MMJ

Malta Medical Journal, 2023; 35(2)
http://mmsjournals.org/index.php/mmj/36

ORIGINAL ARTICLE

Evaluation of HbA1c using High Performance Liquid Chromatography and Capillary Electrophoresis in Type 2 Diabetes Mellitus patients suspected to have haemoglobin variant

Wan Nor Fazila Hafizan Wan Nik, Noorazliyana Shafii, Tuan Salwani Tuan Ismail, Najib Majdi Yaacob

Background

High-Performance Liquid Chromatography (HPLC) is widely used for HbA1c measurement. However, it is prone to haemoglobin (Hb) variant interference. Capillary electrophoresis (CE) is believed to have better performance in patients with Hb variant. This study aimed to compare HbA1c level between HPLC and CE among Type 2 Diabetes Mellitus (T2DM) patients suspected to have Hb variant, determine the type of Hb variant among those patients, and evaluate the agreement between both methods.

Methods

A cross-sectional study conducted at Endocrine Laboratory, Hospital Universiti Sains Malaysia, from June till December 2020. HbA1c results of adults T2DM from HPLC with suspected Hb variant were re-analysed using CE. The comparisons of HbA1c were made using paired t-test and Wilcoxon Signed Rank Test. The correlation and method comparison were made using Pearson correlation, Bland Altman (BA) and Passing-Bablok (PB), whereas the agreement using Intraclass Coefficients Correlation (ICC).

Results

250 patients were included with a median (IQR) age of 52.19 (11.11) years. For reportable results (?3.8% to ?18.5%), both methods showed no difference (p=0.382) whereas the results were difference for HbA1c >18.5% (p=0.048). 26 patients had Hb analysis with majority having Hb E trait 14 (5.6%). HPLC overestimated HbA1c in patients with Hb J and alpha Hb variant while CE able to report. Pearson correlation and PB regression analysis showed good correlation (r=0.987, p<0.001) and good agreement [slope of 1.0 (95% CI: 1.00 to 1.03); intercept of -0.3 (95% CI: ?0.61 to 0.30)]. BA plot revealed a mean difference of 0.30% (95% CI:0.00 to 0.50) with limits of agreement from ?0.54 to +1.14. ICC showed excellent reliability (0.983 (p<0.001).

Conclusion

HPLC and CE can be used interchangeably for HbA1c analysis across the measurement range. CE is the preferred in T2DM with certain Hb variant.

Wan Nor Fazila Hafizan Wan Nik
Department of Chemical Pathology, School of Medical Sciences, Universiti Sains Malaysia, Health Campus, Kelantan, Malaysia

Noorazliyana Shafii
Department of Chemical Pathology, School of Medical Sciences, Universiti Sains Malaysia, Health Campus, Kelantan, Malaysia

Tuan Salwani Tuan Ismail
Department of Chemical Pathology, School of Medical Sciences, Universiti Sains Malaysia, Health Campus, Kelantan, Malaysia

Najib Majdi Yaacob
Biostatistics and Research Methodology Unit, Universiti Sains Malaysia, Health Campus, Kelantan, Malaysia

INTRODUCTION

Haemoglobin A1c (HbA1c) is the major form of all glycated haemoglobin (Hb) species produced by the non-enzymatic addition of glucose residues to valine moieties at the N-terminal end of the β- chain of the Hb.[3]

HbA1c can be measured by various methods and all are based on the principle of Hb fraction separation and quantification. The methods include those based on charge differences (ion-exchange high-performance liquid chromatography [HPLC], electrophoresis, and isoelectric focusing), structural differences (affinity chromatography and immunoassay), or chemical analysis (photometry and spectrophotometry).[7]

HbA1c measurement is subjected to the interference by Hb variant and this is method dependent. Hb variant is abnormal forms of Hb, caused by variations in the genetics. It is defined as Hb with single amino acid substitutions in its globin molecules. It can cause modification in Hb structure and biochemical functions that leads to either insignificant alteration of physiological effects or severe disturbances.[10]

In order to overcome the limitation of HPLC, capillary electrophoresis (CE) has been developed and adapted to the analysis of HbA1c.[13]

This study aims to determine the level of HbA1c in T2DM patients suspected to have Hb variant in Hospital Universiti Sains Malaysia (USM) using ion-exchange HPLC and CE as well as to determine the type of Hb variant among those patients. This study is also to evaluate the agreement of HbA1c results between the two methods. The awareness regarding the interference by Hb variant during HbA1c analysis is crucial in ensuring optimum management of diabetic patient. Concurrent detection of Hb variant can give additional value to routine HbA1c reports and further advice can be given accordingly.

