The comparison of ion exchange-high performance liquid chromatography (IE-HPLC) and capillary electrophoresis (CE) for HbA1C measurement

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ABSTRACT

Background: HbA1c is glycosylated hemoglobin that routinely performed in a patient with Diabetes Mellitus. There are several methods for HbA1c measurement. One of them is based on the molecular charge such as High-Performance Liquid Chromatography (HPLC) and Capillary Electrophoresis (CE). Each method has its advantages and disadvantages. Thus, the selection of HbA1c measurement method is an important thing. This study was aimed to compare HbA1c measurement with HPLC and CE method.

Methods: HbA1c measurement was done by HPLC and CE methods in 110 patient samples which have data of average blood glucose level during three months. We investigate the correlation of HbA1c results with average blood glucose level during three months, precision, methods comparison and interference including Hb variant. This study was conducted in the Central Laboratory of dr. Saiful Anwar Hospital during March-August 2018. Correlation analysis was done by Pearson analysis while precision test and methods comparison were done by Bland Altman Plot and Passing-Bablok Regression analysis.

Results: There were correlation between estimated Average Glucose from HbA1c (eAGHbA1c) calculation with an average blood glucose level during 3 months in both methods. From precision test we found intra-assay coefficient of variation in normal level (HbA1c ± 5%) by HPLC vs CE was 1.7% vs 0.63% and in high level (HbA1c ± 11%) was 0.59% vs 0.61% and inter-assay coefficient of variation by HPLC vs CE was 2.47% vs 2.17%.

Method comparison results showed a strong correlation on both methods. The results of HbA1c in the group without Hb variant by CE method was slightly lower than HPLC. In Hb variant group, the CE method was slightly higher than HPLC, but it was not significant for HbA1c interpretation.

Conclusion: HbA1c measurement by HPLC and CE method are comparable, and there is no significant difference in the results obtained.

Keywords: HbA1c, measurement, HPLC, CE


INTRODUCTION

HbA1c is glycosylated hemoglobin at 1 or 2 N-terminal valine of the β chain of Hemoglobin (Hb) tetramer molecule, which is also named A1c or glycated hemoglobin.1-3 HbA1c is the main fraction of Hb bound to glucose (glyco-Hb) in which in a patient with normal condition there is a low amount of glyco-Hb level. In Diabetes Mellitus (DM) patient with normal Hb, HbA1c level is correlated with blood glucose level. HbA1c can describe the average blood glucose level for 2-3 months by measuring the percentage of hemoglobin glycosylation.3,4

The HbA1c measurement method is divided into two categories. The first method is based on the molecular charge, such as ion exchange/cation exchange High-Performance Liquid Chromatography (IE-HPLC) and capillary electrophoresis (CE). The second method is based on molecular structure such as immunoassays, boronate affinity chromatography, and enzymatic reactions.3,4 Each method has its advantages and disadvantages; thus selection of HbA1c measurement method should be carefully made.3

The IE-HPLC method separates Hb based on the difference in charge between HbA1c hemoglobin and other hemoglobin. It is also a standardized reference method by the National Glycohemoglobin Standardization Program (NGSP) and Diabetes Control and Complications Trial (DCCT).6 The HbA1c examination is less accurate for a patient with anemia, hemoglobinopathy, normal turnover of erythrocytes such as hemolytic anemia and iron deficiency, as well as bleeding. Therefore, it cannot be used for diagnosis of DM.7

Nowadays, with the development of analyzer with IE-HPLC method, it has been able to detect the presence of hemoglobinopathy.6 This is important because the measurement of HbA1c can be false low or false high in patients with Hb variants.8,9 In the HbA1c measurement by IE-HPLC method using a D-10 dual HbA2/ F/A1c analyzer (Biorad), elongation of elution time will give results that are not affected by common Hb traits such as HbAS, HbAC, HbAE, and HbAD, but it can still be affected by other Hb variants such as high HBF
levels (> 10%), HbE disease (very high HbA2), Hb Santa Juana, and Hb Raleigh.8,9,10

