Effect of Hemoglobin E on Measurement of Hemoglobin A1c by Five Different Methods

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Abstract
Patients with hemoglobin (Hb) variants may produce false hemoglobin A1c (HbA1c) measurement leading to misdiagnosis or mistreatment. This study aimed to evaluate the effects of Hb E on five different HbA1c analytical principles. HbA1c levels of 160 EDTA blood samples including Hb type A2A (N=51), heterozygous Hb E (N=104), and homozygous Hb E (N=5) were analyzed by immunoturbidimetric method, HPLC, LPLC, enzymatic assay, and boronate affinity chromatography. The strong positive correlations of HbA1c levels measured by all assays were observed ($r=0.797$ to 0.962, $p<0.001$). The HbA1c results analyzed by immunoassay and enzymatic assay were significant higher than boronate affinity chromatography ($p<0.05$), in Hb type A2A and heterozygous Hb E samples. However, these differences between assays in each hemoglobin typing were comparable. The results by using HPLC were under reportable range in homozygous Hb E group. The mean differences between HbA1c results analyzed by using LPLC and other 4 tested methods were significantly higher in heterozygous Hb E samples with Hb F >2%, compared to samples with Hb F ≤2% ($p<0.05$). This work showed a good comparability of HbA1c assays, although, some methods were influenced by high levels of Hb E and Hb F. Therefore, Hb E and Hb F are interfering factors that laboratories must be aware when reporting results if its presence is suspected.

Keywords: *Boronate affinity chromatography; Enzymatic assay; Immunoturbidimetric method; Hemoglobin A1c; Hemoglobin E; HPLC; LPLC*

1. Introduction

Hemoglobin A1c (HbA1c) is the modified hemoglobin formed by the irreversible non-enzymatic glycation of one or both N-terminal valines of hemoglobin beta chains, as such, HbA1c level in whole blood reflects the average of blood glucose concentration over the red blood cell lifespan. HbA1c has been routinely used in monitoring the long-term glycemic control and assessing the risk of developing complications in diabetic patients. Controlling of HbA1c nearly to normal level prevents the development and slows the progression of microvascular and macrovascular complications (DCCT, 1993: 977-986; DCCT/EDIC, 2005: 2643-2653; UKPDS, 1998: 837-853). In addition, it has also been recommended for use as diagnostic criteria for diabetes, with a threshold level of more than or equal to 6.5%. (ADA, 2018: S13-S27; WHO, 2011: 6-9). For diagnosis, HbA1c tests must be performed using a method certified by the National Glycohemoglobin Standardization Program (NGSP) and standardized to the Diabetes Control and Complications Trial (DCCT). The measurement of HbA1c level can be performed based on different analytical principles such as capillary electrophoresis, isoelectric focusing, high performance liquid chromatography (HPLC), low pressure liquid chromatography (LPLC), affinity chromatography, immunoassays including immunoturbidimetry and immunoturbidimetric inhibition, and enzymatic assay. However, certain methods for HbA1c analysis can be affected by the presence of hemoglobin variants depending on the specific type of variants (Little, 2015: 849-856; Lorenzo-Medina, 2014: 1168-1176; Mongia, 2008: 136-140). This can result in under-, over-, or non-estimation of the HbA1c level and subsequently lead to an inappropriate clinical management. As a result, the NGSP advises that laboratories should consider the likely prevalence of specific

hemoglobinopathies in their population when selecting an HbA1c assay (NGSP, 2018). Among thousand hemoglobin variants identified, the four most common hemoglobin variants worldwide are hemoglobin S (Hb S), hemoglobin E (Hb E), hemoglobin C (Hb C), and hemoglobin D (Hb D). Hb E is the second most prevalent hemoglobin variant worldwide. It occurs at an extremely high frequency in many countries in South-East Asia, particularly in Thailand, of which the prevalence of carrier frequency approximately 50% (Colah, 2010: 103-117; Fucharoen, 2004: 364-372). Hb E contains a substitution of lysine for glutamic acid at position 26 of the beta-globin chain. Subjects who are heterozygous Hb E or homozygous Hb E do not present obvious clinical manifestations whereas subjects who have Hb E with alpha / beta-thalassemia present clinical symptoms with a very wide range of severity (Fucharoen, 2012: 1-15). Thus, laboratories should be aware about the method used in detecting HbA1c in order to provide accurate HbA1c results.