MATERIALS AND METHODS

A cross-sectional study was conducted at Endocrine Laboratory, Department of Chemical Pathology, Hospital USM from June till December 2020. This research has been granted ethical approval by the Human Research Ethics Committee USM (HREC) (USM/JEPeM/19120945).

All HbA1c samples of T2DM patients in Hospital USM sent to the laboratory, which fulfilled the eligibility criteria based on the patient's record, were selected. The inclusion criteria were all samples from patients aged above 18 till 65 years old. The exclusion criteria were samples with Hb F > 10% and urea ≥ 30 mmol/L. Urea level of at least ≥30 mmol/L contributes to carbamylated Hb (cHb) production and cHb ≥3.5% is reported to chromatographically interfere with HbA1c measurement.[14] The largest sample size was obtained with calculation for the agreement objective. After considering 20% anticipated dropout rate, the number of patients required is 250. These samples were selected using the random sampling method.

HbA1c measurement was initially analysed using a Bio-Rad D-10 analyser based on the HPLC principle (main analyser offers for service). First, HbA1c results from HPLC were screened for the presence of the Hb variant by identifying the variant, S and C windows in the chromatogram. Then, the samples with the suspected presence of Hb variant were re-analysed for HbA1c using Sebia Capillarys 2 Flex Piercing analyser based on CE principle. Finally, the results of these two analysers were compared.

Statistical Analysis

Data entry and analysis was done using Statistical Package for the Social Science (SPSS) Version 26.0. P-value of < 0.05 was taken as statistically significant. Descriptive statistics were used to summarise the socio-demographic characteristics of the patients. All the numerical data were presented as mean and standard deviation (SD) or median and interquartile range (IQR) based on their distribution, while categorical data were expressed as frequency (n) and percentage (%). The comparison of HbA1c level between HPLC and CE was analysed using paired t-test and Wilcoxon Signed Rank Test. Correlation, method comparison, and agreement of HbA1c between HPLC and CE were made for the results within the reportable range of HPLC (≥3.8% to ≤18.5%). The correlation was evaluated using Pearson correlation analysis. The method comparison was made using Bland Altman (BA) analysis to assess the bias, whereas Passing-Bablok (PB) regression analysis was done to determine the systematic error between both methods. PB regression and BA analysis were analysed using method comparison regression in R software version 4.0.3. The agreement was evaluated using two-way random effects, absolute agreement, single rater/measurement of Intraclass Coefficients Correlation (ICC).

RESULTS

250 diabetic patients suspected of having Hb variants were included in this study. The baseline characteristics of the participants are summarised in Table 1. The included patients ranged between 19 to 65 years old with the median (IQR) age of 52.19 (11.11) years. The males predominate (52%), and the

Table 1 *Baseline characteristics of the participants (n=250)*

Variables	Median (IQR)	n (%)	
Age (years)	52.19 (11.11)		
Gender			
Male		130	(52)
Female		120	(48)
Ethnicity			
Malay		231	(92.4)
Chinese		15	(6)
Indian		1	(0.4)
Other		3	1.2)

Table 2 *The availability of haemoglobin analysis and the type of haemoglobin variant of the participants (n=250)*

Haemoglobin analysis	n (%)
Not available	224 (89.6)
Available	
Hb E trait	14 (5.6)
HbE disease	7 (2.8)
Hb J	3 (1.2)
Alpha Hb variant	1 (0.4)
HB C	1 (0.4)

Table 3 *Comparison results of HbA1c between HPLC and CE (n=250)*

	Mean (SD) of HbA1c			
	n	*HPLC method*	*CE method*	p-value
Reportable	238	8.6 (2.6)	8.4 (2.6)	0.382[a]
Non-reportable				
No peak	7	-	-	-
>18.5	5	30.5 (5.3)*	7.5 (1.8)*	0.048[b]

[a] Paired Sample Test
[b] Wilcoxon Signed Rank Test, * Median (IQR)

majority are Malay (92.4%). Of all the participants, only 26 patients had done Hb analyses for a confirmatory test for the Hb variant. The majority of these patients have Hb E trait, 14 (5.6%) with Alpha Hb variant and Hb C were the least (Table 2).

