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Effect of hemoglobin F and A_2 on hemoglobin A_{1c} determined by cation exchange high-performance liquid chromatography

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Abstract

Background: The potential effect of increase in hemoglobin (Hb) A_2 and HbF on glycated hemoglobin (HbA_{1c}) measurements were investigated using a high-performance liquid chromatography (HPLC) method compared with an immunoturbimetric assay.
Methods: Samples producing abnormal chromatograms during the measurement of routine HbA_{1c} testing with HPLC were further analyzed to characterize abnormal Hb variants. Patients were divided into three groups that had only high HbF (group 1), only high HbA_2 (group 2), and both high HbA_2 and HbF (group 3). HbA_{1c} values of patients were re-assayed using the immunoturbidimetric method (Advia, Siemens Healthcare, Germany).
Results: HbA_{1c} levels were significantly higher in all groups measured by immunoassay than in HPLC. We found a positive correlation between HPLC and immunoturbidimetry in the group 2 and a slight correlation in the group 1. There was no correlation between the two methods in group 3.
Conclusions: HbA_{1c} measurement by HPLC method interfering with elevated HbA_2 and HbF, especially HbF, should be verified by an immunoturbidimetric method.

Keywords: HbA_{1c}; HbA_2; HbF; HPLC; hemoglobin variant; immunoassay.

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Introduction

A normal hemoglobin molecule is composed of two dissimilar pairs of polypeptide chains (alpha and non-alpha) called globins. The combination of two alpha chains and two non-alpha chains determines the hemoglobin type. Hemoglobin (Hb) A ($a_2\beta_2$) is the major Hb in adult persons, accounting for more than 96% of total Hb. The small part of total Hb constitutes HbA_2 ($a_2\delta_2$) and fetal Hb (HbF) ($\alpha_2\gamma_2$) that do not have β-globin chain. The problems in β-globin chain production, the production of δ-globin and γ-globins may be increased, resulting in higher levels of HbA_2 and HbF. Higher levels of HbA_2 and HbF are biological markers for the β-thalassemia [1]. Carriers of β-thalassemia according to World Health Organization estimates are approximately 1%–5% of the world's population [2]. Also, they are a very common patient group in Turkey, because it originated in the Mediterranean and in the Middle East [3]. In addition, increases in HbF can be observed in some conditions such as hereditary persistence of fetal Hb and some drug treatments [4, 5].

Glycated hemoglobin (HbA_{1c}) is a marker to be correlated with complications of diabetes mellitus (DM). It has been recommended for diagnosing DM as well as for specific treatment goals in this disease [6]. Therefore, accurate measurement of HbA_{1c} is needed. The reference method for the measurement of HbA_{1c} is an ion-exchange high-performance liquid chromatography (HPLC) according to the Diabetes Control and Complications Trial [7]. HbA_{1c} measurements can be adversely affected by conditions that disrupt the Hb composition [8]. Although there are many reports in the literature showing that Hb variant affects the accuracy of methods for HbA_{1c} measurement [8, 9]. HbA_{1c} values measured in patients with only HbA_2 and HbF abnormality were few [10, 11]. HbA_2 is co-eluted with Hb A0, therefore in the case of β-thalassemia carriers, the amount of HbA_2 may affect the elution profile of HbA_{1c}. Even though HbF fraction is eluted a little faster, it is difficult to separate from the HbA_{1c} peak in the case of highly enhanced HbF concentrations. Therefore, we investigated the potential effect of changes in HbA_2 and

HbF on HbA_{1c} measurements by HPLC using the immunoturbidimetric assay as the comparative method.

Materials and methods

The study population consisted of 124 individuals admitted to the Biochemistry Laboratory of Zonguldak Bulent Ecevit University between 2013 and 2014 years. This study was approved by the Ethics Committee of the University of Zonguldak Bulent Ecevit University. The samples were selected from patients who showed a hemoglobin variant during the measurement of routine HbA_{1c} testing. All analyses were performed on a single whole-blood sample taken into an ethylenediaminetetraacetic acid (EDTA) tube. The samples were stored at 4 °C or –70 °C and analyzed within 5 days of the blood being drawn.

