Heterogeneity of Glycated Haemoglobin as Revealed by Chromatography, Isoelectric Focusing and Mass Spectrometry: The A1c Test Standardisation and Assessment of Individual Variabilities in Haemoglobin Glycation

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Glycated Haemoglobin or A1c is used as a marker of long-term glucose control in diabetes patients. For decades, different A1c tests are employed in healthcare for diabetes diagnosis and monitoring patient progress in therapy. The heterogeneous nature of glycated haemoglobin results from the multiple possible glycation sites on each haemoglobin chain and this presents a serious challenge for A1c test standardisation, since different approaches to measuring A1c - such as electrophoretic, chromatographic, or affinity approaches - essentially measure different parts of the total glycated haemoglobin population. High resolution separation techniques such as ion exchange chromatography and electrophoresis, in particular isoelectric focusing, followed by MS - can deliver a complete picture of the glycated haemoglobin which can be of clinical interest. MS based approaches in A1c quantitation also provide an opportunity of considering the individual variabilities in haemoglobin glycation that potentially allows for personalised approach to diabetes patients.

Glycated Haemoglobin

Glycation is a non-enzymatic reaction involving the addition of glycose and/or other reducing sugars to the native protein. Glucose in its aldehyde configuration reacts with primary amines (specifically the side chains of lysine and arginine as well as the N-terminus). The glycated haemoglobin test, referred to as the A1c test, serves as an indicator of long-term glycemic control in diabetes patients since it correlates with the average blood glucose over the life-time of erythrocytes (red blood cells), which is by normal conditions is approximately 120 days. Despite wide use of A1c test in clinical practice, a complex, highly heterogeneous nature of glycated haemoglobin can potentially generate issues in both test standardisation and the results interpretation. As stated previously, the different A1c tests measure different parts of the total population of glycated haemoglobin. Another, perhaps more important issue, consists in the existence of so-called biologic variability in haemoglobin glycation, which may result in different A1c numbers for patients with the same average blood glucose.

Experimental

The chromatography instrument used was Shimadzu Prominence with a VICI Valco switching valve. The absorbance was monitored at 415 nm. The column was Mono S GL 5/50, Supelco (Bellefonte, PA). For capillary isoelectric focusing, an IEF instrument with whole column imagine detection WCID was used (Advanced Electrophoresis Solutions Ltd, Cambridge, Canada, ON). Agilent 3100 OFFGEL Fractionator was also used to collect haemoglobin fractions which were then further analysed using a Sciex API 4000.

Sample preparation: Erythrocytes were isolated from whole blood. Boronate Affinity Chromatography was performed on Glyco-Tec columns (Helena Laboratories, Beaumont, TX, USA), to separate the initial haemoglobin sample into two fractions: boronate reactive (PHB+) and non-reactive (PHA-).

Differential Glycation Index Approach

Patients mean blood glucose was measured with continued blood glucose monitors (Medtronic) by a five day continuous recording. Mass-spectrometric A1c quantitation was used [1]. Finally, the Differential glycation index, DGI_{HB}, was