Table 3 shows the comparison of HbA1c results between HPLC and CE. The reportable range for HbA1c results using HPLC in our laboratory is ≥3.8% to ≤18.5%. Therefore, any chromatogram with no HbA1c peak or outside the reportable range will not be reported. The table showed that both HPLC and CE measurements have no significant difference (p=0.382) in those reportable results, whereas for HbA1c >18.5%, a statistically significant difference (p=0.048) was observed. Thus, HPLC was observed to overestimate HbA1c while CE was able to report HbA1c results. Furthermore, both HPLC and CE gave similar results among 7 patients with no HbA1c peaks, suggesting the absence of HbA1c in those patients, leading to the unmeasurable level of HbA1c by both methods.

Table 4 shows the HbA1c results between HPLC and CE, fasting blood sugar (FBS) and the type of Hb variant for the non-reportable HbA1c. The mean (SD) of FBS for the no HbA1c peak is 10.1 (2.3) mmol/L. Those patients were found to have Hb E disease from Hb analysis. In 5 patients with HbA1c >18.5%, HPLC gave a high result of HbA1c compared to CE, which gave reportable results corresponding to respective FBS. In these patients, 3 have Hb J variant, 1 with Alpha Hb variant and 1 with no documented type of Hb variant.

PB regression with correlation coefficient and BA plot are shown in Figure 1. The Pearson correlation analysis showed a strong positive significant linear relationship between the results of these two methods (r=0.987, p<0.001). PB regression showed a good agreement between HPLC and CE method with a slope of 1.0 (95%CI: 1.00 to 1.03) and an intercept of -0.3 (95% CI: −0.61 to 0.30). The BA plot revealed a mean difference of 0.30 % −0.54 to +1.14. The ICC value of 0.983 (p<0.001) showed excellent reliability between both methods in providing an estimated HbA1c value (Table 5).

DISCUSSION

HbA1c is widely used for the diagnosis, monitoring and complication risk predictor of T2DM. It is an indirect measure of average blood glucose level over the most recent 2-3 months.[11]

Table 4 *Distribution of HbA1c (HPLC and CE), fasting blood sugar (FBS) and type of Hb variant among non-reportable HbA1c based on HPLC (n=12)*

	HbA1c (%)			
n	*HPLC method*	*CE method*	FBS (mmol/L)	Type of Hb variant
7	No peak	No peak	10.1 (2.3) [a]	HbE disease
1	30.9	8.4	10.5	Alpha Hb variant
1	30.7	7.5	9.3	Hb J
1	30.5	6.0	7.5	Hb J
1	27.6	8.1	10.2	Hb J
1	23.4	6.8	8.7	Not available

[a] mean (SD)

