CE-HPLC Testing of Hemoglobin A1c for Assessment of Long Term Glycemic Control in Patients with Diabetes Mellitus

Alina Gabriela Sofronescu

Department of Pathology and Microbiology, University of Nebraska Medical Center Omaha, USA

Abstract

Glycated hemoglobin A1c (HbA1c) is routinely used in the diagnosis of diabetes mellitus (DM) and for monitoring the long-term glycemic control in patients already diagnosed with DM. HbA1c is measured in the clinical laboratory based either on the charge of the glycated molecule by cation exchange high performance liquid chromatography (CE-HPLC) or electrophoresis, or on the physical structure of the glycated hemoglobin via immunaoassays, boronate affinity chromatography or mass spectrometry. The addition of glucose to HbA triggers a decrease in the negative charge of the molecule making chromatographic separation of HbA1c from HbA using CE-HPLC possible. Estimation of HbA1c by CE-HPLC depends upon the separation of each of the patient's hemoglobin species from HbA1c. In this context, the estimation of HbA1c using CE-HPLC is affected by Hb mutations (Hb variants) and posttranslational modifications (e.g. carbamylation and acetylation) that also alter the charge of hemoglobin molecule. Due to the clinical implications of diagnostic of DM and maintaining glucose control, accurate quantification of HbA1c is essential. A thorough understanding of the analytical and clinical limitations of the methods used (e.g. CE-HPLC) for HbA1c evaluation is extremely important. This review discusses the interferences of Hb variants and other factors in the evaluation of HbA1c using CE-HPLC methodology.

INTRODUCTION

Recent ADA and CDC statistics reported that about 8.3% of the USA population is currently diagnosed with Diabetes Mellitus (DM) and about 35% of the adults (age 20 and older) are in the high risk category for developing diabetes ("pre-diabetes" stages). It is also estimated that every year, about 1.9 million new cases of DM are confirmed in USA alone. The cost to society is also high, with about $116 billion medical costs and another $58 billion in compensations for disability, work loss and premature mortality (as per 2007). In the last 15 years the number of adults suffering from DM has almost tripled. The groups with the highest incidence of DM are adults over the age of 45 and individuals belonging to demographic groups with high prevalence, such as: African Americans, Hispanics and Native Americans. One of the most important steps in the diagnosis of DM is the detection of hyperglycemia. ADA defines hyperglycemia as a fasting serum glucose concentration above the upper limit of the reference interval of 70-99 mg/dl; however this does not automatically established the diagnostic of DM. The diagnostic of DM is based on the demonstration of hyperglycemia on two different occasions and, preferably, with two different assessment methods (Table 1). Until recently, the diagnosis of DM was based solely on the assessment of the glucose concentration in the patient's serum and HbA1c measurements were used primarily for monitoring glycemic control in patients already diagnosed. In 2009, the ADA recommended HbA1c evaluation be utilized for the diagnosis of DM and not just for the monitoring of long-term glycemic control [1,2]. This decision was based on the fact that the relative concentration of HbA1c correlates effectively with the average concentration of serum glucose over an immediate previous period of 2-3 months [3,4]. Although there are multiple advantages in assessing HbA1c level, there are also several limitations (Table 2), including multiple interferences and standardization issues when compared with glucose testing. HbA1c is a glycated protein that results from non-enzymatic addition of a glucose molecule to each of the N-terminal valine residues of the β chain of hemoglobin A. The process occurs in two steps: a labile Schiff base is formed initially (labile HbA1c or L-HbA1c), followed by rearrangement of the molecule (Amadori rearrangement) and stabilization of the resulting fructosamine [5]. The process is non-enzymatic and the concentration of serum
Central common hemoglobin variants worldwide are HbS, HbC, HbE and normal and variant hemoglobin peaks from HbA1c [7]. The most estimation of HbA1c depends upon a clear separation of all of the with HbA1c assessment via CE-HPLC and can lead to either falsely substitutions. The presence of variant hemoglobins may interfere been described resulting from point mutations/amino acids mutations [7] as well as some post-translational modifications CE-HPLC methods are affected by the presence of specific Hb cannot completely resolve all potential hemoglobin peaks. Most CE-HPLC methods are affected by the presence of specific Hb mutations [7] as well as some post-translational modifications such as carbylation and acetylation [8] that also alter the change of the hemoglobin molecule. Further, when HbA1c is assessed with CE-HPLC, it is eluted within a time window proximal to that of fetal hemoglobin (HbF). A high concentration of HbF, such as seen in hereditary persistence of fetal hemoglobin, will also interfere with measurement of HbA1c by CE-HPLC [9]. Currently, more than 1000 human hemoglobin variants have been described resulting from point mutations/amino acids substitutions. The presence of variant hemoglobins may interfere with HbA1c assessment via CE-HPLC and can lead to either falsely increased or decreased levels. When evaluated with CE-HPLC, estimation of HbA1c depends upon a clear separation of all of the normal and variant hemoglobin peaks from HbA1c [7]. The most common hemoglobin variants worldwide are Hbs, HbC, HbE and HbD. All of these hemoglobin variants result from point mutations within the coding region of the β-chain. Since these mutations occur at amino acids other than the N-terminal valine the normal glycation process is not affected. The substitution of these amino acids does not lead to a change in the charge of the hemoglobin variant molecule that leads to co-elution or overlap with HbA1c in most CE-HPLC test systems [9]. There is no analytical interference affecting HbA1c evaluation in patients with the common hemoglobin variants traits AS, AC, AE, and AD with the exception of systems manufactured by Menarini (Menarini Diagnostics, Italy) [9]. However, this is not the case in patients who have both β-globin alleles affected by mutation. This includes homozygous mutations such as HbSS-sickle cell disease and HbCC-hemoglobin C disease, or compound heterozygous mutations, such as HbSC. Although the process of hemoglobin glycation still occurs, leading to formation of glycated variant hemoglobins, these patients do not have HbA and therefore do not produce HbA1c. The glycated hemoglobin variants cannot currently be measured using the CE-HPLC method. In contrast, certain beta-chain hemoglobin variants, such as Hb Camden (αβ131(H9)Gln/Glu), Hb Rambam (αβ69(E13)Gly/Asp), Hb Hope (αβ136(H14)Gly/Asp), and Hb) Baltimore (αβ95(FG2) Lys/Glu), cannot be completely separated from HbA or HbA1c via CE-HPLC. Since estimation of HbA1c depends upon clear separation of each hemoglobin variant from HbA1c, co-elution yields significant effects (either falsely low or falsely high) on the

