ORIGINAL ARTICLE

Assessing the Correlation of Capillary Electrophoresis (CE) and High-performance Liquid Chromatography (HPLC) Methods for Measurement of HbA1c in Subjects With and Without Haemoglobin Variant

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ABSTRACT

Introduction: HbA1c measurement is important for diabetes screening, diagnosis, and monitoring. Haemoglobin variants (Hb variants) may interfere with HbA1c measurement with the interference being method specific. This study aimed to determine the correlation between HbA1c results from an HPLC-based method (BioRad D10) and the new CE-based method (Sebia Capillarys 3 OCTA) in samples with and without Hb variants. **Methods:** Samples routinely received for HbA1c analysis and samples with known Hb variants were analysed for HbA1c using both methods. **Results:** 160 samples were analysed out of which 53 (33.1%) were with Hb variants [heterozygous E (n=44, 83%) and HbH (n=9, 17%)]. Mean±SD HbA1c for samples with and without variants were 4.8±0.5% and 6.8±2.0% (BioRad D10) and 5.1±0.8% and 6.9±1.9% (Capillarys 3 OCTA), respectively. Correlation was excellent in those without variants (r=0.997, p<0.001) and moderate in those with variants (r=0.743, p<0.001). HbH peak was noted on electropherograms of Capillarys 3 OCTA for all HbH samples whilst no abnormal peaks were noted on D10. All heterozygous E had atypical profiles on electropherograms whilst 38 (86.4%) had E-window in the chromatograms. The reporting of HbA1c results for all samples was unaffected by the Hb variants. **Conclusion:** Moderate to excellent correlations were demonstrated between the HbA1c results of D10 and Capillarys 3 OCTA. The HbA1c results were not affected by the presence of heterozygous E and HbH.
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Keywords: HbA1c, Haemoglobin variant, Capillary electrophoresis, High-performance liquid chromatography, Interference

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INTRODUCTION

HbA1c is an important biomarker for both diagnosing and monitoring glycaemic control in patients with diabetes mellitus [1,2]. Its level is a strong predictor of diabetic complications and is typically measured every three months as part of ongoing patient management [1]. For the diagnosis of diabetes mellitus, an HbA1c level of ≥6.5% is internationally recognised as the cut-off value, although in Malaysia, a level of 6.3% is applied [1,3]. Given the high prevalence of diabetes mellitus in Malaysia and worldwide, HbA1c has become one of the most frequently requested laboratory tests. Consequently, ensuring accurate and reliable HbA1c measurement is essential [4,5].

Various analytical methods are available for measuring HbA1c, including ion exchange chromatography (HPLC), boronate affinity chromatography, immunoassay, and capillary electrophoresis (CE), each with distinct advantages, disadvantages and performance characteristics. The standardisation of HbA1c measurement and the availability of an international reference method have greatly enhanced the quality and comparability of results across various HbA1c platforms [4]. In Malaysia, the commonly used methods for HbA1c measurements are immunoassays and HPLC-based methods, although CE-based methods are gaining interest. A haemoglobin variant (Hb variant)

may interfere with HbA1c measurement with the interference being method specific. A method may give rise to a falsely high or low HbA1c depending on the type of Hb variant. Clinical laboratories should be aware of the limitations of their HbA1c methods particularly if the population has a widespread Hb variant to allow for the correct interpretation of HbA1c in these patients [6].

This study aimed to determine the correlation between HbA1c results from two different methods: the current Bio-Rad D10, an HPLC-based method, and the Sebia Capillarys 3 OCTA, a CE-based method, which was newly available in the laboratory. The study focused on samples with and without Hb variants

MATERIALS AND METHODS

Study design

This was a laboratory-based study conducted on blood samples routinely received for HbA1c testing by the Pathology Laboratory, Hospital Sultan Aziz Shah, UPM (HSAAS). Additionally, samples for haemoglobin analysis that were reported to have Hb variants from both HSAAS and Hospital Kuala Lumpur (HKL) were included.

Sample collection

HbA1c is routinely analysed in our laboratory bi-weekly using the Bio-Rad D10. Blood samples were collected in potassium ethylenediaminetetraacetic acid (EDTA) vacutainers and refrigerated at 2-8°C pending analysis, in accordance with the manufacturer's recommended storage conditions. For this study, all samples were additionally analysed on the Sebia Capillarys 3 OCTA, within a maximum interval of three hours between the two methods. Samples were selected through convenience sampling, aiming to include a range of HbA1c levels (low, normal, and high) by utilising readily available specimens sent to the laboratory. Additionally, blood samples routinely received for haemoglobin analysis at HSAAS and HKL that were confirmed to have Hb variants during the study period were included for HbA1c analysis. Samples from HKL were initially stored at -80°C transported under controlled conditions to HSAAS, and subsequently analysed for HbA1c on both the Bio-Rad D10 and Sebia Capillarys 3 OCTA systems. All samples were from patients aged ≥18 years and were excluded if the sample volume was insufficient or if haemolysis was present.