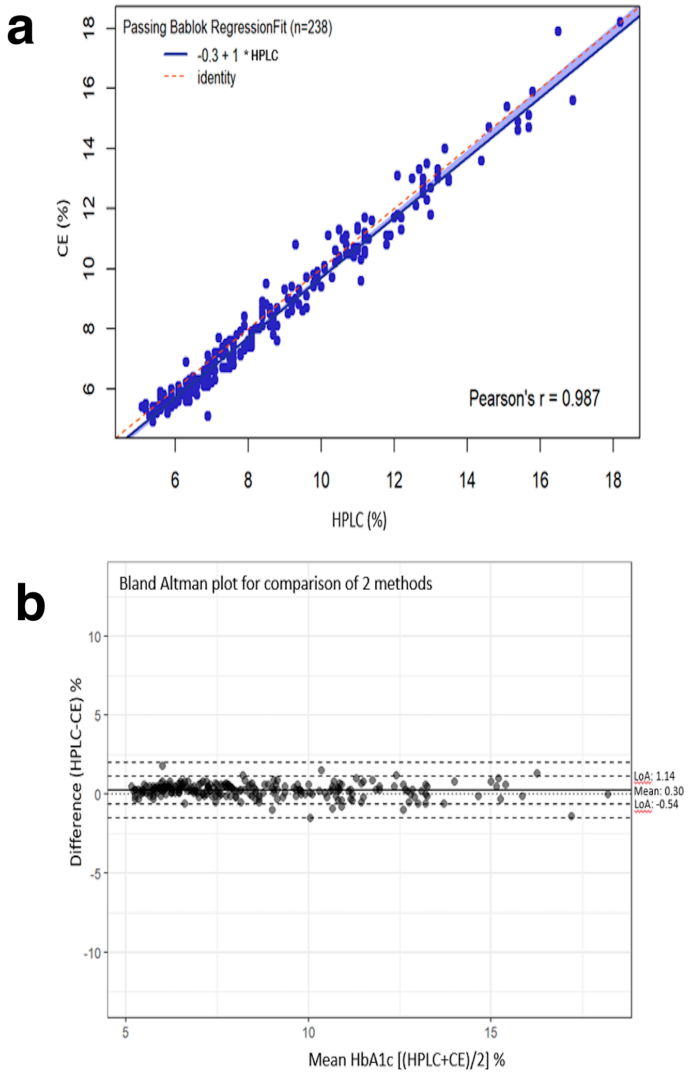

Figure 1 *Method comparison of HbA1c levels between HPLC and CE within the reportable range (n=238) (a) PB regression plot with Pearson correlation coefficient and (b) Bland–Altman plot. The Dotted line represented the 95% limit of agreement.*

The study involved 250 patients with the majority were Malays. This is because in Kelantan, majority of the people are from Malay background (91.3%).[22]

Our study demonstrated most of the patients that undergone Hb analyses are having Hb E variant (trait and disease) followed by Hb J, alpha Hb variant and Hb C. This is in keeping with the study that showed in Malaysia, Hb E is the most prevalent type of Hb variant and it is more commonly seen in Malay with carrier rate of 5% and there are 10 Malays with Hb E to one in Chinese Malaysians.[16]

The HbA1c level within the reportable range showed no significant difference when measured with HPLC and CE. However, inferior performance of HPLC compared to CE can be seen when HbA1c result exceeding linearity limit of HPLC (HbA1c >18.5%) (Table 3, 4). This is attributed to the presence of Hb variant that analytically interfere with measurement of HbA1c using HPLC. For instance, in alpha Hb variant and Hb J, HPLC give misleading result of elevated HbA1c value which significantly different with CE measurement. This is due to this type of Hb variant is carrying similar charge with HbA1c resulted in co-elution of both Hb species thus giving falsely high value of HbA1c with HPLC.[26]

Apart from causing analytical interference, presence of Hb variant may interfere with formation of HbA1c in vivo and this is method independent. Hb variant alters the composition and structure of Hb and resulted in error of measurement HbA1c level. This study found that patients with Hb E disease produced no HbA1c peak both on HPLC and CE (Table 4). It is attributed by the fact that these patients do not have or have very little amount of Hb A and therefore no HbA1c being formed, only the glycated form of the variant can be found, namely HbE1c.[29] This kind of Hb variant impedes the glycation process of Hb in vivo and analytical assay have little influence towards variation of HbA1c result.

This study demonstrated that HPLC has good correlation and agreement with CE for HbA1c values across the measurement range. Pearson correlation analysis showed that the HbA1c level of all 238 patients within the reportable range of HPLC measured by both methods gave a good linear relationship. PB regression proved that there was no significant systematic difference as demonstrated by 95% CI of slope and intercept. The results of the BA analysis showed that the HPLC method measures on average 0.30% more than the CE method with range of agreement between −0.54 to +1.14. However, this difference was not statistically significant given by CI of mean difference which contains 0 (95% CI: 0.00 to 0.50) indicating that HPLC measurement were not

Table 2 *The availability of haemoglobin analysis and the type of haemoglobin variant of the participants (n=250)*

		95% confidence interval		
	Interclass correlation[a]	Lower bound	Upper bound	p-value
HPLC-CE	0.983	0.960	0.991	<0.001