HbA1c measurement using capillary electrophoresis (CE) is a relatively new, yet it is a well-validated method that separates A1c and other Hb fractions through differences in charge at high voltages using electro-osmotic flow. This method can be useful in patients who have a Hb variant because it has a longer runtime, which leads to better resolution so that during the running process Hb variant can be detected directly. Capillary zone electrophoresis is very precise and accurate to estimate the Hb fraction.11

Based on a study conducted by Warade in 2017, out of 157 research subjects that were randomly examined for Hba1c, there were no significant differences in HbA1C results between HPLC methods and CE.1 The study did not examine the presence of interference in hemoglobinopathy. It is important to consider the presence of hemoglobinopathy since the prevalence of hemoglobinopathy is high in Indonesia and Southeast Asia, which is about 1-3% of the entire population.12,13

The secondary data from the laboratory information system (LIS) dr. Saiful Anwar Hospital Malang during August 2012 to March 2018 showed that there were 320 patients or 1% of HbA1c results were accompanied by Hb variants in 32,000 HbA1c examinations using the HPLC method (D-10 dual HbA2 / F / A1c, Biorad). After the time elongation was done in A2 mode, most of the 320 patients obtained high HbA2 results that led to thalassemia β and HbE disease.14

We currently receive a new device from the Ministry of Health, namely Sebia Minicap Flex Piercing with the ability to do capillary electrophoresis method. Based on previous studies, it was stated that the CE method could separate Hb based on its fractions according to zones, so it is suitable for detecting patients with Hb variants.1,2,4,5 In this study we want to compare the HbA1c measurement by the HPLC method (dual D-10 HbA2 / F / A1c, Biorad) which is the reference method with Sebia Flex piercing CE method.4 The purpose of this study was to determine the correlation of both methods with the average blood glucose levels during three months, the precision, and comparison of the two devices method, and also to assess the HbA1c results of the two devices against variants of Hb, especially HbA2 and HbE.

METHODS

This study was an observational analytic method with a cross sectional study design. The study was conducted in the Central Laboratory of Clinical Pathology, dr. Saiful Anwar Hospital Malang. This study was approved by the Ethics Committee of dr.Saiful Anwar Hospital Malang with the number 400/193/K.3/302/2018.

The study population was a sample of DM patients with normal and high HbA1c levels that we took from the examination of patients who routinely visit at the Endocrinology-Metabolic Division of Internal Medicine Department dr. Saiful Anwar Hospital Malang. Blood samples were taken and collected using EDTA tube (5 cc of blood for Hba1c measurement using HPLC and CE methods). The inclusion criteria for samples are DM patients aged 30-90 years, had data of average blood glucose level in the last 3 months in a row (secondary data from LIS), no history of splenectomy, and agreed to take part in the study. The exclusion criteria were the patient without data of average blood glucose level in the last 3 months, history of bleeding in the last 3 months, and patient with blood transfusion in the last 3 months. Samples were taken by consecutive sampling from March through August 2018. The minimum sample size for the device comparison in this study were ≥ 40 samples, which follows the CLSI EP09-A3 guidelines.15

We perform precision tests by repeating multiple examinations in one sample with normal HbA1c levels (± 5%) and high levels (± 11%) using the two methods (IE-HPLC and CE). Measurement of the Coefficient of Variation (CV) was done by intra-assay and inter-assay repetitions of each sample with both methods. Determination of the repetition number of samples for precision (CV) test is determined statistically.9

$$N = \frac{(Za + Zβ) \gamma^2}{\delta^2} = \frac{(1.96 + 0.26)}{1^2} \times 3^2 = 20$$

The expected HbA1c measurement precision / CV is <1% while the highest that can be accepted is 3%, where 1-3% still meets the NGSP criteria.16 The study sample of inter-assay CV was carried out with the control material from the Biorad D-10 (IE-HPLC) factory and the control material from the Sebia minicap (CE) factory every day for 20 days. Precision or CV is Standard Deviation (SD) divided by mean times 100%.