The Bio-Rad D10 employs an HPLC technique to separate haemoglobin fractions based on molecular charge, while the Sebia Capillarys 3 OCTA uses an electrophoresis technique to separate Hb fractions in silica capillaries based on electrophoretic mobility. All instruments and reagents used in this study were operated in accordance with the laboratory's standard operating procedure and the manufacturers' instructions.

Data analysis

Statistical calculations were performed using the IBM SPSS Statistics for Windows, Version 27.0. A paired-sample t-test was conducted to compare HbA1c results measured by the two HbA1c methods. Linear regression analysis was performed to determine the correlation between the Bio-Rad D10 and Sebia Capillarys 3 OCTA in samples with and without Hb variants. Bland-Altman plots were constructed to assess the agreement of HbA1c levels on both methods. The y-axis represents the difference between HbA1c levels on the two methods [Bio-Rad D10 – Sebia Capillarys 3 OCTA], whilst the x-axis represents the average HbA1c level = [(Bio- Rad D10 + Sebia Capillarys 3 OCTA) / 2]. The 95% limits of agreement were calculated as mean ± (1.96 x SD). A p-value of <0.05 (95% confidence interval) was considered statistically significant in all analyses. The Pearson correlation coefficient (r) was used to assess the linear association between HbA1c results obtained from the Bio-Rad D10 and the Sebia Capillarys 3 OCTA.

Ethics

Ethical approval to conduct the study was obtained from the Malaysian Research Ethical Committee (MREC) Ministry of Health (NMRR ID-21-02136-PW) and the Ethic Committee for Research Involving Human Subject (JKEUPM), JKEUPM-2021-198.

RESULTS

A total of 160 HbA1c samples were obtained from 160 subjects, out of which 107 (66.9%) were not known to have Hb variants, while 53 (33.1%) had an Hb variant. The Hb variants included heterozygous E trait (n=44, 83%) and HbH (n=9, 17%). Table I shows the comparison of HbA1c values between the two methods in samples with and without Hb variants. The Sebia Capillarys 3 OCTA produced a slightly higher HbA1c values compared to the Bio-Rad D10 (p<0.001). The mean HbA1c difference between the two methods in the Hb variant and non-Hb variant groups was -0.1 (95% CI: -0.09, -0.03) and -0.3 (95% CI: -0.42, -0.13), respectively.

Table I: Comparison of mean HbA1c between Bio-Rad D10 and Sebia Capillarys 3 OCTA in samples with and without Hb variant

	Mean±SD of HbA1c (%)			
	Bio-Rad D10	Sebia Capillarys 3OCTA	t-value (df)	p-value
Without Hb variant	6.8±2.0	6.9±1.9	-4.26 (106)	<0.001
With Hb variant	4.8±0.5	5.1± 0.8	-3.83 (52)	<0.001

Correlation of HbA1c results between Bio-Rad D10 and Sebia Capillarys 3 OCTA (CE) method in samples without Hb variant (N=107)

The scatter plot in Figure 1A shows a strong positive linear relationship between the HbA1c results obtained using the Bio-Rad D10 and the Sebia Capillarys 3 OCTA, which is confirmed with a Pearson's correlation coefficient (r) of 0.997 (p <0.001). Figure 1B displays the Bland-Altman diagram for the samples in patients without Hb variant. The limits of agreement (± 1.96 SD of difference) were between -0.4 and 0.2. It showed good agreement with minimal dispersion. The mean differences were still within the limit of agreement, supporting the high reliability of the pictogram. Based on regression analysis, there was no systematic bias (b=-0.07, 95%CI=-0.38, 0.24, t=-0.44, p=0.664).

