ORIGINAL ARTICLE

Effect of Hemoglobin E on Glycosylated Hemoglobin Determinations Using Different Commercial Kits

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Summary

The effect of HbE, a hemoglobin variant, on the determination of HbA1/HbA1c using 4 commercial kits based on cation-exchange resin, cation-exchange column chromatography and specific antibody techniques was studied. Fifty-eight normal and 63 HbE heterozygous subjects were tested for HbA1 and HbA1c using 4 commercial kits i.e. Eagles Diagnostics, Boehringer Mannheim (BM), Diastat and Ames DCA 2000. Analyses of the samples by the 4 kits were done within one week and samples were stored at 4°C before analysis. The results showed that HbE affects the determination of glycosylated hemoglobin using cation-exchange based and not kits based on specific antibody techniques.

Key Words: HbE, HbA1, HbA1c determinations, Diabetes mellitus

Introduction

Glycosylated hemoglobin levels have been shown to correlate with the subsequent development of complications of diabetes mellitus[1,2]. Thus glycosylated hemoglobins either as HbA1 or HbA1c are measured routinely to monitor the blood glucose levels of diabetes patients and hence the effectiveness of treatment[3].

Measurements of HbA1 or HbA1c involves separating these from the total hemoglobin fractions. There are several ways in which this can be achieved. Most of the commercially available kits use low pressure cation-exchange column chromatography, high pressure cation-exchange column chromatography, cation-exchange resin and/or specific antibody techniques. The simplicity of the methods correlate with the cost. Generally the fully automated versions are more costly than the manual methods and the methods which employ the use of specific antibody are the most expensive in the market.

HbA1 and HbA1c values are reported as a percentage of the total hemoglobin. Therefore the accuracy of the results obtained is affected by the total hemoglobin concentration. The level of inaccuracy varies with each kit. The same is true regarding the influence of hemoglobin variants. The manufacturers reported that atypical hemoglobins such as HbS, HbC and HbF co-elute with HbA1 or HbA1c during cation-exchange chromatography resulting in higher HbA1 or HbA1c values respectively. The influence of these hemoglobin variants were not seen in methods employing specific antibody. Although extensive studies were done on the effect of HbS, HbC and HbF, no study was done to

determine the influence of HbE on the validity of HbA1 or HbA1c measurements. HbE is an atypical hemoglobin, more commonly present in the South East Asian population. In the North East region of Peninsular Malaysia, 13% of the population was observed to be HbE heterozygotes and 3% were clinically symptomatic homozygous[4].

In this study, the effect of HbE on the determination of HbA1 and HbA1c using commercially available kits employing cation-exchange column chromatography, cation-exchange resin and spesific antibody techniques was studied.

Materials and Methods

Normal subjects

Full blood picture of 91 volunteers with no history of diabetes mellitus was done using Coulter JT hematology analyser (USA). Blood was collected into EDTA tubes and a hemogram consisting of 16 parameters which included total cell counts, hemoglobin; mean corpuscular volume (MCV); mean corpuscular hemoglobin (MCH); mean corpuscular hemoglobin concentration (MCHC); platelet count; packed cell volume (PCV), total hemoglobin and differential white cell counts. Two subjects with low red blood cell count (RBC), total hemoglobin, PCV and MCV were excluded from the study. Only those with normal full blood picture were included in this study.

Blood was collected from the volunteers into lithium heparin containers and tested immediately for random blood glucose using Reflotron (Boehringer Mannheim, Germany). Blood was also collected into EDTA containers and tested for HbA1 and HbA1c using kits from Eagles Diagnostics (USA), Boehringer Mannheim (Germany); Diastat (Bio-Rad Laboratories; USA) and Ames DCA 2000 (Bayer Diagnostics, UK). Whole blood samples were kept at 4°C before analysis as preliminary study on the storage condition showed that whole blood samples stored at 4°C is stable for up to 11 days. The HbA1 and HbA1c determinations were done within one week. Thirty one samples in which the glycosylated hemoglobin determinations by the 4 methods could not be done within the one week period were excluded from this study.

