Rapid Quantitation of Hemoglobin S in Sickle Cell Patients Using the Variant II Turbo Analyzer

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Abstract. A rapid (~90 sec), fully automated method is described for quantifying hemoglobin S (HbS) by high performance liquid chromatography (HPLC) using the Bio-Rad Variant II Turbo analyzer. Although this instrument is designed to quantify only blood hemoglobin A1c (HbA1c), we show that it can also quantify accurately, without modification, HbS levels in sickle cell patients, provided the blood samples meet certain conditions. The samples should contain detectable hemoglobin F (HbF), but should not contain hemoglobin C (HbC). Under these conditions, blood HbS levels obtained by this method correlate well with those obtained by agarose electrophoresis ($r^2 = 0.97$, n = 81 patients). We also show that quantitation of blood HbF in sickle cell patients is more accurate by this method than by agarose electrophoresis when the HbF level is in the range from 0.2 to 10%.

Keywords: hemoglobinopathies, sickle cell disease, fetal hemoglobin, glycated hemoglobin, hemoglobin quantification, electrophoresis, HPLC, HbA1c

Introduction

One of the mainstays of treatment for patients with sickle cell disease is the administration of therapeutic blood transfusions. In addition to correcting the underlying anemia, this intervention is effective in reducing the complications of the disease by lowering the fraction of HbS in the circulation [1-7]. This treatment can prevent sickle cell related complications (eg, stroke, recurrent acute chest syndrome) and reduce the risk of peri- and post-operative complications of surgical procedures. Many studies recommend decreasing the HbS level in these patients to <60% or even to <30% [2-5]. In some studies, patients undergoing surgical procedures who were pre-transfused to a goal of HbS <60% suffered fewer peri- and post-operative complications than did controls who had no transfusion, whereas the more aggressive goal of HbS <30% did not provide additional benefit [5]. These studies point to the need for accurate and rapid methods to quantify pre- and post-transfusion HbS levels for improving the outcome in patients with sickle cell disease [5,8,9]. Three general techniques are used to quantify HbS. The most common is agarose gel electrophoresis [10]. This is a time-consuming technique (~2 hr), which makes it difficult and expensive to perform on an emergency basis. The second method is HPLC based on ion exchange chromatography [11,12]. This method is faster (5 to 10 min) than agarose electrophoresis, but the instrumentation is more expensive [7]. The most recent method is capillary electrophoresis, which is rapid (5-15 min) and more accurate than agarose electrophoresis [13]. However, the instrument is not popular because it is very expensive and relatively new.

This study describes how HbS can be reliably quantified using a Bio-Rad HPLC instrument that is designed mainly for the determination of blood
HbA1c. The instrument is fully automated, rapid (~90 sec), suited for emergency use, and commonly available in clinical laboratories. According to the recent surveys of the College of the American of Pathologists, more than twice as many laboratories use ion exchange chromatography for quantification of HbA1c than those that use electrophoresis for quantification of hemoglobin variants (~700 vs 300). Since the Bio-Rad instrument was not designed or approved for quantitation of HbS, one objective of this work was to explore the factors and pitfalls that affect HbS assays using this analyzer.

Materials and Methods

HPLC instrument. The Variant™ II Turbo (Bio-Rad Laboratories, Hercules, CA) instrument is an automated HPLC that is designed and approved only to separate and quantify HbA1c based on cation exchange chromatography without sample pretreatment. Whole blood is aspirated into the instrument. The red blood cells are hemolyzed and injected onto a short ion exchange column to separate hemoglobin A into fractions with different retention times, such as: HbA1c (glycated), C Hb (carbamyl Hb), L A1C (labile Hb), and P3 (acetylated Hb), based on charge differences. However, other hemoglobin variants such as HbF and HbS also separate based on their respective charges. The different hemoglobins and the various Hb fractions are eluted by a special buffer gradient of increasing ionic strength. Identification of hemoglobin variants by the instrument software depends on their specific retention times (capacity factor), as monitored by a spectrophotometric detector. The buffer gradient can be changed to a different one in order to elute better the different hemoglobin variants, but changing the buffer is inconvenient in routine analysis.

Patients and study protocol. This study was performed in our routine clinical chemistry laboratory on blood samples that were obtained from sickle cell patients for quantitation of HbS by agarose electrophoresis as part of their management. The left-over samples were used for analysis by HPLC for comparison. This study was done under IRB approval of exempt status using the left-over portion of blood samples.

Instrument calibration. The Variant II Turbo instrument was calibrated on a daily basis for HbA1c using the manufacturer’s calibrators and instructions. Two HbA1c controls (low and high) were used to check the analytical precision.

Linearity and recovery studies. Linearity studies were performed by mixing blood samples with high and low levels of HbS and HbA (five point linearity check), taking into account the integration counts, too. The aim of the recovery study was to explain the observed bias in HbF quantitation by HPLC vs agarose electrophoresis. Hemoglobin F in a left-over blood sample from a 4-day-old normal HbA infant was quantified by agarose electrophoresis and then diluted with 2 different samples that did or did not contain HbS, as described in Table 1, to bring the HbF levels to 4.2%.