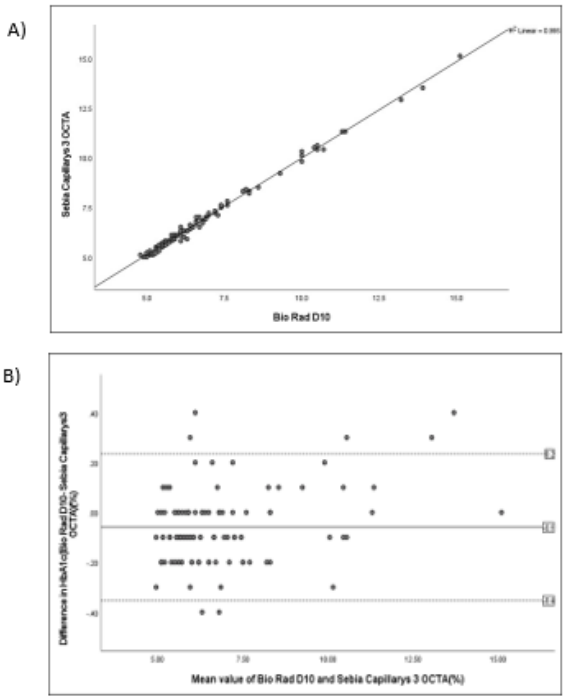

Fig. 1: A) Linear regression analysis of Bio-Rad D10 and Sebia Capillarys 3 OCTA in samples without Hb variant. B) Bland Altman plot displaying the differences between Bio-Rad D10 and Sebia Capillarys 3 OCTA in samples without Hb variant.

Correlation of HbA1c results between Bio-Rad D10 and Sebia Capillarys 3 OCTA (CE) method in samples with Hb variant (n=53)

The scatter plot in Figure 2A shows that the correlation between HbA1c results obtained on Bio-Rad D10 and Sebia Capillarys 3 OCTA for samples with Hb variant was moderate which was also evidenced by Pearson's correlation coefficient (r) of 0.743(p <0.001).

Figure 2B shows the Bland-Altman plot for samples with Hb variant. Although the mean differences were close to zero and within the limit of agreement, there were a few outliers. A regression coefficient (β) was calculated to estimate a relationship between the difference and the mean. Based on regression analysis, there was systematic bias expected (b=-0.42, 95%CI=-0.63, -0.21, t=-4.07, p<0.001).

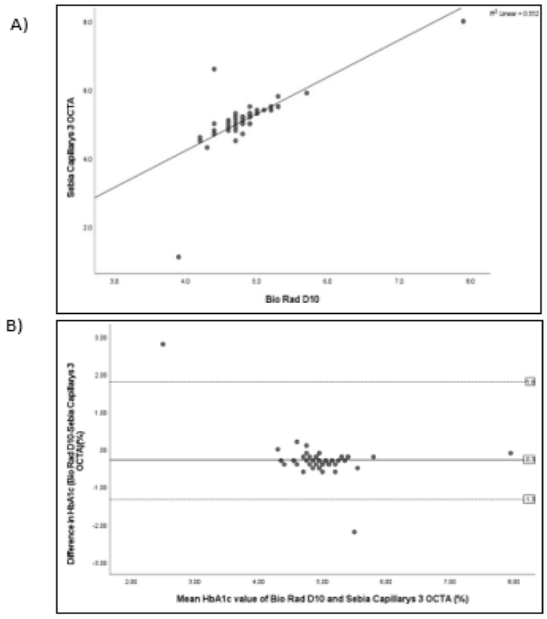

Fig. 2: A) Linear regression analysis of Bio-Rad D10 and Sebia Capillarys 3 OCTA in samples with Hb variant. B) Bland Altman plot displaying the differences between Bio-Rad D10 and Sebia Capillarys 3 OCTA in samples with Hb variant.

Out of 53 samples with Hb variant, nine (17.3%) were samples with HbH. In all the HbH samples, an HbH peak was noted on the electropherogram of Sebia Capillarys 3 OCTA consistent with the haemoglobin analysis result. When these samples were analysed on Bio-Rad D10, no abnormal peak was noted on the chromatogram, and the HbA1c results were reported as usual. The mean HbA1c difference between the two methods was calculated as 0.2% (t =-3.098, p=0.015). There were 44 samples with heterozygous E traits with HbA1c values of 4.2% - 6.6% on Bio-Rad D10 and 4.2 - 5.7% on Sebia Capillarys 3 OCTA. On Bio-Rad D10, an E- window appeared on the chromatogram for 38 (86.4%) of the samples with values ranging between 27.6% and 31.7%, which is within the criteria for reportable HbA1c. For these samples, the HbA1c report included a comment 'presence of abnormal peak on chromatogram which suggests the presence of Hb variant, but it does not interfere with the HbA1c results. There were six (13.6%) samples in which no E-window was noted. Analysis on Sebia Capillarys 3 OCTA revealed all 44 samples had atypical profiles noted on the electropherogram report. A peak was noted before the HbA2, which corresponds to the peak for heterozygous E. The mean HbA1c difference between the two methods was 0.4% (t =-0.723, p-value <0.001).