Correlation analysis was done between average blood glucose level for 3 months and Hba1c that was examined in the fourth month. HbA1c measurement was performed by IE-HPLC and CE methods at the same time. Based on the results of the HbA1c measurement, the average of blood glucose was estimated using the following formula:17

Average estimated blood glucose (mg/dl) = 28.7 × HbA1c levels (%) - 46.7
Then a correlation test between the average blood glucose level for 3 months and an estimate of blood glucose averages from the formula in both methods was conducted.

All data is entered in data tabulation and analyzed. Data is analyzed using the IBM®SPSS® Statistics version 25.0.0, and MedCalc® version 14.8.1 programs. Furthermore, the characteristics between groups (without Hb variants and Hb variants) were compared with independent t-tests. Analysis of correlation of blood glucose means for 3 consecutive months with an estimate of blood glucose average from the results of HbA1c both methods using the Pearson correlation test. Analysis of the comparative test method is done by comparing the results of the IE-HPLC and CE HbA1c on the entire population and analyzing the interference without Hb variant and with the Hb variant using the regression analysis of Passing Bablok, Plot Bland Altman and correlation coefficients using MedCalc® version 14.8.1.

RESULTS

Characteristics of Research Subjects
This study obtained a sample of 110 DM-type 2 patients which fulfilled the inclusion and exclusion criteria during of March-August 2018. Out of 110 patients, 60 diabetic patients were known to have no Hb variants and there were 50 diabetic patients with Hb variant. Data for patient characteristics can be seen in Table 1 and Table 2. From the Table 1, the average age of respondents was 59.6 ± 9.72 years old, males predominant (54.5%), and a similar average of blood glucose during 3 months (171.8 ± 71.86 mg/dL) in both IE-HPLC and CE groups. However, there was no significant difference in the characteristic of respondents between with and without Hb variant groups (P>0.05)

**Correlation test of blood glucose mean and estimated average glucose (eAG)**
The results of the correlation of the blood glucose mean were carried out on the average generated at the last 3 months examination, and the average estimation results obtained from the HbA1c measurements can be seen in Table 3. From Table 3 it can be concluded that there are a statistically strong positive significant correlation among those methods (r>0.6; P<0.05).

**Precision Test**
Following are the precision test results of normal levels of HbA1c (5%) and high levels of Hba1c (11%) without Hb variants. All of the CV results were <3% according to IFCC criteria and <2% based on NGSP criteria (Table 4).

**Comparative Test Method**
Analysis of the comparative test of HbA1c results measured on HPLC and CE Sebia Mini Cap devices can be seen in figure 1a and 1b. Passing-Bablok regression analysis for all researched patients (n = 110) showed that there was a systematic difference

### Table 1  Overall characteristics of research subjects

<table>
<thead>
<tr>
<th>Parameter</th>
<th>IE- HPLC</th>
<th>CE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (Years) (Mean ± SD)</td>
<td>59.6 ± 9.72</td>
<td>59.6 ± 9.72</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male n (%)</td>
<td>60 (54.5%)</td>
<td>60 (54.5%)</td>
</tr>
<tr>
<td>Female n (%)</td>
<td>50 (46.5%)</td>
<td>50 (46.5%)</td>
</tr>
<tr>
<td>HbA1c (Mean ± SD)(%)</td>
<td>7.96 ± 2.72</td>
<td>7.87 ± 2.89</td>
</tr>
<tr>
<td>Hemoglobinopathy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without Hb variant (n(%))</td>
<td>60 (54.5%)</td>
<td>60 (54.5%)</td>
</tr>
<tr>
<td>With Hb variant (n(%)), consist of:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HbE</td>
<td>50 (100%)</td>
<td>50 (100%)</td>
</tr>
<tr>
<td>HbS</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>HbC</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Average of blood glucose during 3 months (mg/dL) in patients without Hb variant (n=60) (Mean ± SD)</td>
<td>171.8 ± 71.86</td>
<td></td>
</tr>
<tr>
<td>eAGHbA1c (mg/dL) patient without Hb variant (n=60) (Mean ± SD)</td>
<td>189.5 ± 83.90</td>
<td>185.6 ± 83.21</td>
</tr>
</tbody>
</table>

- SD: Standard deviation; Hb: Hemoglobin; IE-HPLC: ion exchange-high performance liquid chromatography; CE: capillary electrophoresis.
of -0.443039 and proportional amounting 1.043016. Bland-Altman’s analysis for comparison of HbA1C measurement using HPLC and CE, shows a constant difference of 0.07%. Limit of agreement (± 1.96 SD of difference) between -0.84 - 0.98 mmol/mol. Concordance correlation coefficient r = 0.986 which means there’s a substantial strength of agreement.

