Chromatographic Measurements of Hemoglobin A2 in Blood Samples that Contain Sickle Hemoglobin

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Abstract. In the sickle cell syndromes, Hb A2 measurements aid in the differential diagnosis of sickle cell anemia from sickle-beta-thalassemia. The purpose of this study is to assess the Hb A2 levels in samples containing sickle hemoglobin (Hb S) by the use of an automated high performance liquid chromatography system (HPLC-Variant β-thalassemia Short Program). The blood samples analyzed were from individuals of African descent living in the state of Tennessee who had either sickle cell trait (Hb AS), sickle cell disease (Hb SS), or sickle cell–hemoglobin C disease (Hb SC). Interestingly, the Hb A2 levels determined by HPLC were found elevated in samples containing Hb S. The Hb A2 mean in Hb AS samples (n=146) is 4.09% (SD ± 0.42, range 2.20 to 5.20%); in Hb SS samples (n=33) it is 3.90% (SD ± 1.08, range 0.60 to 5.90%); and in Hb SC samples (n=27) it is 4.46% (SD ± 0.70, range 2.30 to 5.91%). The Hb A2 mean by HPLC in normal individuals (Hb AA, n=70) is 2.57% (SD ± 0.25, range 2.1 to 3.0%), and the Hb A2 range in β-thalassemia carriers is 4 to 9%. Our results show that the Hb A2 levels in Hb S-containing samples partially overlap with those expected from β-thalassemia carriers. The hemoglobinopathy laboratory should be aware of this apparent elevation in Hb A2 levels determined by HPLC in individuals carrying Hb S. Other factors, such as family history and clinical symptoms, should be taken into account before a diagnosis of sickle cell trait, sickle-beta-thalassemia, or sickle cell anemia is made.

Keywords: Hemoglobin A2, hemoglobin S, high-performance liquid chromatography, thalassemia diagnosis

Introduction

The quantitation of Hb A2 is important for the accurate diagnosis of hemoglobin disorders such as the β-thalassemias, or hemoglobin variants in combination with β-thalassemia (eg, sickle-beta-thalassemias). Hb A2 is known to be elevated in β-thalassemia trait [1], sickle-beta-thalassemia [2], and sickle cell disease with α-thalassemia [3]. In sickle cell trait, Hb A2 determined by microchromatography has been found slightly elevated [4], and significantly elevated when determined by capillary isoelectric focusing electrophoresis (IEF) [5]. However, no overlap in Hb A2 levels was seen between Hb S-containing samples and samples from patients with β-thalassemia when determined by capillary IEF. The purpose of this report is to assess Hb A2 levels by HPLC in liquid blood samples containing Hb S. We have found that Hb A2 levels quantitated by HPLC are elevated in samples from non-thalassemic individuals that carry Hb S. We have not observed this increase in Hb A2 levels in samples that contain only Hb AA. HPLC reference ranges for Hb A2 in sickle cell trait, sickle cell anemia, and Hb SC disease should be established in order to prevent an erroneous association of these disorders with β-thalassemia.

