Accepted on : 01/10/2019

(NGSP) certified; implying that results by any method should be equivalent to that reported in the DCCT and UKPDS.

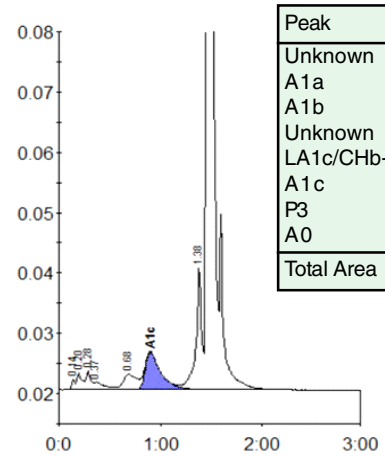
It is the attempt of this article to bring forth the considerations that laboratorians and clinicians need to give while evaluating the HbA1c report on the BioRad D10 HPLC system with special reference to interferences caused by hemoglobin variants.

Materials and Methods

HPLC chromatograms of a predominantly adult population visiting a tertiary care hospital and tested between September, 2016 and December, 2017 on the BioRad D10 (Short Program) were retrospectively studied. BioRad D10 is a standalone instrument based on the principle of cation exchange HPLC for the separation of hemoglobin fractions. These are eluted sequentially based on the properties of size and charge and analyzed for concentration using an inbuilt spectrophotometer at 415 nm. For normal hemoglobin fractions and hemoglobin variants, we recorded and analyzed their retention times, their proportion of the total Hemoglobin (%) and the peak characteristics. Chromatograms with 'Variant' window indicate the presence of an unknown hemoglobin variant. HbS and HbC are reported clearly when detected in the D10 short program. A few representative chromatograms of each variant type were analyzed separately and reported to illustrate the laboratory's experience in dealing with known interferences on the HPLC system.

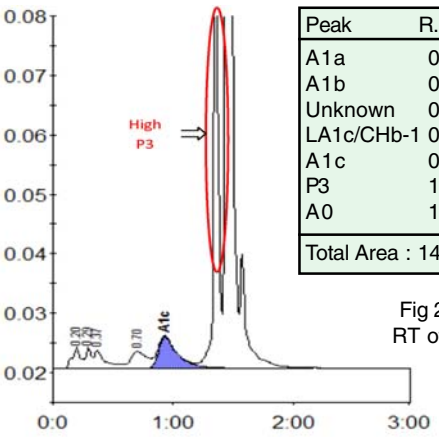
Results

High-performance Liquid Chromatography (HPLC) chromatograms for HbA1c quantification (n=6015) yielded 151 abnormal or suspected 'abnormal' peaks (2.5%). The types of such chromatograms are outlined in Table 1 and



Peak	R. time	Height	Area	Area%
Unknown	0.14	1786	4254	0.3
A1a	0.20	2865	10278	0.8
A1b	0.28	3103	9752	0.7
Unknown	0.37	1266	10032	0.7
LA1c/CHb-1	0.68	2572	23496	1.8
A1c	0.90	6232	66286	6.8
P3	1.38	20260	81517	6.1
A0	1.46	333296	1134396	84.7
Total Area	340011			

Fig 1 — A normal HPLC chromatogram. HbA1c peak (shaded) has eluted at retention time of 0.9; value is 6.8 % of total hemoglobin



Peak	R. time	Height	Area	Area%
A1a	0.20	3658	17579	1.2
A1b	0.29	3593	11657	0.8
Unknown	0.37	3128	18095	1.3
LA1c/CHb-1	0.70	2909	28803	2.0
A1c	0.93	5343	58526	5.8
P3	1.36	73282	206925	14.6
A0	1.44	353627	1078823	76.0
Total Area : 1420409				

Fig 2 — High P3 (>10%) eluted at RT of 1.36, concentration is 14.6% of total hemoglobin

Table1 — *Summary of Abnormal peaks and their characteristics with interpretation on interference in the HbA1c result*

Abnormal Peak	Retention Time (Average, SD)	% Variant of Total Hemoglobin (Average, SD)	Interpretation
P3 > 10% N=2	1.36 (0)	14.3 (0.42)	P3 elevation beyond 10% suggests presence of unknown variant and may affect HbA1c results, interference unknown
HbF = 9.3% N=1	0.51	9.3	HbF near high threshold value of 10%. Interference cannot be ruled out.
S-Window (<60%) HbS Trait N=5	1.65 (0.02)	27.84 (4.34)	HbAS condition. No interference in HbA1c result.
Unknown peak N=18	1.55 (0.02)	30.27 (8.40)	Likely heterozygous condition of unknown hemoglobinopathy. May affect HbA1c results, interference unknown
Variant Window N=122	1.58 (0.019)	29.71 (3.1)	Same as above
No HbA1c Peak N=3	Variant may elute in the HbA0, or as Variant/Unknown peak	>60%, See Chromatogram (Fig 5)	% HbA1c = 0. HbA1c cannot be reported. HbA absent. Likely homozygous or double heterozygous condition

