Laboratory Case Report

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An evaluation of glycated haemoglobin eluting in zone 10 on capillary zone electrophoresis

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Abstract: This is the first report of glycated haemoglobin (Hb) eluting in zone 10 on capillary zone electrophoresis (CZE). In an index case, the aberrant peak was picked up during routine haemoglobinopathy testing using CZE. Mass spectrometry (MS) revealed this aberrant peak to be glycated Hb. Prompted by this initial case, a study was undertaken and the results showed that there was a strong positive linear relationship ($R^2=0.774$; $\rho=0.88$) between the level of glycated Hb peak eluting in zone 10 and the HbA_{1c} value. Furthermore, all samples with an HbA_{1c} of 54 mmol/mol or greater had an HbA_{1c} peak eluting in zone 10.

Keywords: capillary zone electrophoresis; glycated haemoglobin; HbA_{1c}; zone 10 peaks.

Brief summary: A previously undescribed haemoglobin (Hb) peak in zone 10 on capillary zone electrophoresis (CZE) was evaluated and shown to be glycated Hb. A linear relationship between the percentage of glycated Hb peak and HbA_{1c} value was demonstrated. The threshold for detection of glycated Hb eluting in zone 10 is an HbA_{1c} value of 54 mmol/mol.

Introduction

Capillary zone electrophoresis (CZE) is becoming routinely used in clinical laboratories as a reliable method for the detection of haemoglobinopathies (haemoglobin [Hb] variants and thalassaemias). The CZE assay separates the Hb fractions in silica capillaries by their electrophoretic mobility and electro-osmotic flow at a high voltage in an alkaline buffer. The Hb fractions are directly detected at the specific

absorbance wavelength of 415 nm. A grid divides the Hb migration into 15 zones on the x-axis from 0 to 300, with HbA standardised to migrate at position 150 [1]. Hb variants are delineated by their electrophoretic mobility and specific zone positions. A chart describing the Hb variants associated with each zone is available [1, 2].

During routine haemoglobinopathy testing on the newly introduced Capillary 2 Flex Piercing (with Hb variant programme) instrument in our clinical laboratory, we observed a 2% Hb peak eluting in zone 10 at migration position 137, in a patient sample. Comparison with published literature failed to identify this aberrant peak. Subsequent mass spectrometry (MS) testing revealed this aberrant peak to be glycated Hb a finding that has not previously been reported.

After this index case was confirmed as glycated Hb, we observed a similar Hb peak of $\leq 2.0\%$ eluting in zone 10 on further samples during haemoglobinopathy testing using CZE. This prompted an in-depth study with two aims: firstly, to investigate whether there was a relationship between the percentage of glycated Hb eluting in zone 10 and the HbA_{1c} value, and secondly, to determine whether there was a threshold level of glycated Hb which would cause the observed zone 10 peak.

Case presentation

The initial subject of this investigation was a 15-year-old female of New Zealand Māori ethnicity. A haemoglobinopathy screen was requested due to thalassaemic indices in her full blood count: Hb 110 g/L, red blood cells (RBC) 4.64×10^{12} , mean corpuscular volume (MCV) 73 fl, mean corpuscular haemoglobin (MCH) 24 pg, red cell distribution width (RDW) 17.0%. White cell count and platelets were normal.

CZE was performed using the HbE programme on a Sebia Capillary 2 Flex Piercing analyser (Sebia, Lisses, France). The manufacturer's guidelines were followed when performing the analysis. A slightly low HbA₂ of 2.1% (normal range = 2.2–3.3%) and a normal HbF <1% (normal range = <1%) were present. In addition, an aberrant peak

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Figure 1: Capillarys Hemoglobin(E) Electrophoresis chromatogram showing the zone 10 peak of 2.0% eluting just before HbA₀.

constituting 2.0% was detected in zone 10 (Figure 1). α -Thalassaemia testing performed using the immuno-chromatographic (IC) strip test for α -thalassaemia (i + Med Laboratories, Bangkok, Thailand) was positive.

To establish the identity of the zone 10 peak, further investigations were undertaken. Hb electrophoresis was performed on cellulose acetate at pH 8.5 using the Sebia Hydragel Hemoglobin(E) kit and stained with amidoblack. This revealed the presence of two major bands: one with the mobility of HbA and the other a fast band running ahead of HbA.

Examination of lysate by electrospray ionisation (ESI) MS on an Agilent 6230 time-of-flight instrument [3] (Agilent Technologies, Santa Clara, CA, USA) showed a normal pattern of α and β chains but with very high levels of glycation at +162 Da (Figure 2). The mass increase of 162 Da is due to the addition of glucose (180 Da) minus water (-18 Da) [4].

