

Determination of Clinically Silent Hemoglobin Variant by HPLC Method

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HbA_{1c} or glycosylated hemoglobin is a tool widely used to determine the glycemic control of the patients with diabetes. At present, more than twenty different assay methods are being used to measure the level of the HbA_{1c} in clinical biochemical laboratories. Among them HPLC is widely used method. The aim of this study is to analyze HPLC findings of the clinically silent hemoglobin variants detected during HbA_{1c} estimation. This prospective study was performed on adult type 2 diabetic patients receiving treatment at BIRDEM General Hospital over a period of three months from May 2014 to July 2014. Samples were analyzed by HPLC method using VARIANT-II TURBO- Bio Rad Laboratories (USA) according to the manufacturer's instructions. Samples that showed abnormal HbA_{1c} levels by HPLC method as well as a variant window on the HPLC chromatogram were selected for this study. 52 diabetic patients were diagnosed with hemoglobin variant Among them 26 cases (50%) elevated HbF, 24 (46%) HbE trait and 2 (4%) cases showed HbE disease. HPLC can be used as a diagnostic tool for identification of clinically silent hemoglobin variant.

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Key words: Hemoglobin variant, erythrocyte, HPLC, diabetes mellitus.

Introduction

The measurement of glycosylated hemoglobin (gHb) serves as a powerful tool in the evaluation and management of patients with diabetes mellitus.¹ Concentrations of gHb provide a means of assessing long-term glycemic control and correlate well with the risk for the development of chronic complications related to diabetes.¹⁻³ HbA_{1c} is the result of

irreversible nonenzymatic glycation at one or both N-terminal valines of the Hb β chain. This irreversible nonenzymatic reaction between glucose and HbA, the main type of Hb in normal adults, occurs during the life span of the erythrocyte. The total amount depends on the average glucose concentrations within 2–3 months prior to the measurement.⁴

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Although several methods based on different principles (high-performance liquid chromatography (HPLC), immune agglutination, boronate-affinity assays, and electro-phoresis have been developed,^{5,6} the designated Diabetes Control and Complications Trial comparison method utilized an ion-exchange HPLC(Diamat; Bio-Rad, Richmond, CA, USA).⁷ Therefore, the majority of HbA_{1c} measurements are still performed by ion-exchange HPLC.

More than 700 characterized Hb variants have been reported the majority arise from point mutations in the α , β , γ , or δ Hb chain.⁸ In addition, certain genetic abnormalities can also cause the switch to adult hemoglobin synthesis to fail, resulting in a condition known as the presence of high fetal hemoglobin (HbF).⁹ The widespread measurement of gHb has identified new variants, many of which produce no phenotypic abnormalities.⁸ Many hemoglobinopathies, including sickle cell disease, homozygous HbC disease, HbSC disease, and beta-thalassemia, frequently show increased amounts of minor Hb species, i.e. HbA₂ and HbF, which interfere with some gHb methods. In addition, pathologic conditions affecting red cell half-life, including hemolysis,¹⁰ iron deficiency anemia,^{11,12} or red cell transfusion affect gHb values.¹³

The aim of the present study is to analyze HPLC findings of the hemoglobin variants detected during HbA_{1c} estimation, and to discuss problems that we faced in diagnosis in a routine clinical laboratory.

Method

This prospective study was performed on adult type 2 diabetic patients receiving treatment at BIRDEM General Hospital over a period of three months from May 2014 to

July 2014. HbA_{1c} measurements were performed on EDTA blood samples. Samples were analyzed by HPLC method using VARIANT-II TURBO- Bio Rad Laboratories (USA) according to the manufacturer's instructions. Samples that showed abnormal HbA_{1c} levels by HPLC method as well as a variant window on the HPLC chromatogram were selected. Patients with uremia detected either by renal function test or samples showing presence of Carbamyl-Hb (CHb) by HPLC based HbA_{1c} analysis were excluded from the study. Hb% and Mean Corpuscular Volume (MCV) was measured on an automated hematology analyzer(Sysmex 1800i, USA) according to the manufacturer's instructions. All shows MCV level less than 80 fl. Therefore study population mostly showed microcytic hypochromic red cell morphology. Hemoglobin electrophoresis was performed by alkaline method on agarose gel. Data were expressed as Mean \pm SD, number (percent) as applicable. Students' "t" test by using MedCalc Statistical software respectively.