HbA_{1c} was assayed by ion-exchange HPLC (Spectra System, Thermo Scientific, Boston, MA, USA) using the commercially available kit ClinRep (Recipe Chemicals – Instruments GmbH, Munich, Germany) according to the manufacturer's instructions. Calibration of instruments was done according to the manufacturer's instructions. The method is linear over the whole range of physiologically relevant HbA_{1c} values (3–16% HbA_{1c}). The presence of abnormal patterns on the chromatograms were cross-checked using by ClinRep hemoglobin variant and β-thalassemia screening kit (Recipe Chemicals – Instruments GmbH, Munich, Germany) on same HPLC analyzer. Patients were identified according elevated HbA_2 (>3.5%) and/or HbF (>2%) and divided into three groups (Figure 1). HbA_{1c} values of groups were re-assayed by immunoturbidimetric method using manufacturer kit on an ADVIA 2400 analyzer (Siemens Healthcare, Erlangen, Germany). HbA_{1c} values are reported with both the National Glycohemoglobin Standardization Program (NGSP) (%) and International Federation of Clinical Chemistry (IFCC) (mmol/mol) units. In this article, HbA_{1c} results will be reported in percentage of HbA_{1c} units. Conversion of HbA_{1c} units is by the following equation [7]:

$$HbA_{1c}(\text{mmol/mol}) = [HbA_{1c}(\%) - 2.15] / 0.092$$

The intra- and inter-assay coefficient of variation (CV) values in determination of HbA_{1c} by HPLC were 1–2% and 2–3%, respectively. The intra- and the inter-assay CVs for the immunoturbidimetric method were 2.2% and 2.6%, respectively.

Statistical analyses

All statistical analysis were performed using SPSS 12.0 for Windows (Microsoft, Seattle, WA, USA), except the Passing-Bablok regression analysis which was performed using Medcalc Software version 12. Statistical analysis was evaluated using nonparametric techniques. Intergroup comparisons of HbF and HbA_2 levels were analyzed by employing the Kruskal-Wallis nonparametric test, followed by post hoc group comparisons with the Bonferroni-adjusted Mann-Whitney U-test. Regarding the Bonferroni correction, $\alpha = 0.05/3 = 0.016$ was taken to be statistically significant. HbA_{1c} values were compared using Wilcoxon signed-rank test between the two methods. We used Spearman's correlation coefficient and Passing and Bablok regression analysis for method correlation. p-Values of less than 0.05 were considered statistically significant.

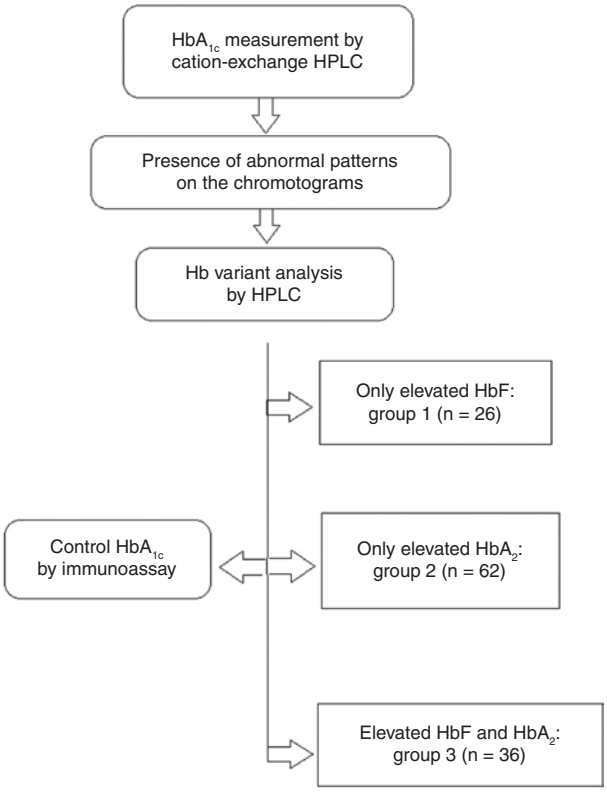

Figure 1: Flowchart demonstrating the procedure of the study.