Retention time (RT, min) is the amount of time a particular hemoglobin type spends on the cation exchange column after it has been injected onto the column. It is highly characteristic of the hemoglobin and helps in its identification, NA = Not Applicable

representative chromatographs follow with findings.

Elution chromatograms of patient specimens on the Bio-Rad D10 HPLC System using the Short Program are presented in Figs 1-5. X axis represents the retention time in minutes for each fraction to elute. The Retention Time (RT) for each fraction is shown with the peak.

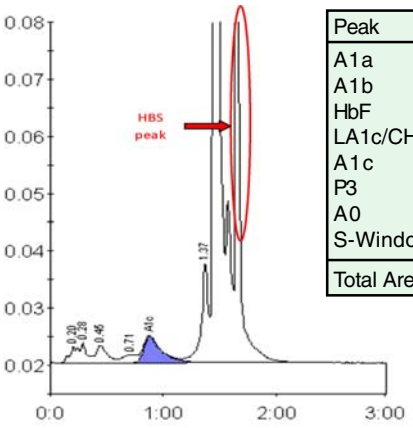
Unknown/ Variant peak detected may interfere with the HbA1c result. If the variant is HbE or HbD, it does not interfere with the HbA1c result on the D10 short program as shown in Fig 4.

Homozygous or double heterozygous conditions can occasionally be detected when evaluating for HbA1c. In such cases, HbA is absent and therefore HbA1c is not detected (Fig 5).

Discussion

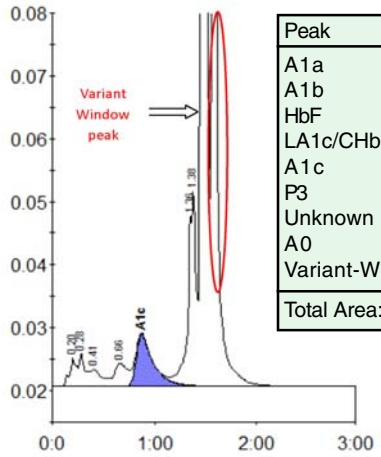
The short program on BioRad D10 measures HbA1c using HPLC and remains one of the most accepted methods of HbA1c quantification[4]. As it separates the hemoglobin fractions chromato-graphically, it lends the extra benefit of identifying the presence of hemoglobinopathy over other methods[5]. This is particularly useful in the presence of a variant which may have interference in the HbA1c measurement. An unknown peak thus warns the laboratorian to report the HbA1c result carefully.

Our survey of HbA1c chromatograms on 6015 adult patients, revealed the presence of 'other than normal peaks' in 151 cases. The most common finding was the Variant Window (2.0%) with an average retention time of 1.58 minutes. In the current methodology adopted for HbA1c testing, the manufacturer authorizes reporting for cases of HbC and HbS variants, which is identified in the chromatogram itself. There is no clear cut instruction from the manufacture's end to report on the other variants eluted as the 'Variant Window' peak. In such cases, it becomes difficult for the laboratory personnel to satisfy the customer on their request for HbA1c. This also includes the 'Unknown peak' which elutes close to the Variant Window. As per manufacturer's direction, laboratory personnel are not to report the HbA1c values obtained[3]. But, contrary to this, NGSP[6,7] has issued a guideline that HbA1c can be reported even in the presence of HbE and HbD Variants. Therefore in this light, the BioRad D10 short program should have the provision of identifying HbE and HbD peaks which are common in Eastern India. Due to the lack of this provision, the laboratory has to rerun the specimen on a BioRad A2/ F extended program which entails a financial



