ORIGINAL ARTICLE

Glycated Haemoglobin (HbA$_{1c}$) Peak in Capillary Zone Electrophoresis (CZE) among Diabetes Mellitus (DM) Individuals

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ABSTRACT

Introduction: Capillary zone electrophoresis (CZE) is a method of haemoglobin (Hb) analysis for the screening of haemoglobinopathies or variant haemoglobins. In our centre, few samples sent for Hb analysis showed Hb peak at zone 10 requiring further evaluation. This pattern was observed in patients with history of diabetes mellitus (DM); hence it was postulated the peak observed is glycated Hb (HbA$_{1c}$). The objective of this study was to determine whether the expression of Hb peak at zone 10 among blood samples from DM patients correlate with HbA$_{1c}$ values measured using high performance liquid chromatography (HPLC). **Methods:** This was a cross-sectional study involving blood samples from DM patients. Samples with HbA$_{1c}$ values ≥ 6.5% by HPLC were selected and subsequently analysed by CZE method. Presence of Hb peak in zone 10 were correlated and analysed with the HbA$_{1c}$ levels measured by HPLC method. **Results:** A total of 131 samples were analysed. Hb peak was detected at zone 10 in 50/131(38.2%). Out of 50 samples, 47 (94%) were from patients with HbA$_{1c}$ level > 10%. Correlation study showed it was 18 times more likely for Hb peak to appear in zone 10 in patients with HbA$_{1c}$ > 10%. Cut off point for HbA$_{1c}$ to appear in CZE is 10.5% with AUC of 0.965. **Conclusion:** Hb peak detected at zone 10 of CZE was most likely to be HbA$_{1c}$. However, it is recommended that for every primary method of Hb analysis used be confirmed by secondary method. Therefore, both zone 10 in CZE and P2 peak in HPLC must be correlated together to achieve final diagnosis.

Keywords: Glycated haemoglobin, Capillary zone electrophoresis, Diabetes mellitus, High performance liquid chromatography, Hb analysis

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INTRODUCTION

In the Pathology Department, patients' samples go to different sections for screening, diagnosis, and monitoring of disease. There are instances when disease markers shown in common test methods may cause interpretation dilemma by pathologists reporting the case. This issue was recently encountered in our centre which uses capillary zone electrophoresis (CZE) as the first method for haemoglobin (Hb) analysis to screen for haemoglobinopathy disorders.

Many government hospitals in Malaysia have moved to using CZE as the primary screening method in Hb analysis, whilst the secondary method is high performance liquid chromatography (HPLC). Both methods are intended for the identification of haemoglobinopathies. Using HPLC, HbA$_{1c}$ is located at P2 window where even at low concentrations, it can be detected and do not share

the same window as HbA (1). The presence of P2 peak in HPLC with levels ≥ 6.5% suggest presence of DM (2). However, unlike HPLC, CZE does not separate the various posttranslational fractions of HbA (3). These modified variants are not separated by CZE until their concentration exceeds the normal threshold. Using CZE, for instance, HbA$_{1c}$ will not dissociate from HbA until it reaches a concentration above ~10% (3).

It was observed that few patient' samples sent for Hb analysis expressed abnormal Hb peak at zone 10 when screened via CZE, requiring further evaluation by HPLC. Further investigation revealed samples were from patients with history of diabetes mellitus (DM) and had high HbA$_{1c}$ and fasting plasma glucose. Further investigation using HPLC showed presence of P2 peak which was glycated haemoglobin (HbA$_{1c}$), and not due to haemoglobinopathy disorders. To our knowledge, there is limited study on Hb subtype in zone 10 of CZE among DM patients.

Therefore, considering the above observation, this study was conducted to determine expression of Hb peak at zone 10 among blood samples from patients

with DM and correlate with HbA$_{1c}$ results from HPLC. It also hoped that findings of this study are potentially to be included in local laboratory guideline to prevent unnecessary additional testing which incur cost and human resource.

MATERIALS AND METHODS

Ethical approval
This was a cross-sectional study conducted in Department of Pathology, Hospital Malacca (HM). Institutional ethical approval was granted by Universiti Putra Malaysia (JKEUPM-2018-045) and Ministry of Health Medical Research Ethical Committee (NMRR-17-2810-38408). It was carried out in accordance with the guidelines of the Malaysian Good Clinical Practice (GCP).

Study population and sampling
Study population were HbA$_{1c}$ blood samples from patients with underlying DM who were over 18 years of age and had samples analysed in HM between February 2018 to May 2018. Sample size for this study was calculated to be 98 based on observation where Hb peak in zone 10 was detected in CZE at average of 5 per 100 cases. In this study, HbA$_{1c}$ samples with concentration $\geq$ 6.5% from DM patients were selected. Inclusion criteria include samples which were less than seven days old and with volume of more than 1 ml. Subsequently, these samples were sent to haematology laboratory for Hb analysis using CZE method.