Results

The present study was carried out among 124 subjects and categorized into three groups based on their hemoglobin variant analysis. The mean ± standard deviation (SD) HbA_2 levels were significantly higher in the group 2 (4.8 + 0.5%) and 3 (4.7 ± 0.5%) than the group 1 (2.1 ± 0.5%) ($p < 0.016$). HbF levels were significantly higher in the group 1 (4.2 ± 2.3%) and 3 (4.3 ± 2.2%) compared with

Table 1: Differences of HbA_{1c} levels measured by HPLC and immunoassay for each group with different Hb variant.

Groups	HbA_{1c}, %		
	HPLC	Immunoassay	p-Value
Group 1 (high HbF) (n = 26)	4.3 ± 1.4 (2.3–8.4)	6.1 ± 1.8 (4.0–12.7)	<0.05
Group 2 (high HbA_2) (n = 62)	4.8 ± 1.1 (2.7–9.7)	5.7 ± 0.9 (4.4–10.1)	<0.05
Group 3 (high HbA_2 and HbF) (n = 36)	3.9 ± 0.9 (2.1–5.9)	5.3 ± 0.7 (3.9–6.9)	<0.05

Data are expressed as mean ± SD (range: minimum–maximum). HbA_{1c}, glycated hemoglobin; HPLC, high-performance liquid chromatography; SD, standard deviation.

the group 2 (1.0 ± 0.5%) ($p < 0.016$). The differences of the HbA_{1c} levels between HPLC and immunoturbidimetry for each group are shown in the Table 1. HbA_{1c} concentrations in all groups were showed lower results in HPLC methods compared to immunoturbidimetry ($p < 0.05$).

With Passing and Bablok regression analysis, group 1 (Figure 2A) yielded the equation of $y = 1.11 \times$ (95% CI, 0.67–2.27)–2.64 (95% CI, −9.1 to −0.11); group 2 (Figure 2B) yielded the equation of $y = 1.46 \times$ (95% CI, 1.11–1.94) − 3.31 (95% CI, −6.03 to −1.36); group 3 (Figure 2C) yielded the equation of $y = 1.62 \times$ (95% CI, 0.79–3.11) − 4.18 (95% CI, −11.6 to −0.05). Calculation of Spearman's correlation coefficient showed a positive correlation ($r = 0.49$, $p < 0.0001$) between the two methods in the group 2. A slight correlation ($r = 0.38$, $p = 0.06$) in the group 1 and no correlation ($r = 0.04$, $p = 0.78$) in the group 3 was observed between two methods.