In this study, we investigated the possibility of interference by hemoglobin E on five HbA1c methods including HPLC, LPLC, boronate affinity chromatography, immunoturdibimetric method and enzymatic assay. All methods evaluated in this work have not been previously reported for interference from the presence of hemoglobin E.

2. Materials and methods

The study was approved by the ethics review committee at Huachiew Chalermprakiet University (อ.432/2559). Total of 160 whole blood samples from pregnant women and/or their spouses were collected in EDTA-containing tubes; 51 hemoglobin (Hb) type A2A, 104 heterozygous Hb E, and 5 homozygous Hb E. After routine hemoglobin typing has been completed using Variant™ II hemoglobin testing system (Bio-Rad, USA) at Regional Medical Sciences Center 5 Samut Songkhram, small aliquots (500 µL) of each sample were made and stored at 4 ^{o}C until they were shipped on dry ice to faculty of Medical Technology at Huachiew Chalermprakiet University for HbA1c analysis. Hb type A2A samples with Hb F >1% were excluded. HbA1c levels in all samples were analyzed by the following assays and instruments: cation-exchange high performance liquid chromatography (HPLC), H9 hemoglobin analyzer (Lifotronic Technology, China); low pressure liquid chromatography (LPLC), GH-900 HbA1c analyzer (Lifotronic Technology, China); immunoturbidimetric method, HbA1c Turbidimetric reagent (Linear chemicals, Spain) was used on XL-200 automated chemistry analyzer (Erba Mannheim, Germany); enzymatic assay, A1care™ analyzer (i-SENS, Korea); boronate affinity chromatography, Labona Check™ A1c HbA1c analyzer (Green Cross Medis, Korea). All results were interpreted according to the manufacturers' instructions. Results for all methods were reported as NGSP HbA1c equivalents. Means and 95% confidence intervals of HbA1c results obtained by each method were calculated. Relationships between different assays were performed using Pearson's correlation test. For each type of sample, multiple-group comparison was made by one-way ANOVA/Friedman test and comparisons between different methods were made by paired t-test/Wilcoxon signed-rank test. The mean difference (%) between assays of interest were calculated for each hemoglobin type and compared by using independent t-test/Mann-Whitney U test.

3. Results

The characteristics of 160 samples including 51 samples of Hb type A2A, 104 samples of heterozygous Hb E (EA) and 5 samples of homozygous Hb E (EE) were shown in Table 1. As shown in Table 2, there were strong positive correlations of HbA1c levels, both in hemoglobin type A2A and heterozygous Hb E samples, determined by H9 (HPLC), GH-900 (LPLC), Labona Check™ (boronate affinity chromatography), XL-200 (immunoturdibimetric method) and A1care™ (enzymatic assay), $p<0.001$.

Table 1. Characteristics of the study samples

Characteristics (mean ± SD)	Hemoglobin type		
	A2A (N=51)	EA (N=104)	EE (N=5)
%Hb A	86.67 ± 1.38	62.32 ± 2.61	4.92 ± 0.90
%Hb A2	3.01 ± 0.65	—	—
%Hb F	0.40 ± 0.21	1.00 ± 0.60	2.68 ± 0.81
%Hb E	—	26.86 ± 2.82	82.88 ± 2.83

— indicates no test result.