Principle of separation of Hb subtypes using HPLC
Hb subtypes including HbA$_{1c}$ were separated based on elution of haemoglobin bound to a solid phase over time by buffers with a pH gradient, using Bio-Rad Variant II analyser (Bio-Rad Laboratories, USA) located in chemical pathology. VARIANT II β- thalassaemia Short Program utilizes principles of ion-exchange liquid chromatography (HPLC). The VARIANT II Sampling Station (VSS) mixes and dilutes samples automatically before injecting them into an analytical cartridge. The dual pumps in the VARIANT II Chromatographic Station (VCS) supply a pre-set buffer gradient of increasing ionic strength to the cartridge, separating the HbA2/F based on their ionic interactions with the cartridge material (11). In HPLC, HbA$_{1c}$ is detected in P2 window at retention time of 1.25-1.43. Window (e.g., ranges) have been defined for the most often occurring haemoglobins based on their typical retention times to aid in the interpretation of results. The elapsed time between the injection sample and the apex of the haemoglobin peak is known as retention time. The retention time of each haemoglobin is unique.

Principle of separation of Hb subtypes using CZE
As for CZE, Hb analysis were performed on Capillarys 2 Flex Piercing analyser (SEBIA, France) located in haematology laboratory. CZE works on the principle of electrophoretic mobility to separate charged molecules (Hb subtypes) in an alkaline buffer with a specified pH and electroosmotic flow. The electropherogram results are examined visually for pattern irregularities based on zone (zone 1-zone 13). Direct detection allows for precise relative quantification of individual haemoglobin fractions, such as haemoglobin A2 for β thalassaemia diagnostics (4). This procedure's high resolution enables for the detection of haemoglobin variants, and in particular, the differentiation of haemoglobins S from D, and E from C. When haemoglobin E is present, the haemoglobin A2 quantitation can also be done (4). For HbA$_{1c}$, it as postulated this Hb subtype would appear in zone 10.

The above analysers have been evaluated and validated and took part in External Quality Control (EQC) by RCPA. Internal Quality Control (IQC) runs periodically as stated by the standard operating procedure.

Statistical Analysis
Data were entered and analysed using statistical package programme SPSS version 25.0 windows. Descriptive statistics for categorical variables were expressed as frequency and percentage. The association between HbA$_{1c}$ level by HPLC and its presence in zone 10 of CZE in blood samples from patients with DM was assessed using the Chi-square test. Meanwhile, receiver operating characteristics (ROC) curve was used to determine the most appropriate cut-off value of HbA$_{1c}$ for its presence in zone 10 of CZE in blood samples from patients with DM. A p- value of < 0.05 was considered significant.

RESULTS

The sociodemographic characteristics of patients are summarised in Table I. There were 131 patients with HbA$_{1c}$ $\geq$ 6.5%. Majority were in the 46-60 years age of group, (n=54, 41.2%). There were more females (n=68, 51.9%) compared to males (n=63, 48.1%). Majority were Malays, (n=95, 72.5%), followed by Chinese, (n=19, 14.5%) and Indians, (n=17, 13%). Patients with HbA$_{1c}$ between 6.5%-10%, were more (n=70, 53.4%) as

Table I: Demographic characteristics of DM patients and HbA$_{1c}$ level

Characteristics	n (%)	HbA$_{1c}$ level > 6.5-10% n (%)	HbA$_{1c}$ level > 10% n(%)	Mean ± SD HbA$_{1c}$ (%)
Age (years)				
18-25	2 (1.5)			
26-45	23 (17.6)			
46-60	54 (41.2)			
>60	51 (38.9)			
		70(53.4)	61(46.6)	10.2 ± 2.0
Gender				
Male	63 (48.1)			
Female	68 (51.9)			
Race				
Malay	95 (72.5)			
Chinese	19 (14.5)			
Indian	17 (13)			
Total	131 (100)			

compared to those with HbA$_{1c}$ > 10%, (n=61, 46.6%). The overall mean ± SD HbA$_{1c}$ was 10.2% ± 2.0.

When these blood samples were run in CZE, there were Hb peak detected in zone 10 from 50 patient samples. Out of the 50 samples, 47 were from patients with HbA$_{1c}$ > 10%, and 3 were from those with HbA$_{1c}$ between 6.5% and 10%. It was 18 times more likely (95%CI:5.9-54.9) for Hb peak in zone 10 to appear in patients with HbA$_{1c}$ > 10% compared to those with HbA$_{1c}$ between 6.5% and 10% Results were summarised in Table II. Examples of P2 peak and presence of Hb peak in zone 10 were showed in Figure 1.