Two-way random effects, absolute agreement, single rater/measurement

[a.] Type A intraclass correlation coefficients using an absolute agreement definition, the value taken of single measure

significantly higher than CE measurements. To further support the good comparability of HPLC and CE, ICC analysis demonstrated excellent reliability of the measurements given by these two methods. This is consistent with reports from previous studies have shown that HbA1c result measured from HPLC and CE methods are comparable and there are no significant different observed.[31]

The clinical laboratory must aware of the effect of locally prevalence Hb variant when choosing the analytical assay for HbA1c measurement. Laboratories personnel must take extra caution on reporting results when the presence of a Hb variant is suspected. As with other laboratory test, any discordant result with clinical finding should be investigated further. In those patients with Hb variant that are not eligible for HbA1c measurement, non-Hb-based methods such as continuous glucose monitoring, serum fructosamine or glycated albumin can be alternative way to access long term glycemic control.

CONCLUSION

The HbA1c levels between HPLC and CE are comparable and have good agreement that can be used interchangeably for the analysis of HbA1c across the measurement range. However, CE has advantages in the presence of Hb variant. Special attention should be given during interpretation of HbA1c in the presence of Hb variant to prevent mismanagement of these patients.

SUMMARY BOX

What is already known?

- HbA1c can be measured by various methods with each method having limitations and advantages.
- HPLC is considered as gold standard for HbA1c analysis. However, it is prone to analytical interference by Hb variant.
- Laboratories personnel must take extra caution on reporting HbA1c results when the presence of a Hb variant is suspected.

New findings from this study

- HPLC and CE showed excellent agreement for the analysis of HbA1c across the measurement range.
- In patients with Hb J and alpha Hb variant, CE showed better performance in the measurement the HbA1c value compared to HPLC. CE was able to report a more accurate result which corresponding to FBS of those patients, meanwhile HPLC overestimated HbA1c value.
- For patients with Hb E disease, the absence of HbA in vivo leading to the unmeasurable level of HbA1c by both methods as indicated by absence of HbA1c peak.

ACKNOWLEDGEMENTS

This study received no external funding. We thank Utas Maju for the support and providing the kits for HbA1c measurement using CE without charge. We thank Endocrine Laboratory Hospital USM staffs for the assistance.

REFERENCES

1. 1Higgins T. HbA1c—An analyte of increasing importance. Clinical Biochemistry. 2012;45(13-14):1038-1045.
2. Sikaris K. The correlation of hemoglobin A1c to blood glucose: SAGE Publications; 2009.
3. Sherwani SI, Khan HA, Ekhzaimy A, Masood A, Sakharkar MK. Significance of HbA1c test in diagnosis and prognosis of diabetic patients. Biomarker Insights. 2016;11:BMI. S38440.
4. Burtis CA, Ashwood ER. Tietz textbook of clinical chemistry: Amer Assn for Clinical Chemistry; 2015.
5. Goodall I. HbA1c Standardisation Destination–Global IFCC Standardisation How, Why, Where and When: A Tortuous Pathway From Kit Manufacturers, via Inter-laboratory Lyophilized and Whole Blood Comparisons to Designated National Comparison Schemes. Clin Biochem Rev. 2005;26(1):5.
6. Organization WH. Use of glycated haemoglobin (HbA1c) in diagnosis of diabetes mellitus: abbreviated report of a WHO consultation: World Health Organization;2011.
7. Chandrashekar V. Hb A1c separation by high performance liquid chromatography in hemoglobinopathies. Scientifica. 2016;2016.
8. Thom CS, Dickson CF, Gell DA, Weiss MJ. Hemoglobin variants: biochemical properties and clinical correlates. Cold Spring Harb Perspect Med. 2013;3(3):a011858.