Result

A total of 52 samples were initially found to show abnormal HbA_{1c} (less than 4%) and variant window by HPLC method and thus included in this study.(Figure : 1) . Among them 26 cases (50%) shows elevated HbF , 24 (46%) showed HbE trait and 2 (4%) cases showed HbE disease (Table 1, Figure 2). HbE trait and HbE disease was confirmed by electrophoresis. Mean \pm SD FBG was 11.31+1.08, 11.54+1.08 and 12.65+4.85 in elevated HbF, HbE trait and HbE disease respectively. Mean \pm SD HbA_{1c} value was 3.27+0.10, 1.99+0.32, 3.60+0.20 in in elevated HbF, HbE trait and HbE disease respectively. In elevated HbF cases, Mean \pm SD MCV and Hb% was 75.83+1.24 and 12.31+0.54 respectively, in HbE trait Mean \pm SD MCV and Hb % was 72.96+2.38

and 10.90±0.44 respectively and in HbE disease Mean±SD MCV and Hb % was 66.60±1.90 and 13.95±0.65 respectively (Table 1).

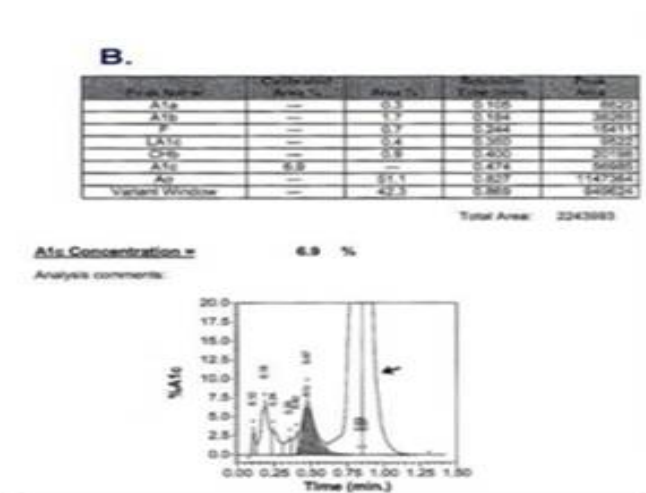


Figure 1. Chromatogram from the ion exchange high performance liquid chromatography Bio- Rad variant turbo II showing variant window.

Table I: Types of hemoglobin variant with number and percentage

Hemoglobin(Hb) variant	Number	Percentage (%)
Elevated HbF ($\alpha 2\gamma 2$)	26	50
HbE trait (AE)	24	46
HbE disease (EE)	2	4

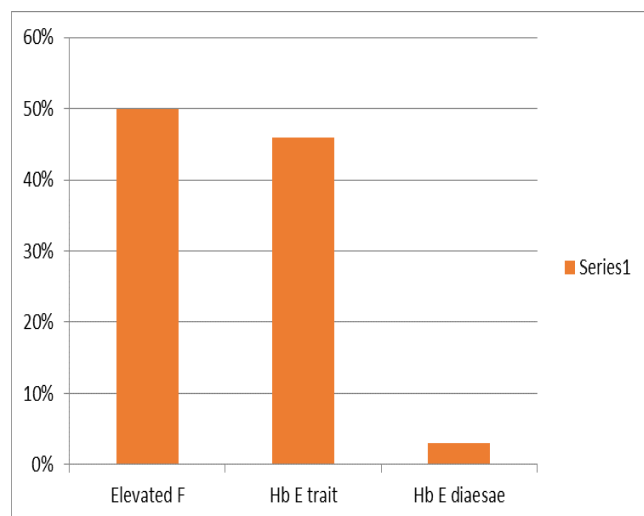


Figure 2. Percentage of hemoglobin variant

Table II: Variable changes in hemoglobin variant study subjects

Hemoglobin(Hb) variants	HbA _{1c} (%)	FBG (mmol/L)	MCV (fl)	Hb%
Elevated HbF ($\alpha 2\gamma 2$)	3.27±0.10	11.31±1.08	75.83±1.24	12.31±0.54
HbE trait (AE)	1.99±0.32	11.54±1.08	72.96±2.38	10.90±0.44
HbE disease (EE)	3.60±0.20	12.65±4.85	66.60±1.90	13.95±0.65

HbA_{1c}: Glycosylated hemoglobin, FBG: fasting blood glucose, MCV: Mean Corpuscular Volume.