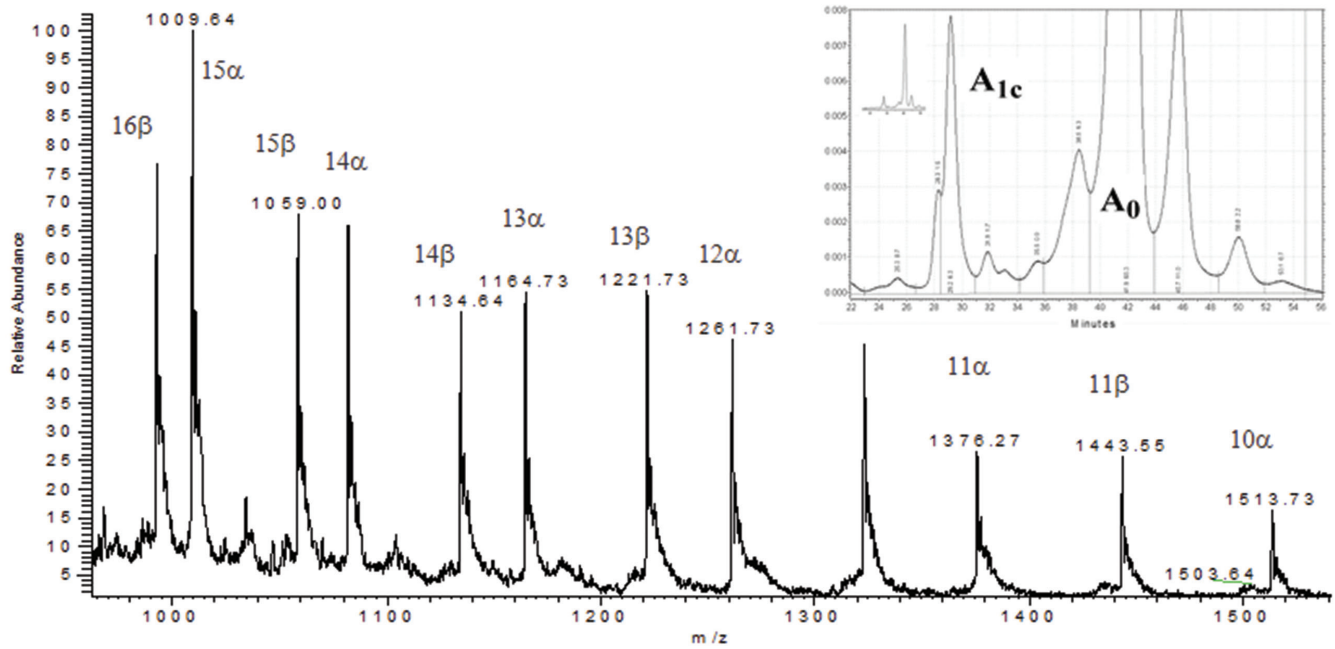

Figure 1A. Ion exchange separation of human hemoglobin and mass spectrometry showing predominant alpha and beta chains (unmodified). Part of human haemoglobin mass-spectra showing 1000-1500 m/z range (A0). Six ionisation states for each globin chain are visible. On the insert the ion exchange chromatogram of the initial human haemoglobin preparation.

calculated by the formula:

DGI_{Hb} = *[A1c(real)-A1c(expected)]/A1c(real),*

where A1c(expected) was estimated according to ADA recommendations [2].

Results and Discussion,

Ion exchange chromatography at present provides the highest resolution in analysing haemoglobin heterogeneity. It's also one of the most suitable methods for the total haemoglobin screening and preparative isolation of single fractions. Electrophoretic methods are less widely used so far in haemoglobin analysis, although they have great potential, especially Isoelectric Focusing (IEF).

Ion exchange chromatography with strong cation exchanger MonoS

In the previous studies of haemoglobin heterogeneity using Mono S cation exchanger reported by different researchers the buffers used were with a relatively low pH, below pH6, [3]. Under these conditions the different post-synthetically modified haemoglobin isoforms do not obtain sufficient relative charge differences for optimal separation because the pI differences are not pronounced as they are, when the pH of the running buffer is closer to the isoelectric points (pI) of the proteins of interest. By increasing pH it was possible to increase the resolution. Highest resolution (Figure 1) was achieved in the pH range 6.6-6.8 which is very close to the pIs of the haemoglobin isoforms of interest (pH7-7.2, approximately). The specified conditions allowed for additional gain in resolution. Here, at least three components of the A0 peak are clearly detected, which normally are non-resolved. (A0 haemoglobin peak is a major haemoglobin that usually considered as unmodified protein). Also, the 'A1c' peak appears to be composed in two components and with slower salt gradients it became possible to increase the resolution between them. A very complex pattern of haemoglobin glycation is illustrated by relatively vide 'chromatographic spectra' both for glycated and non-glycated haemoglobin fractions and by sophisticated distribution of glycated/non-glycated alpha- and beta chains in single peaks as revealed by MS.