HbE subjects

HbE subjects were identified from in-patients and patients attending the out-patients department at "Klinik Perubatan Masyarakat" and "Klinik Pakar Perubatan", Hospital Universiti Sains Malaysia. Full blood picture profiles of these patients' samples sent to the hematology unit were screened for 'thalassaemic profile' i.e. low levels of MCV, MCH and MCHC with or without anemia. Blood films of these samples were then made using Wright's stain. This was done on the same day that the samples were collected. The peripheral blood smears were studied and those with microcytic hypochromic red cell, fragmented red cell, anisocytosis (different shapes of red cells) and poikilocytes (tear drop shaped cells) were identified and subjected to hemoglobin electrophoresis (Beckmann, USA) to differentiate HbE carriers from iron deficiency anaemia. Hemoglobin electrophoresis were done within 4 weeks of the time of sample collection (manufacturer's recommended time is within a month from date of collection). Sample hemolysates were stored at 4°C until analysis.

The subjects above were further selected to include only those patients who had no history of diabetes. One hundred and three blood samples which met the above requirements were confirmed for the presence of HbE using hemoglobin electrophoresis- (presence of E/A2 band without the F band).

Ninety-one subjects were thus included in this study and informed consent was obtained to collect blood for HbA1/HbA1c, total hemoglobin and RBS as above for the normal subjects. Only the samples whose HbA1 and HbA1c determinations by all 4 kits were done within one week were included in the study. Samples whose HbA1 and HbA1c determinations could not be carried out within this time period were discarded. The total hemoglobin levels was the next inclusion criteria. Only data from those subjects having hemoglobin levels greater than 7g/dl were included in the study. This is because the performance of some of the commercial kits used for the measurements of HbA1 and HbA1c have been reported to be affected by low levels of hemoglobin[5].

Using the above inclusion criteria, complete data were obtained from 58 normal volunteers and 63 HbE heterozygous subjects.

The mean HbA1 and HbA1c levels ± s.d. were then calculated for the normal and the HbE heterozygous groups (Microsoft Excel, USA). The effect of HbE on the determination of HbA1 and HbA1c were then statistically compared using non-parametric Wilcoxon Signed Ranks test.

Reagent kits

Four commercial kits were used for the measurements of HbA1 and/or HbA1c. They are the cartridge style method based on specific antibody (Ames DCA 2000 HbA1c reagent kit, Bayer Diagnostics, UK), a manual cation-exchange resin method (Eagles Diagnostics, USA), a manual low pressure cation-exchange column chromatography method (Boehringer Mannheim GmbH; Germany) and a fully automated low pressure cation- exchange column chromatography (Diastat hemoglobin kit, Bio-Rad Laboratories, USA).

Results

Subjects

Table I shows the means ± standard deviation and range of the age, random blood sugar (RBS) and hemoglobin levels of the normal and HbE heterozygous subjects. All subjects were euglycaemic and not anaemic.

Table I
Demographic, Biochemical and Hemoglobin Profile of Subjects

Subjects	Normal	HbE
No. of Subjects	58	63
No. of Males	23	31
No. of Females	35	32
Age	24±8.2 years (range 17 - 47)	23.6±15.6 years (range 1 - 64)
Random blood sugar	5.1 ± 1.1mmol/l (range 3.6 - 7.8)	4.3 ±1 .3mmol/l (range 3.4 - 7.4)
Hemoglobin	13.8 ± 0.7g/dl (range 10.6 - 16.6)	10.8 ± 2.1g/dl (range 7.2 - 15.6)

Performance of reagent kits

The Ames DCA 2000 measures HbA1c. Its within run precision for both normal and elevated quality control samples (Eagles Diagnostics, USA) were found to be 2.3% and 2.8% respectively (n=6) while the coefficient of variation (CV) for the inter-assay were 1.2% and 0.8% (n=12) respectively.

The Hemoglobin A1 kit from Boehringer Mannheim measures HbA1. Its cv for the within run precision were 2.5% and 7.4% and for the inter-assay were 11.5% and 8.4% at normal and elevated ranges respectively.

The Eagles Diagnostic kit measures HbA1 manually. It has a within run precision of 2.7% and 1.7% and inter assay precision of 6.8% and 5.0% at normal and elevated ranges respectively.

The Diastat kit measures both HbA1 and HbA1c simultaneously and automatically. Its within run precision were 1.8% and 2% and inter assay precision were 9% and 4.7% at normal and elevated levels respectively.

Effect of HbE on glycosylated hemoglobins

Table II shows HbA1 and HbA1c levels for the normal volunteers. The normal levels obtained in this study agreed with those published in the literature confirming the validity of the kits used in this study.