Table 2. Correlation coefficient (r) of HbA1c test methods based on hemoglobin type

Method	Correlation coefficient (r)	
	A2A	EA
H9 vs GH-900	0.944 *	0.873 *
H9 vs XL-200	0.890 *	0.952 *
H9 vs A1care™	0.948 *	0.962 *
H9 vs Labona Check™	0.864 *	0.957 *
GH-900 vs XL-200	0.886 *	0.850 *
GH-900 vs A1care™	0.927 *	0.835 *
GH-900 vs Labona Check™	0.815 *	0.837 *
XL-200 vs A1care™	0.863 *	0.954 *
XL-200 vs Labona Check™	0.797 *	0.946 *
A1care™ vs Labona Check™	0.824 *	0.954 *

* Significance level at $p<0.001$

The results of HbA1c measurements using five different methods were presented in Table 3. In Hb type A2A samples, the mean HbA1c levels determined by boronate affinity chromatography were statistically significant lower than immunoturbidimetric method and enzymatic assay ($p<0.05$). In heterozygous Hb E samples, the HbA1c levels measured by enzymatic assay were significant higher than the results performed by HPLC, LPLC, and boronate affinity chromatography ($p<0.05$). The HbA1c results analyzed by immunoturbidimetric methods also showed significant higher than boronate affinity chromatography ($p<0.01$). In homozygous Hb E samples, the HbA1c results obtained by using HPLC method were not in the reportable range (3.8%-18%) and were statistically significant lower than all HbA1c values determined by other four tested methods ($p<0.05$).

Table 3. HbA1c results measured by 5 different methods

Hemoglobin type	%Mean HbA1c (95% Confidence interval)					p-value
	H9	GH-900	XL-200	A1care™	Labona Check™	
A2A	5.80 (5.62, 5.97)	5.76 (5.56, 5.97)	5.84 (5.70, 5.99)	5.84 (5.65, 6.02)	5.72 (5.54, 5.90)	0.848
EA	5.68 (5.49, 5.87)	5.63 (5.42, 5.84)	5.72 (5.57, 5.87)	5.77 (5.60, 5.95)	5.64 (5.47, 5.81)	0.780
EE	2.12 (1.61, 2.63)	5.26 (4.13, 6.39)	4.82 (3.98, 5.66)	5.16 (4.87, 5.45)	5.10 (4.66, 5.54)	0.023 *

* Significance level at p<0.05

As shown in Table 4, though some assays showed statistically significance differences of HbA1c results in Hb type A2A and heterozygous Hb E sample groups, the difference between assays in the presence of heterozygous Hb E were comparable to Hb type A2A group (p>0.05). In homozygous Hb E samples, the mean differences of HbA1c results between HPLC and other tested methods were significantly greater than the differences observed in Hb type A2A group (p<0.001). We also found the greater mean differences of HbA1c results between immunoturbidimetric method and LPLC, boronate affinity chromatography (p<0.05).

Notably, the mean differences between HbA1c results obtained from LPLC and other analytical principles were significantly higher in heterozygous Hb E samples with Hb F >2%, compared to samples with Hb F ≤2% (p<0.05) (Table 5). We found that, in this group, the mean LPLC-derived HbA1c (6.38%) were higher than results determined by HPLC, immunoturbidimetry, enzymatic assay and boronate affinity chromatography (5.40%, 5.48%, 5.43%, 5.40%, respectively), whereas HbA1c results analyzed by using LPLC were lower than results by other assays, in heterozygous Hb E samples with Hb F ≤2% (HPLC 5.70%, LPLC 5.58%, immunoturbidimetric assay 5.74%, enzymatic assay 5.79%, boronate affinity chromatography 5.65%).