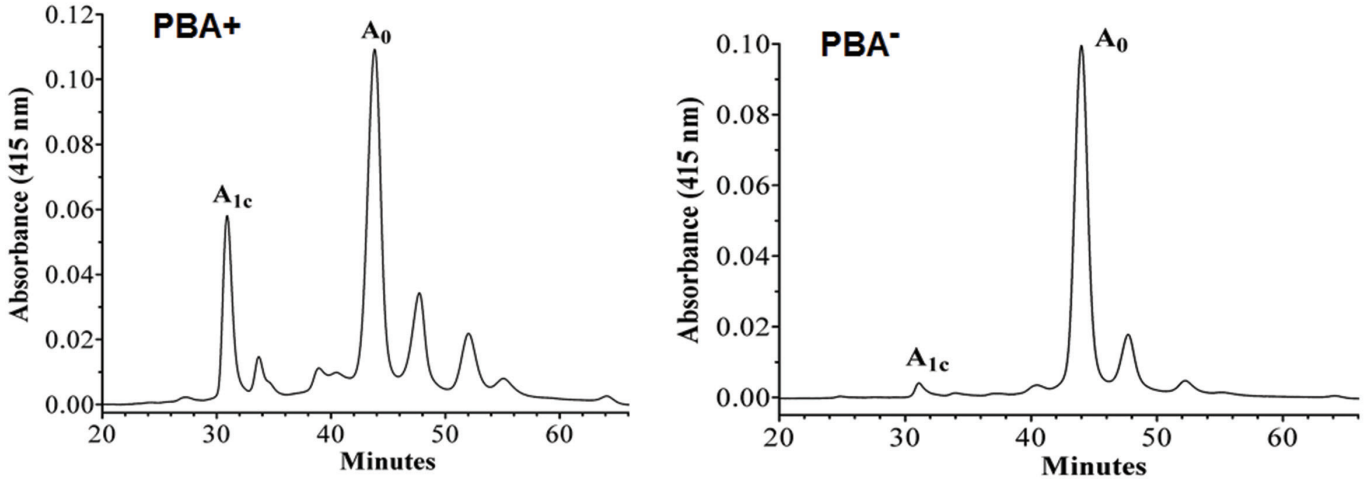

Figure 1B. Boronate affinity separation of haemoglobin followed by cation-exchange chromatography. Mono S chromatography of haemoglobins present in glycated (PBA+) and non-glycated (PBA-) fractions obtained by boronate affinity chromatography. Note the marked heterogeneity present in the PBA+ fraction. Cation exchange chromatography at pH 6.8 in acetate buffer, 20mM.