A comparative test of HbA1C results measured on HPLC and CE Sebia Mini Cap devices for the patient with and without Hb variants can be seen in Figure 1c and 1d. Regression analysis of Passing-Bablok in patients without Hb variant (n = 60) indicates a systematic difference of 0.200 and proportional at 1.000. In the distribution of data through the Bland-Altman plot, it also shows that the average data shows a constant and significant difference of 0.14%. Limit of agreement (± 1.96 SD of difference) was between -0.41 - 0.68. Concordance correlation coefficient r = 0.995 which means its correlation is almost perfect.

Passing-Bablok regression analysis for patients with Hb variant (n = 50) indicates that there is a systematic difference of -1.013333 and proportional bias of 1.133333. In the distribution of data through the Bland-Altman plot, it is also shown that the mean data shows a constant and significant difference of 0.00%. Limit of agreement (± 1.96 SD of difference) was between -1.2 - 1.2; concordance correlation coefficient r = 0.986 which means there is a substantial strength of agreement (Figure 1e and 1f).

### Table 2 Demographical characteristic of patients in groups without and with Hb variant

<table>
<thead>
<tr>
<th>Variable</th>
<th>Without Hb variant (n=60)</th>
<th>With Hb variant (n=50)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years) (Mean ± SD)</td>
<td>58.2 ± 8.69</td>
<td>61.32 ± 10.65</td>
<td>0.274</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>29 (51.7 %)</td>
<td>21 (48.3 %)</td>
<td>0.507</td>
</tr>
<tr>
<td>Female, n (%)</td>
<td>31 (58.0 %)</td>
<td>29 (42.0 %)</td>
<td>0.507</td>
</tr>
<tr>
<td>HbA1c method</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPLC (%)</td>
<td>8.20 ± 2.92</td>
<td>7.63 ± 2.45</td>
<td>0.286</td>
</tr>
<tr>
<td>CE (%)</td>
<td>8.09 ± 2.89</td>
<td>7.64 ± 2.88</td>
<td>0.816</td>
</tr>
</tbody>
</table>

### Table 3 Average Blood Glucose Correlation and Estimation of Mean of Blood Glucose from Calculations, in HbA1c measured by HPLC (D10) and CE (Sebia)

<table>
<thead>
<tr>
<th>Variables</th>
<th>Correlation Coefficient (r)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average blood glucose level during 3 months</td>
<td>eAG HbA1c method of HPLC (D10)</td>
<td>0.687</td>
</tr>
<tr>
<td></td>
<td>eAG HbA1c method of CE (Sebia)</td>
<td>0.719</td>
</tr>
<tr>
<td>eAG HbA1c of CE (Sebia) method</td>
<td>eAG HbA1c method of HPLC (D10)</td>
<td>0.989</td>
</tr>
</tbody>
</table>

*Statistically Significant if p<0.05

### Table 4 Precision Comparison between HPLC Method (D10) and CE (Sebia Minicap) in DM Patients without Hb variant

<table>
<thead>
<tr>
<th></th>
<th>HPLC (D10)</th>
<th>CE (Sebia Minicap)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>CV intra-assay for normal concentration</td>
<td>20</td>
<td>4.84 ± 0.305</td>
</tr>
<tr>
<td>CV intra-assay for high concentration</td>
<td>20</td>
<td>11.1 ± 0.065</td>
</tr>
<tr>
<td>CV inter-assay with the same factory control (normal concentration)*</td>
<td>20</td>
<td>5.25 ± 0.130</td>
</tr>
</tbody>
</table>

*Different materials cannot be compared (*) ; SD: standard deviation; high-performance liquid chromatography; CE: capillary electrophoresis.
The purpose of this study is to compare the two methods, CE methods and HPLC which is the reference of NGSP. All the study subjects involved in this research were routinely controlled adults outpatient with DM in internal medicine endocrine polyclinic with age mean 59.6 ± 9.72 years old and there is a nearly balanced sex distribution between men and women.