Based on the ROC curve (Figure 2), the optimal cut-off HbA$_{1c}$ level in predicting the presence of Hb peak in zone 10 in CZE was 10.5%. It provides sensitivity of 92% and specificity of 86.4% with area under curve of 0.965.

Table II: Association of HbA$_{1c}$ level in HPLC with presence of Hb peak in zone 10 in CZE

	Presence of Hb peak in zone 10 in CZE, n (%)		X² statistic (df)	p value	Prevalence ratio (95% CI)
	Yes	No			
HbA$_{1c}$ level (>10%)	47 (94%)	14 (17%)	73.1	<0.001	17.9 (5.9-54.9)
HbA$_{1c}$ level (6.5%-10%)	3 (6%)	67 (83%)			

A

B

Figure 1: Examples of two patients [A& B] with HbA$_{1c}$ level of more than 10.5% measured using HbA1c program in Bio Rad Variant II and Hb peak (black arrow) is eluted in zone 10 of CZE. (A) P2 peak (glycated haemoglobin) level of 10.9% using beta thalassaemia program in Bio Rad Variant II (black arrow head) and Hb peak of 0.6% in CZE (B) P2 peak (HbA1c) level of 12.8% using beta thalassaemia program in Bio Rad Variant II(black arrow head) and Hb peak of 1.0% in CZE.
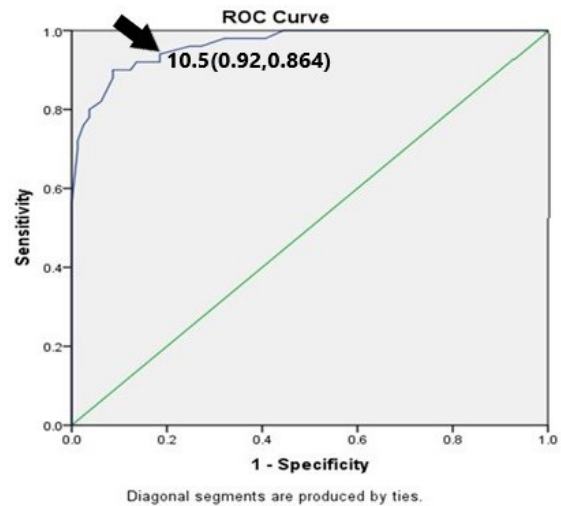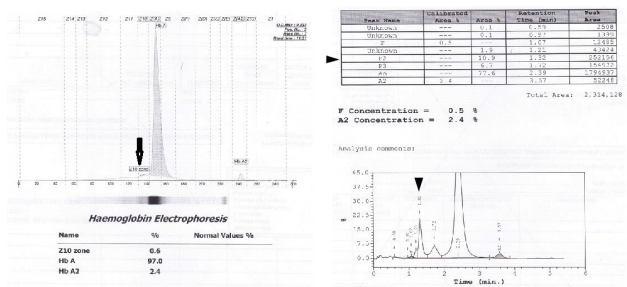

Diagonal segments are produced by ties.

Figure 2: ROC curve indicating HbA$_{1c}$ value of 10.5% in HPLC (black arrow) can be used to predict presence of Hb peak in zone 10 in CZE with sensitivity of 92% and specificity of 86.4% with area under the curve of 0.965.
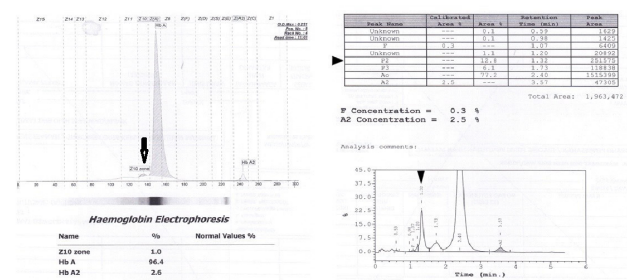
DISCUSSION

In this study, the presence of Hb peak at zone 10 of CZE is more noticeable in the group of patients with higher HbA$_{1c}$ level (>10%), which was 94% as compared to the other group of patients with HbA$_{1c}$ level of 6.5% to 10% which was only 6%. Based on these findings, there was significant association between presence of Hb peak in zone 10 of CZE with the level of HbA$_{1c}$ in HPLC (Pearson Chi-square, p<0.001). It was 18 times more likely (95%CI:5.9-54.9) for presence of Hb peak in zone 10 of CZE is due to HbA$_{1c}$. As these findings occurred in the group of patients with HbA$_{1c}$ level of more than 10% as compared to the group of patients with HbA$_{1c}$ level of 6.5% to 10%, there is a possibility that HbA$_{1c}$ in patients with 6.5%-10% still co-elute with the HbA peak in CZE, thus not detected in zone 10.