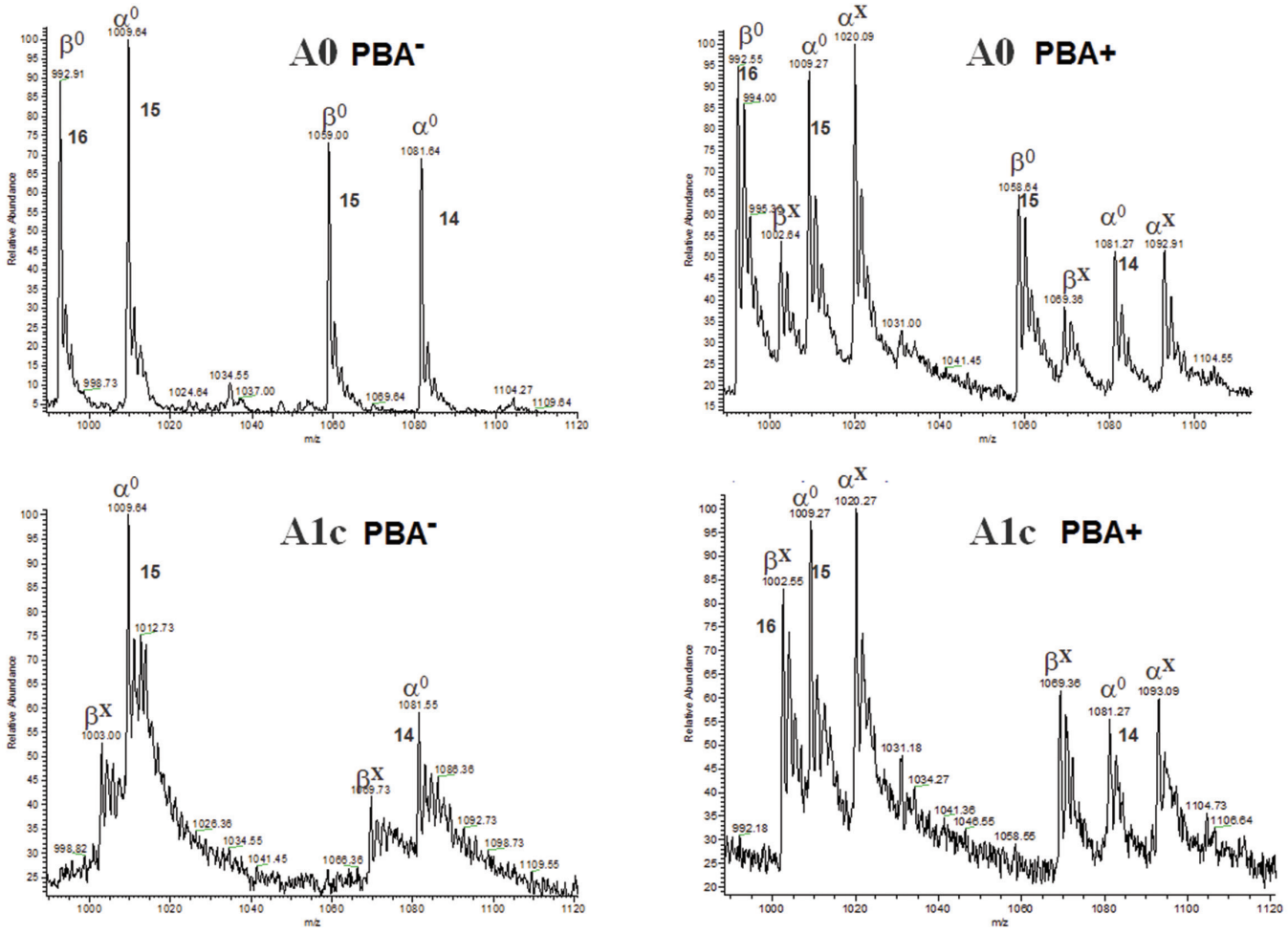

Figure 1C. Mass-spectrometry analysis of globin chains of A1c and A0 peaks from 'Non-Glycated, PBA-' and 'Glycated, PBA+' fractions, obtained by boronate affinity chromatography. The left and right panels represent the A0 and A1c peaks obtained by cation-exchange chromatography analysis of PBA- and PBA+ fractions. Only a part of mass-spectrogram is shown: alpha and beta chains with ionisation states: +14a, +15b and +15a, +16b. The modified (Mw-shift of 160 Da, approx.) and unmodified globin chains are marked by the chain symbol with right superscripts 'X' or '0', respectively.

Capillary Isoelectric Focusing with whole column imagine detection (WCID).

Capillary electrophoresis with WCID provides essential advantage in analysis of complex biological mixtures due to its opportunity to observe the whole separation path. Since most traditional CE devices are equipped with just a narrow window at the very end of the capillary, the stage of so-called mobilisation is required to scan a steady-state distribution achieved in IEF process. The latter essentially reduces the resolution and can even potentially result in artificial peaks appearing. Figure 2 shows an electropherogram of human glycated haemoglobin. Relatively high resolution, comparable to ion exchange separation, is achieved due to narrow range carrier ampholytes (CAs) preparations. Further improvement in resolution can be connected with using of modified pH gradients- the gradients containing higher number of CA species per pH unit.

Preparative Gel Electrophoresis.

Agilent OFFGel fractionator employs Immobilised pH gradients (IPG) for isoelectric focusing. Due to its original design the sample fractions can be easily collected at the end of experiment. The absence of CA chemicals makes it possible to analyse collected protein fractions without further purification. The OFFGel fractionation combined to MS, perhaps, is the easiest way for the total haemoglobin/ glycated hemoglobin analysis with the possibility of detecting minor components, Figure 3.

Clinical applications: A1c tests comparison and standardisation

The heterogeneity of glycated haemoglobin was first observed in the nineteen fifties, although it was only with MS that it became possible to give a reasonable qualitative definition to A1c [4,5]. A1c was deemed to be broadly a 'major' component of glycated haemoglobin population with a strong belief that represents N-terminal b-chain fructosyl valine. Recently, IFCC has come with a reference method that gives precise definition to A1c [6]. The quantitation is based on a MS reference method, and despite some uncertainties associated with the digestion procedure, allows a degree of confidence in the final data produced. Still, even the IFCC definition describes essentially heterogeneous population of glycated haemoglobin (a limited number of glycated haemoglobin 'isomers'), although not all researches do realize this.

The above heterogeneity is connected with the fact of the small glycated hexapeptide originates from a number of different dimer combinatory (a-chain plus b-chain), each of which can be differently glycated on different possible glycation sites. The same can be said about modern Immunoassays: they, in theory, should determine exactly the same population of glycated haemoglobin, provided one can neglect cross reactivity.

The MS detection based on haemoglobin

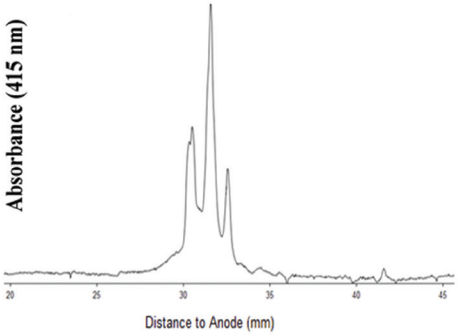

Figure 2. Experimental IEF steady-state profile for the human haemoglobin sample in the narrow pH-gradient 4-8. Run conditions: 1 min at 1500 V and 6 min at 3000V. A resolution close to ion exchange chromatography as achieved. Please note that the IEF electropherogram does not resemble the ion exchange chromatogram, since the two separations are based on different mechanisms.

chain analysis provides the easiest way to quantify A1c, even if does not identify a single component. It should be noted, however, that the use of collision induced dissociation techniques would allow for this to happen. Many commercial A1c methods do not isolate a single component, (as it is clearly visible from Figure 1, p.e.) that results in all the A1c standardisation efforts having some certain limits. This is also valid when an MS reference method is going to be used.