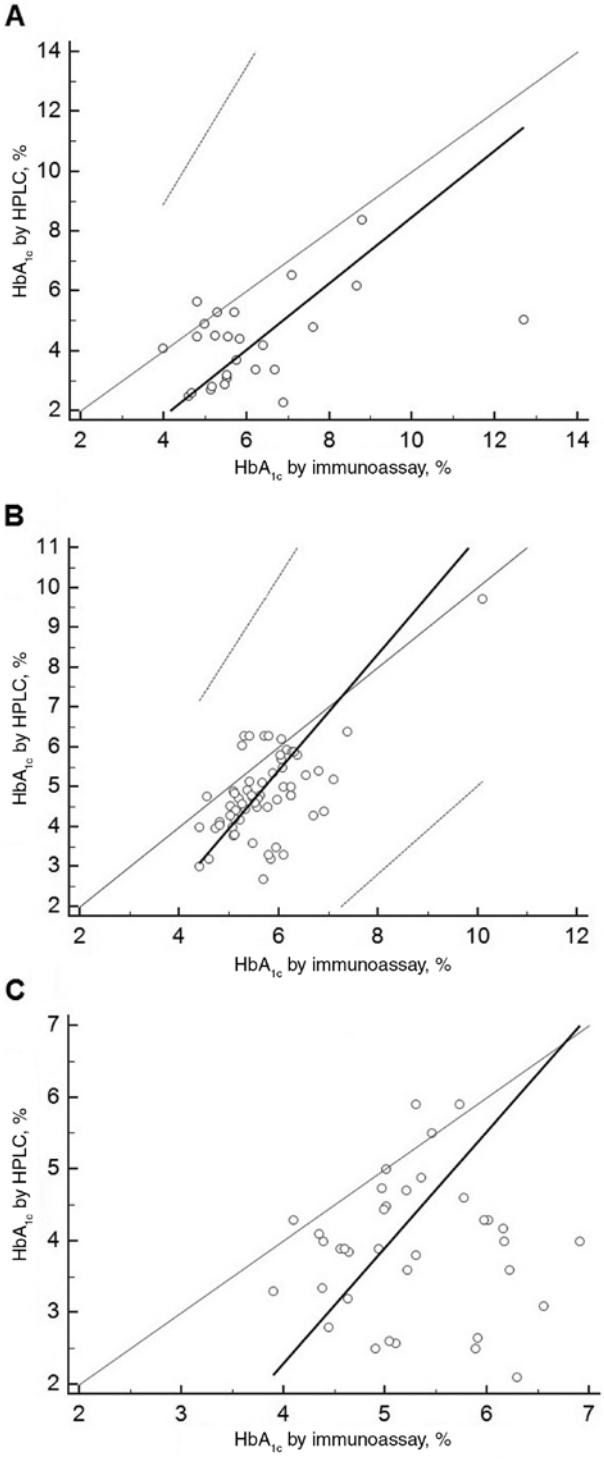

Figure 2: Spearman's correlations coefficients of 2A, 2B and 2C, respectively ($r = 0.38$, $p = 0.06$; $r = 0.49$, $p < 0.0001$; $r = 0.04$, $p = 0.78$). Passing-Bablok regression analysis of HbA_{1c} results obtained with the HPLC compared with immunoassay method in group 1 (A), 2 (B) and 3 (C).

Discussion

HbA_{1c} is glycated hemoglobin in which glucose is bound specifically to the N-terminal valine residue of one or both beta chains of hemoglobin via a nonenzymatic process. It reflects the mean glycemic level in the lifespan of the erythrocyte. The Diabetes Control and Complications Trial showed that a reduction of 1% HbA_{1c} corresponded to an approximate 30% reduction in the risk of diabetes complications [12].

Hemoglobin variants may interfere with the HbA_{1c} result in three ways; by decreasing the life span of red blood cells, by influencing the binding of glucose to Hb and by affecting the net charge of the hemoglobin [8, 13]. Ion-exchange HPLC separates Hb species based on charge differences, while immunoassay methods use antibodies that recognize the N-terminal glycated amino acids in the first four to 10 amino acids of the β-globin chain of the hemoglobin. In our study, we demonstrated that elevated

HbA_2 and/or HbF can affect determination of HbA_{1c} by our HPLC method and might result in falsely lower HbA_{1c} concentrations. Analytic interference of immunoturbidimetric methods is extremely low in heterozygous Hb variant conditions in which the red blood cell lifespan may be normal [14]. However, they do not provide any information about the proportions of the different Hb fractions. Most ion exchange chromatography techniques separate HbF and Hb A_2 from HbA_{1c} and this can be a very precious information on medically silent patients [15]. Routine inspection of chromatograms allowed us to identify the presence of HbF and HbA_2, although it could not be presented in this paper.

In conclusion, chromatograms must be carefully examined to identify possible interference in HbA_{1c} measurement using HPLC. HbA_{1c} measurement by HPLC method interfering with elevated HbA_2 and HbF, especially HbF, should be verified by an immunoturbidimetric method.

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