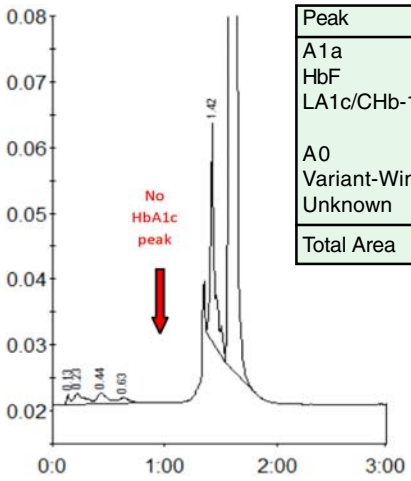
Peak	R. time	Height	Area	Area%
A1a	0.20	2841	15548	1.0
A1b	0.28	3495	13326	0.9
HbF	0.45	3091	21135	1.4
LA1c/CHb-1	0.71	1416	11861	0.8
A1c	0.88	4652	51176	6.4
P3	1.37	17536	74635	5.0
A0	1.45	290671	940707	62.5
S-Window	1.65	213506	377354	25.1
Total Area 1505642				

Fig 3 — HbS Variant (trait) detected. HbS has eluted at RT of 1.65 with Area% of 25.1%. HbS trait does not interfere with the HbA1c result



Peak	R. time	Height	Area	Area%
A1a	0.20	4564	22799	1.0
A1b	0.28	5261	16383	0.9
HbF	0.41	2667	23814	1.4
LA1c/CHb-1	0.66	3671	33995	0.8
A1c	0.87	8248	95701	6.4
P3	1.36	26058	96965	5.0
Unknown	1.38	30876	74962	3.5
A0	1.46	373032	1150687	54.5
Variant-Window	1.58	214964	597692	28.3
Total Area: 2112998				

Fig 4 — *Chromatogram of 60 year old male diabetic patient with HbE trait. HbA1c of 8.6% reported*



Peak	R. time	Height	Area	Area%
A1a	0.23	1775	11808	0.9
HbF	0.44	1650	11417	0.8
LA1c/CHb-1	0.63	860	6301	0.5
	No HbA1c peak			
A0	1.42	33375	84051	6.2
Variant-Window	1.58	210140	403303	29.7
Unknown	1.62	579127	837426	61.7
Total Area	1357447			

Fig 5 — Chromatogram of a 65 year old male patient with a rare HbDE double heterozygous condition (combined area% of variant and unknown peaks is 91.4%). HbA1c peak is missing.

implication on the patient. Our experience of re-examining Variants on the A2/F extended program revealed that majority of patients are HbE trait; occasionally HbD trait was encountered (data not shown). This agrees with the prevalence of hemoglonopathies seen in this region[8]. Considering the above demographic distribution of hemogloniopathies in this region, composition of abnormal hemoglobin in the eluted variant window will have more than 90% of HbE and HbD trait. Therefore, HbA1c result can be reported with a reasonable accuracy with a disclaimer of presence of other hemoglobinopathies in rare cases which may be present in the eluted Variant Window. Clinicians should be aware of this fact and in cases where clinically suspected, he/she recommend alternative measures such as serum glycated albumin[9] to index glycemic control.

An occasional chromatogram may show no HbA1c peak indicating the absence of HbA and the presence of a homozygous or double Heterozygous Hemoglobinopathy. These may be clinically silent HbE disease or rare double heterozygous conditions such as HbED as found in our study. Increased P3 peak or high HbF are also indicators of presence of hemoglobin variants and warrant careful reporting of HbA1c[10].

There may be some variants such as the heterozygous hemoglobin, hope which spuriously increases the levels of HbA1c, without showing any variant peak, asking for cross verification on alternate systems[11]. Thus, while it is safe to assume that HbE/HbD/HbS/HbC traits do not interfere, presence of other variants may inadvertently affect HbA1c results.

Our finding of about 2.5% of the population having a hemoglobin variant agrees with the picture of high prevalence of hemoglobinopathies in Eastern India[8]. The inclusion of HbA1c in screening healthy population for diagnosis[12] of Diabetes has resulted in an upsurge of HbA1c testing. Chromatograms with hemoglobin variants will demand careful inspection against an increasing test load. Clinicians reading the chromatogram will need to make well informed decisions regarding the validity of HbA1c in such a setting.

Conclusion

In summary, the laboratory may encounter several hemoglobin variants depending on the patient population it serves. Careful scrutiny of chromatograms may help identify the presence of aberrant peaks produced by variants. The HbE, HbD, HbS and HbC traits do not interfere with HbA1c results on the BioRad D10 short program. Others can interfere. Patients with a homozygous hemoglobinopathy will show no HbA1c result and alternate methods are required to ascertain glycemic control. Laboratorians need to be aware of the limitations and strengths of their methods of reporting HbA1c especially with an increasing workflow of the biomarker. A laboratory – clinician interaction becomes essential for solving cases that do not align to the apparent clinical picture.

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