HbA1c examination performed on 110 subjects with HPLC (tool D10) and CE (Sebia devices) showed slightly higher results on HPLC than CE with mean ± SD 7.96 ± 2.72 and 7.87 ± 2.89 but did not change the clinical meaning of the interpretation of results and there was no significant difference between HbA1c measured in HPLC (D10) with CE (Sebia). This is consistent with a

DISCUSSION

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previous study by Warade J (2017) which also states that there is no difference in HbA1c HPLC results (D10) and CE (Sebia) P value 0.954 (p<0.05).1

Correlation test of 3-month blood glucose means with the results of estimated average glucose calculated by the formula showed a fairly good correlation of HPLC (r = 0.687) and CE (r = 0.719) in patients without Hb variant and no significant differences for the estimated average glucose of HbA1c examination results in both devices (p <0.05). This means that from both devices, the results of HbA1c examination can describe the average level of blood glucose in the patient for the last 3 months and can be used for diagnosis and long-term glucose monitoring.19,20

We perform precision tests for normal patients without Hb variants in both devices. At normal levels of HbA1c ± 5% it shows the intra-assay CV of HPLC (D10) 0.63% and CE (sebia) 1.7%. Meanwhile the intra-assay CV of high level HbA1c (± 11%) showed the intra-assay CV of HPLC (D10) 0.59% and CE (sebia) 0.61%. Both CV meet IFCC criteria of <3% and NGSP criteria for <2%.16,17,21 Intraassay CV at high levels HbA1c of CE (sebia) were closer to CV of HPLC (D10) than normal levels of HbA1c, indicating that the higher the HbA1C levels measured in both devices, the higher the similarity and the better the accuracy is. The limitation of our study is that we did not measure the difference in precision and linearity of the two devices for the Hb variant group (HbA2 and HbE).

A comparative test of the two methods that were done with bland Altman and passing bablok analysis in all subjects showed a strong HbA1c correlation. But in comparison with the two groups without Hb variants and with Hb variants, HbA1c examination results showed a slightly higher mean on HPLC than in CE, but in the group with Hb variant, HbA1c examination results showed a slightly lower value in HPLC than CE. One of the advantages of the CE method is that it can separate the Hb fraction based on zones so that it can detect better compared to HPLC. The HPLC turbo II variant detects the Hb variant in this study, but it only shows the total area of A2 which might be the total area of A2 and HBE, whereas CE can separate A2 zone with E zone. So when interpreting HbA1c on HPLC with high Hb variant with A2, we cannot confirm the Hb type of the variant which causes a false low level of HbA1c. Thus we issue the results with comments on Hb variant or hemoglobinopathy. Meanwhile, the advantage of using the CE method is that in the interpretation of results, we can ensure a high zone identification in which if A2 alone is high, a thalassemia β can be the conclusion, whereas if E zone is high, it leads to HbE disease.

**CONCLUSION**

The measurement of HbA1C using HPLC (D10) and CE method (Sebia minicap Flex piercing) has a good correlation. It is also correlated well with the 3-month blood glucose mean. Both normal and high levels of intra-assay CV and inter-assay CV have met NGSP and IFCC standards. Comparison of methods on both devices shows no significant difference, but each method has its advantages and disadvantages. One of the advantages of CE (Sebiaminicap Flex piercing) is that it can detect Hb variants (HbA2 and HbE) because it can separate the two zones and other Hbpati meanwhile the HPLC methods shows the total area of HbA2 and HbE. Thus it is insignificant for HbA1c interpretation.

**ETHICAL CLEARANCE**

This research had been approved by the Ethics Committee of Saiful Anwar Hospital Malang, with the number 400/193/K.3/302/2018

**CONFLICT OF INTEREST STATEMENT**

The authors declare that there was no conflict of interest in this research.

**AUTHOR CONTRIBUTION**

All authors have contributed to all process in this research, preparation, drafting, review and approval of this manuscript.

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