Table 4. Mean differences (%) of HbA1c between assays

Method	Hemoglobin type		
	A2A	EA	EE
H9 vs GH-900	± 0.04	± 0.06	± 3.14 **
H9 vs XL-200	± 0.05	± 0.04	± 2.70 **
H9 vs A1care™	± 0.04	± 0.09	± 3.04 **
H9 vs Labona Check™	± 0.08	± 0.05	± 2.98 **
GH-900 vs XL-200	± 0.08	± 0.10	± 0.44 *
GH-900 vs A1care™	± 0.08	± 0.14	± 0.10
GH-900 vs Labona Check™	± 0.04	± 0.01	± 0.16
XL-200 vs A1care™	± 0.01	± 0.05	± 0.34
XL-200 vs Labona Check™	± 0.12	± 0.09	± 0.28 *
A1care™ vs Labona Check™	± 0.12	± 0.13	± 0.06

* Significance level at p<0.05, compared to hemoglobin type A2A

** Significance level at p<0.001, compared to hemoglobin type A2A

Table 5. Mean differences (%) of HbA1c between assays for samples containing heterozygous Hb E with various Hb F levels

Method	Hb F ≤ 2% (n=98)	Hb F > 2% (n=6)
H9 vs GH-900	± 0.12	± 0.98 **
H9 vs XL-200	± 0.04	± 0.08
H9 vs A1care™	± 0.09	± 0.03
H9 vs Labona Check™	± 0.05	± 0.00
GH-900 vs XL-200	± 0.16	± 0.90 **
GH-900 vs A1care™	± 0.21	± 0.95 **
GH-900 vs Labona Check™	± 0.07	± 0.98 *
XL-200 vs A1care™	± 0.05	± 0.05
XL-200 vs Labona Check™	± 0.09	± 0.08
A1care™ vs Labona Check™	± 0.14	± 0.03

* Significance level at $p<0.05$, compared to Hb F ≤ 2%
** Significance level at $p<0.01$, compared to Hb F ≤ 2%

4. Discussion

HbA1c plays an important role in diagnosis and treatment of diabetes, as it directly is related to diabetes complications. The current common methods used to quantify HbA1c are ion-exchange chromatography, affinity chromatography, enzymatic and immunoassays. Several reports have shown that Hb variants can affect determination of HbA1c levels with some methods by causing falsely decreased, increased or non-estimation of HbA1c results, potentially leading to misdiagnosis and undertreatment or overtreatment of diabetic patients. In present study, we investigated the possibility of interference by Hb E, the second most common Hb variant worldwide, particularly in Thailand, on five different HbA1c methods including ion-exchange HPLC, LPLC, boronate affinity chromatography, enzymatic assay and immunoturbidimetric method. We demonstrated the strong positive correlations of HbA1c obtained by these five different assays both in hemoglobin type A2A and heterozygous Hb E groups. The statistically significance higher HbA1c results obtained by immunoturbidimetric and enzymatic assays, in comparison with boronate affinity chromatography, were observed in both heterozygous Hb E and hemoglobin type A2A samples. However, the mean differences of HbA1c results between tested methods in the presence of heterozygous Hb E showed no significant difference, compared to the hemoglobin type A2A group, suggesting that heterozygous Hb E did not interfere with the tested methods.

Previous studies also showed that the presence of Hb E trait does not interfere with HbA1c assays including immunoassay, enzymatic assay, and boronate affinity chromatography at clinical decision point of 6% and 9% HbA1c, although the HbA1c results for each method were statistically different (Azizi, 2015: 495-497; Little, 2008: 1277-1282). On the other hand, several ion-exchange HPLC based methods were clinically affected by Hb E trait at varying degree such as non-quantitation and significant low or high HbA1c results (Lin, 2012: 819-821; Little, 2008: 1277-1282; Rohlfing, 2016: 80-83; Sthaneshwar, 2013: 417-419; Wu, 2016: 353-364; Zhang, 2018: 1-7). The different values of HbA1c obtained for the same blood samples measured by HPLC methods depends on the chromatographic system such as the kind of resin, resin lot variation, column size, buffer composition and elution times (Jeppsson, 2002: 78-89), suggesting that the influence of Hb E variant are principle- and method-specific.