9. Gillery P, Hue G, Bordas-Fonfrede M, et al. Hemoglobin A1c assays and hemoglobinopathies: problems and strategies. Ann Biol Clin (Paris) 2000;58:425-9.
10. Nasir NM, Thevarajah M, Yean CY. Hemoglobin variants detected by hemoglobin A1c (HbA1c) analysis and the effects on HbA1c measurements. Int J Diabetes Dev Ctries 2010;30:86.
11. Urrechaga E. High-resolution HbA1c separation and hemoglobinopathy detection with capillary electrophoresis. Am J Clin Pathol 2012;138:448-56.
12. Warade J. Comparison of glycated hemoglobin with HPLC and capillary electrophoresis. Int J Res Med Sci. 2017;5(5):1976-1979.
13. Pundir CS, Chawla S. Determination of glycated hemoglobin with special emphasis on biosensing methods. Anal Biochem. 2014;444:47-56.
14. Paisey R, Banks R, Holton R, et al. Glycosylated haemoglobin in uraemia. Diabet Med. 1986;3(5):445-448.
15. Association AD. Standards of medical care in diabetes—Diabetes care. 2009;32(Suppl 1):S13.
16. George E. HbE β-Thalassaemia in Malaysia: Revisited. Journal of Hematology & Thromboembolic Diseases. 2013.
17. Lin C-N, Emery TJ, Little RR, et al. Effects of hemoglobin C, D, E, and S traits on measurements of HbA1c by six methods. Clin Chim Acta. 2012;413(7-8):819.
18. Jaisson S, Leroy N, Desroches C, Tonye-Libyh M, Guillard E, Gillery P. Interference of the most frequent haemoglobin variants on quantification of HbA1c: comparison between the LC–MS (IFCC reference method) and three routinely used methods. Diabetes Metab J. 2013;39(4):363-369.
19. Chu C-H, Lam H-C, Lee J-K, et al. Common hemoglobin variants in southern Taiwan and their effect on the determination of HbA1c by ion-exchange high-performance liquid chromatography. J Chin Med Assoc. 2009;72(7):362-367.
20. Thevarajah M, Nadzimah M, Chew Y. Interference of hemoglobinA1c (HbA1c) detection using ion-exchange high performance liquid chromatography (HPLC) method by clinically silent hemoglobin variant in University Malaya Medical Centre (UMMC)—A case report. Clin Chem Lab Med. 2009;42(4-5):430-434.
21. Kelantan Statistics. Department of Statistics Malaysia (DOSM). 2016.
22. Chandran A, Abdullah M, Abdul F. National diabetes Registry report 2013-Section NCD, editor. Malaysia: Ministry of Health. 2020.
23. Radin MS. Pitfalls in hemoglobin A1c measurement: when results may be misleading. J Gen Intern Med. 2014;29(2):388-394.
24. Florida S. A rare hemoglobin variant which interfered hemoglobin A1c result: hemoglobin South Florida [β1 (NA1) Val> Met, GTG> ATG; HBB: c. 4G> A]. Turk Biyokim Derg. 2014;39(2):226-230.
25. Little RR, La'ulu SL, Hanson SE, Rohlfing CL, Schmidt RL. Effects of 49 different rare Hb variants on HbA1c measurement in eight methods. J Diabetes Sci Techno. 2015;9(4):849-856.
26. Little RR, Rohlfing CL, Hanson S, et al. Effects of hemoglobin (Hb) E and HbD traits on measurements of glycated Hb (HbA1c) by 23 methods. Clin Chem Lab Med. 2008;54(8):1277-1282.
27. Bry L, Chen PC, Sacks DB. Effects of hemoglobin variants and chemically modified derivatives on assays for glycohemoglobin. Clin Chem Lab Med. 2001;47(2):153-163.
28. Mitchai M, Suwansaksri N, Seanseeha S, et al. Misleading HbA1c Measurement in Diabetic Patients with Hemoglobin Variants. Medical Sciences. 2021;9(2):43.
29. Azizi A, Sthaneshwar P, Shanmugam H, Arumugam S. Effect of HbE trait on measurement of HbA1c by three different methods. Pathology-Journal of the RCPA. 2015;47(5):495-497.
30. Sutrisnani CS, Darmawan, E., Widijanti, A., Soehita, S. The comparison of ion exchange-high performance liquid chromatography (IE-HPLC) and capillary electrophoresis (CE) for HbA1C measurement. Bali Medical Journal 2019;8(2): :316-322.
31. Genc S, Gurdol F, Kanmaz-Ozer M, Ince N, Ozcelik F. The analytical performances of four different glycated hemoglobin methods. Med Chem. 2014;4(501):5.