### Table 1: The diagnosis of DM is based on detection of at least two ADA criteria.

<table>
<thead>
<tr>
<th>Type of Test</th>
<th>ADA Diagnosis of Diabetes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting serum glucose</td>
<td>≥ 126 mg/dl</td>
</tr>
<tr>
<td>2h Oral Glucose Tolerance Test (OGTT)</td>
<td>≥ 200 mg/dl</td>
</tr>
<tr>
<td>Random serum</td>
<td>≥ 200 mg/dl and presence of symptoms (polyphagia, polydipsia, polyuria, blurred vision, poor wound healing, etc.)</td>
</tr>
<tr>
<td>Hb A1c</td>
<td>≥ 6.5%</td>
</tr>
</tbody>
</table>

### Table 2: Comparison of glucose and HbA1c testing modality advantages/disadvantages.

<table>
<thead>
<tr>
<th>Glucose evaluation</th>
<th>HbA1c evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting</td>
<td>No fasting necessary</td>
</tr>
<tr>
<td>Multiple draws (e.g. OGTT)</td>
<td>One draw</td>
</tr>
<tr>
<td>Reflects immediate glycemic level</td>
<td>Reflects long-term glycemic level (2-3 months)</td>
</tr>
<tr>
<td>No hemoglobin variants interferences</td>
<td>Hb variants can interfere</td>
</tr>
</tbody>
</table>