The conventional Capillarys Hb (E) used in this centre was unable to separate post-translational modification of HbA (eg: HbA$_{1c}$) from HbA until the HbA$_{1c}$ reaches a concentration greater than ~10% (3). However, using HPLC, HbA$_{1c}$ will be separated from HbA even at low concentration (2), hence the presence of Hb peak at zone 10 required further evaluation which cause additional human resource and cost to exclude presence of haemoglobinopathy.

Based on instruction manual released by the manufacturer (4), a total of eight listed Hb variants may eluted in zone 10 which include three α-chain variants (Hb Nouakchott, Hb Wayne, Hb M-Iwate [M-Kankakee]) and five β-chain variants (Hb Camden [Tokuchi], Hb Hope, Crน̃teil, Hb Complutence, Hb Stockholm). Amongst other Hb variants that are reported to coelute with HbA$_{1c}$ fraction on HPLC, causing falsely high HbA$_{1c}$ is Hb Hope (5). Mean (SD) percentage of Hb

Hope in HPLC is 45 (2.2)% (6). Hb Hope is a clinically silent beta globin chain mutation involving change of amino acid Gly to Asp in position 136 (α2 β2 136(H14) Gly/Asp). Hb Hope is also detected in zone 10 of CZE; however, Hb Hope if present will cause Hb percentage to be as more than 50% as compared to HbA1c which ranges from 0.5% to 1.6%. These values are very much higher as compared to our observed Hb peak seen in zone 10 of CZE, therefore, the peak observed is most likely of glycated Hb (Fig 1).

Other beta-chain haemoglobin variants co elute with HbA1c are Hb Camden (α2 β2 131(H9)Gln/Glu), Hb Rambam (α2 β2 69(E13)Gly/Asp), and Hb J Baltimore (α2 β2 95(FG2) Lys/Glu) (7). A number of α-globin chain variants, such as Hb Tatras β2 α2 7(A5)Lys/Asn), Hb O Padova β2 α2 30(B11)Glu/Lys) and Hb J-Meerut β2 α2 120(H3) Ala/Glu) have also been reported to interfere with HbA1c evaluation (6). These Hb variants may cause false elevation or low value of HbA1c. Hb Raleigh β2 α2 1(NA1)Val/Ac-Ala) is a point mutation in N terminal residues and if presence will cause false elevation of HbA1c in P2 peak in HPLC. Other Hb variants that produce similar interferences include Hb Graz β2 α2 2(NA2)His/ Leu), Hb Sherwood Forest β2 α2 104(G6) Arg/Thr), Hb South Florida β2 α2 1(NA1)Val/Met– amino terminus extended with a methionyl residue), Hb Niigata β2 α2 1(NA1)Val/Leu) and carbamylated Hb A (7). Another new Hb variant detected which has peak adjacent to HbA1c is Hb Belluno (8). Hb Shantou also will cause false elevation of HbA1c (9), however these variants are not detected in zone 10 of CZE hence, it may not cause an issue in our centre.

From this study, based on receiver operating characteristic (ROC) curve, it is identified that the optimal cut-off level of HbA1c in predicting the presence of Hb peak in zone 10 in capillary electrophoresis is 10.5%. However, another recent study found the cut-off point for HbA1c to elute in zone 10 is 7.1% (equivalent to 54 mmol/mol) (10). In the reported study, all samples with an HbA1c of ≥ 7.1% (54 mmol/mol) had a glycated Hb peak eluting in zone 10 and this effect was not seen in HbA1c levels of less than 7.1 % (54 mmol/mol). Hence, the study determined the threshold value that would produce glycated Hb peak eluting in zone 10 was HbA1c of 7.1% (54 mmol/mol) or greater (10). The cut off point were lower than seen in our study which could be attributed by different population served. In our study, only 3 patients with HbA1c level ranges from 6.5 to 10% had glycated Hb eluted in zone 10 of CZE. Therefore, in our population, it can be postulated that the higher the level of HbA1c specifically more than 10.5%, the higher the chance of glycated Hb peak detection in zone 10 of CZE.

One limitation of this study was no further molecular diagnosis or mass spectrometry study was sent if Hb peak was detected at zone 10 in CZE to exclude possibility of existing variant haemoglobin or other forms of glycated haemoglobin. Therefore, the findings of small Hb peak at zone 10 of CZE in our centre must be analysed by HPLC as the second method, reassuring that the small peak belongs to HbA1c. This procedure involves two different methods and is more costly.

CONCLUSION

The peak detected at zone 10 of CZE was most likely to be HbA1c. The optimal cut off point for glycated Hb to appear in zone 10 of CZE is 10.5%. Both zone 10 in CZE and P2 peak in HPLC must be correlated together and this may help to alert clinicians in identifying patients with DM.

ACKNOWLEDGEMENT

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