Methods

Blood specimens were collected in heparinized capillaries or in ethylene diaminetetraacetate (EDTA) microvettes (Sarstedt, Inc., Newton, NC). The criteria for sample inclusion were: (1) presence of Hb S, (2) sample age ≤ 7 days after collection, (3) symmetrical
Hb A2 chromatogram peak (Fig. 1), and (4) subjects from 1 to 60 years of age. Samples from subjects being treated with hydroxyurea, a medication prescribed for treatment of sickle cell disease that increases Hb F levels, were excluded from this study, as were samples diagnosed as sickle-beta-thalassemia (Fig. 1) or samples with asymmetrical Hb A2 peak (Fig. 2). Blood specimens were received in the laboratory by postal service, courier, or walk-in. Red cell hemolysates were run by alkaline electrophoresis (pH 8.6) (Helena Laboratories, Inc., Beaumont, TX) and by isoelectric focusing electrophoresis (IEF) (Wallace Laboratories, Inc., Akron, OH) before HPLC analysis. The Bio-Rad Variant β-thalassemia Short Program was used for Hb quantitations. The Variant (Bio-Rad Laboratories, Inc., Hercules, CA) is a fully automated HPLC system that separates a hemoglobin mixture into its individual components by pumping liquids through a column at high pressure. It is a fast and reproducible method that can be used to separate and determine area percentages for various hemoglobins, including Hb F and Hb A2. It can also provide qualitative determinations of abnormal hemoglobins. The β-thalassemia Short Program utilizes the principle of cation exchange HPLC. The resin of the column has a negatively charged surface group that will bind the positively charged protein (Hb), while the more negatively charged molecules will elute readily from the column. A higher cation concentration will be required to elute the positively charged hemoglobin from the column. The ionic strength of the elution buffer mixture is increased by raising the percent contribution of elution buffer #2. As the ionic strength of the mixture increases, more strongly retained hemoglobins elute from the column [8].

Results

For this study, 146 Hb AS samples, 33 Hb SS samples, and 28 Hb SC samples were analyzed by HPLC. Results showed that in Hb AS samples, the mean for Hb A2 is 4.09% (SD ± 0.42, range 2.20 to 5.2%). In Hb SS samples, the mean for Hb A2 is 3.90% (SD ± 1.08, range 0.60 to 5.90%). In Hb SC samples, the mean for Hb A2 is 4.46% (SD ± 0.70, range 2.30 to 5.91%). The corresponding hemoglobin quantitations for Hb F, Hb A, Hb S, and Hb C are shown in Tables 1, 2, and 3 for the sample populations mentioned.

Fig 1. Bio-Rad HPLC chromatogram showing a typical Hb A2 peak (symmetrical peak). This patient was diagnosed as sickle-beta-thalassemia.

Fig 2. Bio-Rad HPLC chromatogram showing an atypical Hb A2 peak (asymmetrical peak, having a shoulder). This patient was diagnosed as sickle cell trait.
HPLC analysis of Hb A2 in blood that contains Hb S

above. The reference range for Hb A2 in normal adults is 2.1 to 3.0%, with a mean of 2.57% [8] using the Bio-Rad Variant system.

Discussion

The sensitivity of HPLC has significantly contributed to the accurate quantitation of hemoglobin variants. In this report we have established reference ranges for Hb A2 quantified by HPLC (Bio-Rad Variant β-thalassemia Short Program) in samples containing Hb S. We found that blood samples containing Hb S usually have increased Hb A2 levels when compared to previously published HPLC reference ranges [8,9]. The HPLC manufacturer states that Hb S byproducts could coelute with Hb A2 [8]. We have not observed increased Hb A2 values by HPLC in normal Hb AA samples.

Table 1: Hemoglobin percentages in Hb AS samples (sickle cell trait), based on HPLC analysis.

<table>
<thead>
<tr>
<th>Hb</th>
<th>N</th>
<th>Mean ± SD (%)</th>
<th>Range (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>146</td>
<td>53.80±2.93</td>
<td>(47.70 - 63.60)</td>
</tr>
<tr>
<td>S</td>
<td>146</td>
<td>34.66±3.71</td>
<td>(21.20 - 42.10)</td>
</tr>
<tr>
<td>A2</td>
<td>146</td>
<td>4.09±0.42</td>
<td>(2.20 - 5.20)</td>
</tr>
<tr>
<td>F</td>
<td>98</td>
<td>0.98±0.67</td>
<td>(0.20 - 3.90)</td>
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</tbody>
</table>

Table 2: Hemoglobin percentages in Hb SS samples (sickle cell disease), based on HPLC analysis.