Differential Glycation Index concept as a tool for evaluation of individual variability in haemoglobin glycation

It has been shown that individual variability in haemoglobin glycation between diabetes patients occurs. It would, therefore, be beneficial for physicians to have a parameter which can quantify such a variability in order to modify the therapy accordingly.

The Differential Glycation Index (DGI) is the difference between the experimentally measured A1c minus the expected A1c, normalised by A1c measured. Here, the expected A1c is theoretically calculated based on average blood glucose level [2]. The index is dimensionless and it directly shows how any given individual differs from an average patient. In contrast to previous attempts to connect the average blood glycose and A1c [7-9], the approach is based on simultaneous mass-spectrometric A1c quantitation and continuous glucose monitoring CGM for average blood glucose measuring, providing the highest possible precise evaluation of the individual variability of haemoglobin glycation in diabetes patients. While blood glucose - A1c relationship is a result of multiple gene influencing both glycose metabolism and erythrocytic pathways [10], the DGI_{Hb}, which connects the same values, can be obtained with two easily measurable biochemical parameters. Based on the index value the physician becomes able to modify the therapy according to individual patient needs, in particular, to set the optimal A1c goals [11]. The A1c goals, should be essentially different, for example, for the two patients with high negative and high positive DGIs.

Conclusions

Glycated haemoglobin represents essentially heterogeneous populating circulating in human blood. Mass spectrometry provides an opportunity for better A1c test standardisation and quantitative evaluation of the individual variabilities in haemoglobin glycation. Further progress in electrophoresis and chromatography can potentially facilitate the establishment of the complete pattern of glycated haemoglobin. This goal can be achieved only with extensive pre-fractionation, since the concentration range of different glycated haemoglobin isoforms varies essentially. Mass spectrometric A1c quantitation methods combined with continuous glucose monitoring allows for evaluation of the individual variability in haemoglobin glycation that can lead to personalised therapy for diabetes patients.

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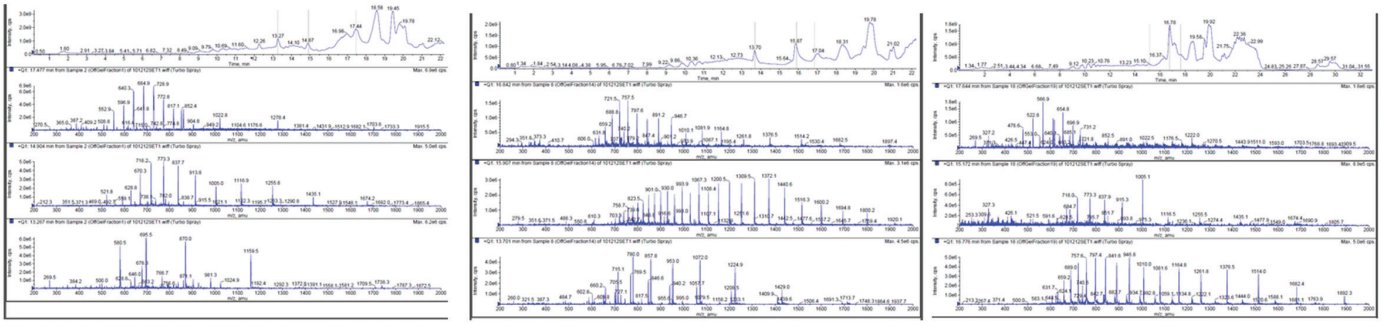

Figure 3. MS data for selected hemoglobin fractions separated with preparative isoelectric focusing (Agilent OFFGel Fractionator). The three collected fractions represent different parts of a narrow pH gradient 4-7. The upper panel on each figure corresponds to a Total Ion Count (TIC), or continuous density profile. Lower panels correspond to the individual peaks as pointed by vertical lines on the TIC curve. MS analysis was performed using an API-4000 triple quadrupole mass spectrometer (AB Sciex) coupled to a Shimadzu Prominence LC system integrated with a switching valve (VICI Valco). Reverse-phase chromatography was performed using a Jupiter 5µ C18 column (Phenomenex). Two mobile phases were used for elution: (A) 0.4% formic acid in water and (B) 0.4% formic acid in acetonitrile. The total flow rate was 0.5 ml /min. Each fraction represents an essentially heterogeneous populations including modified and partially truncated haemoglobin chains as revealed by MS.