For samples with homozygous Hb E, Hb A is very low, with over 80% hemoglobin being Hb E itself, thus, the level of HbA1c may also very low. Despite of the limited samples, our data

showed that HbA1c results obtained from HPLC were under the reportable range and nearly half of all other different analytical principle methods (LPLC, immunoturbidimetric method, enzymatic assay and boronate affinity chromatography). These data were consistent with the study by Goce Dimeski (2012: 1479-1482) which showed the unreportable HbA1c results by HPLC analyzers. Our data showed that the mean differences of HbA1c between HPLC and other four tested methods in homozygous Hb E samples were significant greater than in hemoglobin type A2A samples, suggesting that homozygous Hb E obviously interfered HPLC method. In addition, the significantly greater mean differences of HbA1c between immunoturbidimetric method and other assays (LPLC and boronate affinity chromatography) in homozygous Hb E samples were observed, compared to hemoglobin type A2A samples, indicating that these tested methods may also be interfered by homozygous Hb E. Nonetheless, as most of the samples in routine HbA1c analyses are unknown hemoglobin type and patients with homozygous Hb E can be asymptomatic, the HbA1c results analyzed by using HPLC could raise awareness to laboratories about the existence of hemoglobin variants whereas the other methods could not.

Hemoglobin F is one factor which can interfere with HbA1c analysis (Khajuria, 2015: 85-88; Nitta, 2015: 569-575; Shu, 2012: 1712-1713). Some methods/instruments showed the interfering from Hb F in a range of 5 - 30% (NGSP, 2018). In this study, we have seen the influence of Hb F on HbA1c measurement by LPLC. Our data showed the significant differences of HbA1c values between LPLC and other tested methods in heterozygous Hb E samples with Hb F >2% ($p<0.05$), but not in samples with Hb F ≤2%, implying that the measurement of HbA1c by LPLC method was interfered by an increased in Hb F. This could be due to the co-migration of Hb F with the HbA1c, causing overlapped peaks of Hb F with that of HbA1c. Thereby, the falsely high HbA1c results by LPLC may be found in patients with clinical conditions presenting with elevated Hb F (>1% Hb F), such as β-thalassemia (1–5% Hb F), pregnancy (3% Hb F), leukemia (5–17% Hb F), and hereditary persistence of fetal hemoglobin (≤30% Hb F) (Carrocini, 2011: 231-236; Manca, 2008: 94-111). Further study is needed for evaluating the effect of varying of %Hb F on these tested methods.

In addition to hemoglobin variants, the HbA1c can be inaccurate in people with certain conditions that alter the red blood cell lifespan, conditions with abnormal red cell turnover, such as anemia. In such cases, American Diabetes Association (ADA) recommends laboratory to consider alternate diagnostic tests (fasting plasma glucose test or oral glucose tolerance test) if there is disagreement between HbA1c and blood glucose levels (ADA, 2018: S13-S27), alternative methods for blood glucose monitoring such as more frequent and/or different timing of self-monitoring blood glucose (SMBG) or continuous glucose monitoring (CGM) use, and alternative tests of assessing average glycemia such as fructosamine and 1,5-anhydroglucitol (ADA, 2018: S55-S64). However, there were no reports about red cell lifespan changes in subjects with Hb E variant, so Hb E may or may not affect the HbA1c results through the red cell lifespan changes (Rhea, 2014: 5-16).

In summary, this work presents a good comparability in samples with hemoglobin type A2A and heterozygous Hb E, and the influences of high levels of Hb E and Hb F on five different HbA1c measurement methods. GH-900 (LPLC-based HbA1c method) were interfered with an increasing in Hb F. H9 (HPLC-based HbA1c method) gave the results under reportable range, in homozygous Hb E samples. Therefore, Hb E and Hb F are interfering factors that laboratories must be cautious when reporting results if its presence is suspected. However, HPLC is the only method that could detect the Hb variants in the samples, whereas immunoassay, enzymatic assay, and boronate affinity chromatography could not, this could be helpful for clinical interpretation particularly in cases of which the red cell lifespan is altered.

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