<table>
<thead>
<tr>
<th>Hb</th>
<th>N</th>
<th>Mean ± SD (%)</th>
<th>Range (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S</td>
<td>33</td>
<td>86.74±3.62</td>
<td>(80.40 - 94.70)</td>
</tr>
<tr>
<td>A2</td>
<td>33</td>
<td>3.90±1.08</td>
<td>(0.60 - 5.90)</td>
</tr>
<tr>
<td>F</td>
<td>33</td>
<td>6.80±2.95</td>
<td>(1.30 - 11.40)</td>
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Table 3: Hemoglobin percentages in Hb SC samples (hemoglobin SC disease), based on HPLC analysis.

<table>
<thead>
<tr>
<th>Hb</th>
<th>N</th>
<th>Mean ± SD (%)</th>
<th>Range (%)</th>
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</thead>
<tbody>
<tr>
<td>S</td>
<td>28</td>
<td>46.46±1.27</td>
<td>(43.70 - 49.20)</td>
</tr>
<tr>
<td>C</td>
<td>28</td>
<td>44.25±1.74</td>
<td>(40.40 - 46.90)</td>
</tr>
<tr>
<td>A2</td>
<td>27</td>
<td>4.46±0.70</td>
<td>(2.30 - 5.91)</td>
</tr>
<tr>
<td>F</td>
<td>27</td>
<td>2.17±1.51</td>
<td>(0.50 - 6.10)</td>
</tr>
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</table>

It has been postulated that, in the presence of β-chain hemoglobin variants, the association of the α-globin chain with the β-globin chain variant is impaired, which fosters the association of α-chains with δ-chains, increasing the Hb A2 levels [10,11]. Other factors that play a role in this increase are the accumulation of Hb S adducts and sample aging. The Hb S adducts include glycohemoglobin [11] and other post-translational modification products of Hb S. The effect of sample aging is observed in the accumulation of glycohemoglobin in blood samples over time [7]. If Hb S-containing blood samples are stored for 2 weeks, Hb A2 levels are increased, possibly due to accumulation of glycohemoglobin [7,12]. We did not determine glycohemoglobin in our samples. Since the blood samples used for this study were relatively fresh (1 week) and we still observed a marked increase in Hb A2, we postulate that other factors may be involved in the increase of Hb A2 in Hb S-containing samples when determined by HPLC.

Concurrently elevated levels of Hb A2 and Hb F offer a practical way to diagnose carriers of the β-thalassemia gene or other Hb disorders that lead to an increase in this hemoglobin. The HPLC manufacturer indicates that a range for Hb A2 of 4 to 9% is typical of heterozygous β-thalassemia [8]. Our results show that in samples containing Hb S, the Hb A2 values are elevated above the normal reference ranges given by the manufacturer [8] and also above those that we previously reported in a healthy African American population (Hb A2 range = 0.05 to 3.4%, mean 1.2%) [9]. With the system used in the present study, Hb A2 levels in samples containing Hb S partially overlap with those expected for β-thalassemia carriers. When Hb A2 quantitations in Hb S-containing samples are reported, it should be recognized that the apparently elevated Hb A2 values may well be within the normal range for those samples. The elevated Hb A2 values should be correlated with the clinical picture and family studies to help establish the diagnosis.

Conclusions

The HPLC Hb A2 levels in subjects older than 1 year and younger than 60 years of age having sickle cell trait (Hb AS) or sickle cell disease (Hb SS and Hb SC) have been assessed. These results confirm the elevation
of Hb A2 levels in individuals with Hb AS, Hb SS, and Hb SC, as determined by the BioRad Variant HPLC System. We conclude that in Hb AS, levels of Hb A2 lower than 5.2% are normal. In Hb SS and Hb SC samples, levels of Hb A2 lower than 5.9% may also be normal if there is no other sign of β-thalassemia. If further increased Hb A2 levels are found in a sickle cell disease patient, one should also consider family history, clinical symptoms, drug treatment, and sample aging before the diagnosis of an associated β-thalassemia disorder is made.

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References