Discussion

HPLC or high performance liquid chromatography in simple terms is a form of column chromatography. HbA_{1c} and other hemoglobin fractions can be separated by HPLC. The principle of the HPLC assay is cation exchange chromatography.¹⁴ The method utilizes the difference in molecular weight and charge to separate hemoglobin on a column. The HPLC method is able to distinguish between glycated and non-glycated fetal hemoglobin and HbA.¹⁵

The blood plasma components, including HbA_{1c}, get separated due to a variety of chemical interactions between plasma molecules and the column of the HPLC instrument. The levels of detected substances are plotted in the form of a series of peaks. The area under the peak is proportional to the amount of analyte present and by calculating the area of the peak using the mathematical function of integration.¹⁴

According to 2007 CAP proficiency surveys, the ion-exchange chromatography method, in which Hemoglobin species are separated based on charge differences, accounted for a little more than 32% of the methods used for the measurement of HbA_{1c}. Typically, these methods used cation-exchange chromatography, in which Hb species elute from the cation-exchange column at different times, depending on their charge, with the

application of buffers of increasing ionic strength.¹⁵ The concentration of Hb is measured after elution from the column, which is then used to quantify HbA_{1c} by calculating the area under each peak. Consequently, changes or mutations in the Hb molecule that result in a charge difference from the wild-type Hb species, may or may not alter the normal elution time of those Hb species when using cation-exchange chromatography. Although alterations in the ionic strength of mobile phases used in some ion-exchange chromatography methods have reduced the level of co-elution of some of the more common Hb variants (HbS and HbC), it is recommended that clinicians consider methods other than HbA_{1c} for determining average blood-glucose monitoring, such as glycated albumin or fructosamine, given the confusing factors in interpreting HbA_{1c} results for these individuals.¹⁶

Elevated HbF

HbF estimation is particularly important in diagnosis of carriers of thalassemia and hereditary persistence of fetal hemoglobin (HPFH) as well as the major thalassemia syndromes. However, erroneously low levels may be obtained when HbF levels are very high.¹⁷

In Fetal hemoglobin (HbF) is the main hemoglobin component throughout fetal life and at birth, accounting for approximately 80% of total hemoglobin in newborns. After birth, HbF synthesis rapidly declines and HbF is gradually substituted by HbA in the peripheral blood, so that within the first two years of life, the characteristic hemoglobin phenotype of the adult with very low levels of HbF (less than 1%) is found.¹⁸⁻²⁰ HbF($\alpha_2\gamma_2$) is formed by two α - and two γ -globin chains consisting of 141 and 146 amino acid residues respectively. Changes in this ratio were observed in some hemoglobin disorders.^{21, 22}

Our finding is similar to one study who found low HbA_{1c} using HPLC(Bio red variant) due

to elevated HbF.²³ Where another study found elevated HbF not affect HbA_{1c} by HPLC.²⁴ Higgins et al found that Hb F up to 8% produce only small effect on HbA_{1c} by HPLC. Similar result was found in our study.²⁵

Hemoglobin E

Hemoglobin E, is the commonest structural hemoglobin variant globally.²⁶ Hemoglobin E (HbE) is mainly found in the eastern half of Indian subcontinent and throughout South East Asia, where in some areas, carrier rates are as high as 60% of the population.²⁷ The HbE gene is a mutant form of the β -globin gene that encodes lysine instead of glutamate at position 26.²⁶ HbE may be present in the heterozygous state (genotype AE or HbE trait) and the homozygous state (genotype EE or HbE disease). Heterozygosity (HbE trait) and homozygosity (HbEE disease) are clinically silent, whereas compound heterozygosity for Hb E and HbS (HbSE) and compound heterozygosity for HbE and β -thalassemia (HbE- β -thalassemia) are clinically severe.²⁸

A recent study little et al. showed that most HPLCs give low HbA_{1c} result when compared to others in patients with HbE trait.²⁹ A study also showed significantly lower HbA_{1c} values measured by HPLC, in patients with HbE trait.²³ Other studies on a large range of abnormal hemoglobins also showed similar results.³⁰

Conclusion

Analysis using a HPLC based method may lead to the identification of the carrier status for a Hb-variant in diabetic patients. Often, the identified Hb-disorders are clinically silent but may not be biochemically silent when it comes to HbA_{1c} measurement. In managing diabetic patients, knowledge of hemoglobin variant influencing the locally used HbA_{1c} method is essential because unidentified hemoglobin variants may cause

mismanagement of diabetes resulting from false HbA_{1c} results.

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