DISCUSSION

This laboratory-based study evaluated the agreement between the current method (Bio-Rad D10, HPLC) and the new method (Sebia Capillarys 3 Octa, CE) for measuring HbA1c in subjects with and without Hb variants. The correlation study in samples without Hb variants showed strong agreement and excellent correlation between the two methods across the HbA1c reporting range. This finding was supported by previous studies, although different CE or HPLC-based methods were used [6-9]. Jalali et al. compared HbA1c results between HPLC (Tosoh G8) and CE (Capillarys 2 Flex Piercing), in addition to an immunoassay (Roche Cobas C311) HbA1c method in samples without Hb variants [7]. They showed good agreement with narrow scattering at the regression line for all three methods and suggested that the interchangeability of these methods is an advantage, favouring economic measures in laboratories. Similarly, good correlation between the results of CE and HPLC-based methods, with an R value close to 1 (0.99), was obtained in another study [6]. In their study, HbA1c results from one CE-based method (Sebia Capillarys 2 Flex Piercing) and two HPLC-based methods (G8 Tosoh Biosciences and Bio-Rad Variant II) were compared using normal samples. Excellent concordance between the results of CE and HPLC methods ($R = 0.99$, $P < 0.0001$ for G8 HPLC; $R = 0.99$, $P < 0.0001$ for Variant II HPLC) was obtained. Sutrisani et al., also showed a strong correlation between a CE (Sebia Minicap Flex Piercing) and HPLC (Bio-Rad D10), with a concordance coefficient correlation of $r = 0.995$ [8]. In a study involving more than 1,000 samples that compared three HbA1c methods—enzymatic, turbidimetric, and CE versus HPLC—it was demonstrated that although the mean values obtained by these methods showed statistically significant differences, they were considered clinically irrelevant [10].

With regards to Hb variants, a moderate correlation was obtained between Bio-Rad D10 (HPLC) and Sebia Capillarys 3 OCTA (CE). This was consistent with previous studies that showed good correlation between the two methods in samples with Hb variants. In their samples with Hb variants, namely HbE, an excellent correlation of $r = 0.995$ was obtained [8].

HbH and heterozygous HbE variants do not interfere with HbA1c measurements by the two HbA1c methods used in this study. This concurs with both manufacturers' claims, as the abnormal peaks for the Hb variants (HbH and HbE) are expected to elute separately from the A1c peak on the chromatogram and electropherograms of Bio-Rad D10 and Sebia Capillarys 3 OCTA, respectively, and therefore should not interfere with HbA1c values. In this study, the Sebia Capillarys 3 OCTA was able to detect the variant peaks for all the samples with HbH and heterozygous E. This was consistent with another study that used a different CE-based method [11]. It has been suggested that a CE-based HbA1c method is useful in patients with Hb variants due to its long runtime and better resolution [9]. It may offer the advantage of passive surveillance of haemoglobinopathies [12]. In another study, the Sebia Capillarys 2 Flex Piercing was only unable to provide an HbA1c result in three out of 98 samples from thalassemia patients due to overlap of the HbH peak with the HbBart's within the HbA1c fraction [13]. For the rest of the samples, there were no issues in reporting the HbA1c results. They concluded that the Capillarys 2 Flex Piercing could report an accurate HbA1c value while also screening for the presence of thalassemia traits [13].

Although not all samples with variants had a peak appearing on the chromatogram of Bio-Rad D10, it did not affect the interpretation of the HbA1c result, as the peaks for HbH and heterozygous E, as previously mentioned, are not expected to co-elute with the A1c peak for Bio-Rad D10. However, there have been a few case reports showing falsely high HbA1c results in the presence of HbH, but these used a different HPLC-based method [14]. Thus, all manufacturers' claims must be verified whenever possible by the laboratory.

In this study, statistically significant differences in mean HbA1c values were observed between the Bio-Rad D10 and Sebia Capillarys 3 OCTA in samples with Hb variants. These differences may be due to method-specific variability in the presence of Hb variants. Sample storage may also have influenced the results, although all samples were stored and handled according to manufacturer guidelines. This highlights the need to review chromatograms or electropherograms for abnormal peaks and be aware of method limitations when interpreting HbA1c in such cases.

A limitation of this study is the use of convenience sampling, which, although intended to capture a broad range of HbA1c values, yielded only a limited number of samples with elevated HbA1c levels. As the samples were derived from routine laboratory samples during the study period, representation of diabetic patients with markedly increased HbA1c values was limited. Additionally, only two haemoglobin variants, HbH and heterozygous HbE were included in the analysis. As such, the findings may not be generalisable to other Hb variants.

CONCLUSION

There was excellent correlation between HbA1c results obtained from the Bio-Rad D10 and Sebia Capillarys 3 OCTA in samples without Hb variants ($r = 0.997$, $p < 0.001$), and a moderate correlation in samples with variants ($r=0.743$, $p<0.001$). The presence of HbH and heterozygous HbE did not affect the HbA1c measurements by either method, as the variant peaks

appeared separately from the HbA1c fraction on both chromatograms and electropherograms.

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