An HbA_{1c} assay undertaken on a Bio-Rad D100 HPLC system (Hercules, CA, USA), performed on the same day as her haemoglobinopathy screen was markedly raised at >166 mmol/mol, consistent with a history of poorly controlled type 1 diabetes mellitus.

The subsequent study used patient samples which had been tested concurrently for both haemoglobi-nopathy screening and HbA_{1c} quantitation. There were

154 samples; ages ranged from 7 to 91 years, with 75 (48.7%) females and 79 (51.3%) males. Seventy-three of 154 (47.4%) samples yielded glycated Hb peaks between 0.1 and 2.0% eluting in zone 10 at migration position 136.5 ± 1.0 on CZE (Figure 3). The HbA_{1c} values ranged from 18 to 166 mmol/mol with a median of 49 mmol/mol (nr < 40 mmol/mol).

Discussion

This study reports the investigation of an aberrant zone 10 peak on CZE. To elucidate Hb variants known to elute in zone 10, the Sebia Capillarys Hemoglobin(E) Electrophoresis instruction manual [1] and Sebia website customer extranet Hemoglobin Atlas [2] were consulted. There were a total of eight listed: three α -chain variants (Hb Nouakchott, Hb Wayne, Hb M-Iwate [M-Kankakee]) and five β -chain variants (Hb Camden [Tokuchi], Hb Hope, Créteil, Hb Complutence, Hb Stockholm). The chromatograms, percentage variant and x-axis position were reviewed for the reported variants for which information was available. The zone 10 peak characterised in our index case, and in the samples further investigated, did not correlate with any of these reported variants.

Figure 2: Transformed electrospray mass spectrum of whole lysate showing normal α and β chains at 15,127 and 15,867 Da. Glycation causes a mass increase of 162 Da and the glycosylated α and β chains are shown at 15,289 and 16,029 Da, respectively.

Figure 3: Graph showing the relationship between HbA_{1c} value (mmol/mol) and percentage of peak in zone 10 for the 154 samples collected for this study.

MS determined the aberrant peak to be glycosylated Hb. The major fraction of glycosylated Hb, constituting approximately 80% of HbA_{1c}, is HbA_{1c} in which glucose is attached to the N-terminal valine residue of the β chain of HbA [5]. Glycation may also occur at sites other than the end of the β chain, such as lysine residues or the α chain of Hb. MS measures all types of glycosylated Hb, whilst methods that measure HbA_{1c}, such as the Bio-Rad D100, specifically measure glucose that is attached at the N-terminal valine [6]. Previous MS studies have established that every sample with an increased HbA_{1c}, i.e. observable β -chain glycation at the N-terminal valine, also had

α -chain glycation [7]. Additionally, some samples had glycation at other β -chain glycation sites, such as lysine, as well. Furthermore, as glycation increased, the ratio of β -chain to α -chain glycation increased, and the number of glycation sites on the β chain increased [7]. This case had an elevated HbA_{1c} value corresponding to high glycation levels as measured by MS, representing 11% and 19.5% glycosylated α and β chains, respectively, of the total α - and β -chain material. Based on the study by Peterson et al. [7], it seems reasonable to assume that the observed zone 10 peak may represent glycosylated Hb that has been glycosylated on other sites of the α and β chains, not only HbA_{1c}.

Data from the investigation initiated by the index case showed the lowest level of glycosylated Hb eluting in zone 10 was 0.1%. This level was associated with HbA_{1c} values of 54, 55, 56, 57, 60, 64, 70, 76 and 85 mmol/mol. The highest level of glycosylated Hb eluting in zone 10 was 2.0%, which was associated with an HbA_{1c} level of 166 mmol/mol. These results showed there was a strong positive linear relationship ($R^2=0.774$; Pearson's correlation coefficient $\rho=0.88$) between the percentage glycosylated Hb peak in zone 10 and the HbA_{1c} value.

All samples with an HbA_{1c} of ≥ 54 mmol/mol had a glycosylated Hb peak eluting in zone 10, this effect was not seen in HbA_{1c} levels of less than 54 mmol/mol. This determined that an HbA_{1c} of 54 mmol/mol or greater was the threshold value that would produce the observed glycosylated Hb peak eluting in zone 10 on CZE (Figure 3).

Conclusions

This report describes for the first time a glycated Hb peak eluting in zone 10 on CZE, demonstrating a strong positive linear relationship to the HbA_{1c} value. These results would suggest that if an HbA_{1c} value is less than 54 mmol/mol, the zone 10 peak is unlikely to represent HbA_{1c} and could be indicative of an Hb variant.

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