HbA1c is proportional to the prevailing glucose concentration encountered during the lifetime of the protein (Hemoglobin A) in the red blood cell. HbA1c, in the case of patients with normal RBC life spans, will reflect the average glucose level over the prior 8-12 weeks [3, 4]. It has to be noted that the glycation process is not restricted to hemoglobin A and/or only to first N-terminal valine residue of the β-globin chain. Glycation occurs on other types of serum and structural proteins (e.g. albumin and collagen) and also occurs at other amino acid residues on the hemoglobin molecule (e.g. resulting in minor glycated hemoglobins, such as HbA1a and HbA1b) (Figure 1)[6]. There are multiple methods for assessing HbA1c in the clinical laboratory and they can be divided into two categories: methods based on molecular charge and those based on structure. The former category includes cation exchange-high performance liquid chromatography (CE-HPLC) and electrophoresis, and the latter includes immunosassays, boronate affinity chromatography, and mass spectrometry [7]. The addition of glucose to HbA triggers a decrease in the net negative charge of the molecule allowing for separation of HbA1c from HbA using electrophoretic or charge-based chromatographic methods. Chromatographic separation using CE-HPLC, however cannot completely resolve all potential hemoglobin peaks. Most CE-HPLC methods are affected by the presence of specific Hb mutations [7] as well as some post-translational modifications such as carbylation and acetylation [8] that also alter the charge of the hemoglobin molecule. Further, when HbA1c is assessed with CE-HPLC, it is eluted within a time window proximal to that of fetal hemoglobin (Hbf). A high concentration of Hbf, such as seen in hereditary persistence of fetal hemoglobin, will also interfere with measurement of HbA1c by CE-HPLC [9].

![Figure 1 CE-HPLC chromatogram (Tosoh HLC-723 GHb V A1c system, Tosoh, San Francisco, CA, USA) for evaluation of HbA1c in a patient with DM, Hba, Hbf and glycated hemoglobins (HbA1a, HbA1b, L-HbA1c and S-HbA1c) are eluted separately. The relative concentration of HbA1c is expressed as percentage of HbA1c reported to total Hba (glycated and non-glycated), taking into account the area peak of each of the hemoglobins involved in the calculation of HbA1c concentration.](image-url)
quantification of HbA1c [10,11]. Furthermore, this type of interference is not restricted to β-globin variants. A number of α-globin chain variants, such as Hb Tatras βα1(5)Glu/Asn, Hb Padova βα1,30(B11)Glu/Lys and Hb J-Meerut βα1,120(H3) Ala/Glu have also been reported to interfere with HbA1c evaluation [11,12]. Of particular interest is the interference by Hb Raleigh βα1,1(NA1)Val/Ac-Ala. Hb Raleigh is a hemoglobin variant resulted from a point mutation (T to C) at the second base of the codon encoding the first amino acid of the β-chain, changing the N-terminal valine to an alanine residue. This substitution by itself would not induce a change in HbA charge, except that the N-terminal alanine is immediately acetylated to acetylalanine soon after translation [13,14]. The addition of the carboxyl group on the terminal nitrogen yields an overall charge on the hemoglobin molecule identical to that of HbA1c. The result is that the retention times of HbA1c and Hb Raleigh are indistinguishable and the peaks co-elute on the chromatogram. Thus, the presence of Hb Raleigh produces a falsely elevated HbA1c value when the latter is assessed by CE-HPLC [15]. Other Hb variants that produce similar interference in the HbA1c assay are Hb Graz βα1,7(5)A5(Lys/Asn), Hb O Padova βα1,30(B11)Glu/Lys and Hb J-Meerut βα1,120(H3) Ala/Glu [16].

The presence of Hb Raleigh produces a falsely elevated HbA1c value when the latter is assessed by CE-HPLC [15]. Other Hb variants that produce similar interference in the HbA1c assay are Hb Graz βα1,7(5)A5(Lys/Asn), Hb O Padova βα1,30(B11)Glu/Lys and Hb J-Meerut βα1,120(H3) Ala/Glu [16].

Other Hb variants that produce similar interference in the HbA1c assay include Hb Graz βα1,7(5)A5(Lys/Asn), Hb O Padova βα1,30(B11)Glu/Lys and Hb J-Meerut βα1,120(H3) Ala/Glu [16].

REFERENCES