Table II
Comparison of HbA1 and HbA1c Levels of Normal and HbE Heterozygous Subjects as Determined using Eagles Diagnostic (Eagles), Boehringer Mannheim (BM), Diastat and Ames DCA 2000 (Ames) kits

	Eagle HbA1(%)	BM HbA1 (%)	Diastat HbA1 (%)	Diastat HbA1c (%)	Ames HbA1c (%)
Normals	6.9 ± 1.1	7.1 ± 1.2	8.3 ± 3.8	6.1 ± 1.9	5.1 ± 0.5
HbE	9.5 ± 3.9	14.8 ± 9.3	13.5 ± 10.8	10.7 ± 8.7	5.3 ± 2.7
p value	<0.0001	<0.0001	<0.0001	<0.0001	0.69

Table II also shows the comparison between HbA1 and HbA1c levels in normal versus HbE heterozygous subjects as determined by the 4 commercial kits tested in this study.

The results clearly show that there was no significant difference between HbA1c levels of normals and HbE subjects as determined by the Ames DCA 2000 kit. However all the cation-exchange chromatography based kits were affected by the presence of HbE. The HbA1 and HbA1c results determined using the Eagles Diagnostics, Boehringer Mannheim and Diastat kits were significantly higher in the heterozygous HbE group compared to the normal subjects.

Discussion

HbE involves point mutation at position 26 of the ß-chain in which glycine is replaced by lysine[6]. It is estimated that 3.5% of the Malaysian population are homozygous HbE thalassaemia which is associated with hemolytic anaemia[4]. These patients have hemoglobin levels less than 5.0g/dl. However there is a larger number of heterozygous HbE (13%) who are clinically asymptomatic. The presence of HbE was reported to affect the determination of hemoglobin by giving falsely elevated levels, often masking the anaemic states of the patients[4]. Since the determination of glycosylated hemoglobin is expressed as a percentage of the total hemoglobin, any factor affecting the total hemoglobin levels will also affect the glycosylated hemoglobin levels.

The present study was done on non-diabetic subjects and clearly shows that the presence of HbE affects the determination of HbA1 and HbA1c levels using the cation-exchange techniques.

The interference HbE exerts on the cation exchanged based methods could be due to several factors: HbE may affect the total hemoglobin determination. HbE may also co-elute with HbA1 and HbA1c fractions. HbE was reported earlier to affect the results of total hemoglobin by giving a falsely raised value[4]. If this is the main interference, then the HbA1 or HbA1c results measured would be low. However, we noticed an increase in the level of glycosylated hemoglobin measured using the cation-exchange chromatography based kits.

The co-elution with HbE on cation-exchange columns is another possibility. Other hemoglobin variants such as HbS and HbC had been reported to co-elute with the glycosylated hemoglobins resulting in the raised HbA1 or HbA1c levels. In this study, all three kits used low pressure chromatography. Perhaps the use of high performance liquid chromatography or different columns such as PolyCAT A[7] might be able to resolve HbE from HbA1 or HbA1c.

The Ames DCA 2000 kit is an immunoassay kit. It uses specific antibody directed to the first few amino acids of the N-terminus of the ß-chain. The specificity of the antibody for the first few amino acid of the ß chain is illustrated by its inability to measure HbF in which the ß-chain of HbA is replaced by γ-chain and the N-terminus of the γ is different from that of the ß-chain in the first few amino acids (gly-his-phe replacing val-his-leu). It seemed that even point mutations at position 6 of the ß-chain (such as in HbS and HbC) did not affect the specificity of the binding of the antibody. From the results of the present study it may well be that point mutation at position 26 of the ß-chain (as in HbE) is not involved in the antibody binding site.

Therefore the present study shows that HbE behaves similarly to HbS and HbC in regard to its influence on the glycosylated hemoglobin determinations. All three involve point mutations in the ß-chains which affect the glycosylated hemoglobin determinations using cation-exchange techniques.

The present study was conducted on normoglycaemic subjects. The effect of HbE at hyperglycaemic state is not known but should be determined in a future study as glycosylated hemoglobin is measured as an index for monitoring diabetes mellitus patients.

Conclusion

This study shows that HbE affects the determination of HbA1 and HbA1c using kits as determined by cation-exchange chromatography with low-pressure system. HbE does not affect the determination of HbA1c using methods based on specific antibody to the glycosylated terminus of the ß-chain. Thus it is recommended that for HbE positive individuals, the determination of their

HbA1 (or HbA1c) should be carried out using kits based on specific antibody and not on cation-exchange chromatography.

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