 Carryover. The carryover of HbS in the HPLC method was measured by assay of samples free of HbS immediately after samples with high HbS levels (>75%). The percentage of HbS carryover was measured as the (false) increase of HbS in the samples free from HbS.

Agarose electrophoresis. The semi-automated alkaline agarose gel electrophoresis system of Sebia, Inc. (Norcross, GA) is the method routinely used in our laboratory to quantitate HbS and screen for other hemoglobin variants. Samples were prepared and hemoglobin was separated and quantified according to the manufacturer’s procedure. This method requires about 2 hr for analysis. The precision (CV) for HbS assay is 2.9% (mean = 17%, n = 10).

Results

Hemoglobin variant detection by HPLC is seldom performed in routine clinical laboratories because of the small number of samples and the relatively high cost of the instrument. On the other hand, use of the Bio-Rad HPLC instrument with a fast elution gradient and different software for analysis of HbA1c is much more common. We noticed that if two major problems are addressed, the instrument can give reliable quantification for hemoglobins S, F and A: First, the blood sample should not contain HbC; and second, HbF should be detectable directly by the instrument. Under these conditions, the correlation of HbS levels in 81 patients by the HPLC assay and by agarose electrophoresis is very good (correlation coefficient r² = 0.97; Fig 1).

Fig. 1. Correlation of HbS assays by HPLC and electrophoresis (n = 81 patients).
Fig. 2. (Top panel): HPLC of a sample with HbF level of >10% (arrow). (Middle panel): HPLC of a sample with HbF level of 0.7% (arrow). (Bottom panel): HPLC of a sample with HbF level of 0% (arrows: HbF retention time = 0.27 min). The shaded peak in each panel is HbA1c.

Fig. 3. Correlation of HbF assays by HPLC and electrophoresis for 43 patients with HbS.

Fig. 4. Electrophoresis of a blood sample containing HbS (43%) and HbC (42.4%) illustrating an apparent increase in the area of HbF (9.7%). The dotted line indicates the projected corrected area.

Fig. 5. Correlation of %HbA levels calculated from HPLC data and those obtained by electrophoresis (n = 81 patients).
HbS values outside the range from -5 to 95% exhibit slight differences that are of academic concern rather than clinical concern and can be reported as such. Both instruments have their own limitations for quantification of values at the extremes, with agarose electrophoresis being more affected than HPLC. Quantification by electrophoresis has many limitations [14] especially for small bands such as HbA$_2$ [15] and HbF. At very low levels these bands become undetectable or sometimes change their migration [16]. Denatured hemoglobin migrates in front but very close to the different hemoglobins, rendering peak integration difficult. The HPLC instrument, as measured in this study, suffers from a carry-over of 4.7%.

Although the major emphasis in transfusion of sickle cell disease patients is on quantitation of HbS itself [1-5,17], it is also helpful to quantify HbA and HbF in these patients. For example, quantitation of hemoglobin F is very important in sickle cell patients treated with hydroxyurea. When HbF is ≤10% by HPLC (or ≤15% by electrophoresis), it elutes as a sharp single HPLC peak over a 5 sec period, (Fig 2, middle panel), which is identifiable as HbF by the instrument. On the other hand, when HbF is ≥10% it elutes over a 10 sec period as a wide peak that overlaps, co-migrates, and interferes with the labile fraction and HbA1c peaks. Under this condition (Fig. 2, top panel) the instrument erroneously identifies HbF as the labile fraction. Thus, whenever HbF is not identified, the sample should be analyzed by electrophoresis or the chromatogram examined for the absence of HbF (based on the retention time of HbF (Fig. 2, bottom panel) or the presence of elevated (>10%) labile hemoglobin.

A large labile hemoglobin peak (>10%) on samples collected recently is an indication of elevated HbF (Fig. 2, top panel). Such samples can be confirmed and re-analyzed on the HPLC instrument after mixing with an adult sample (low HbF in absence of HbS). The majority of adult sickle cell patients have HbF levels of 0.2-10% [18] which the HPLC instrument can identify and quantify directly and accurately (Fig. 3). The correlation coefficient for HbF analyses by HPLC vs agarose electrophoresis in sickle cell patients with HbF of <10% is good ($r^2 = 0.89$). However, the regression analysis for patients with HbS yields a slope of 0.72. In other words, the HbF values by HPLC average about 30% less than those by agarose electrophoresis for patients with HbS.

Fig. 4 shows that the area under the F peak by electrophoresis is falsely affected by the HbS area. Almost one-third of the area under the F peak belongs to that of HbS. The % HbF for the sample in Fig. 4 is 9.7% by electrophoresis, but only 5.9% by HPLC. The artefactitious increase in HbF by electrophoresis causes an equivalent false decrease in the area of HbS; however, because the HbS level is usually high this effect is not as significant as that for HbF.

To confirm this observation further, a known amount of HbF (4.2%) was added to two samples very low in HbF; one contained HbS and the other did not (only HbA), Table 1. The sample that contained HbS showed a falsely elevated recovery of HbF (141%) in the electrophoretic method while it yielded a slightly lower recovery (89%) in the HPLC method. The recovery was almost the same by the two methods for the sample that contained only HbA, indicating that the artefactitious increase in HbF by electrophoresis is not observed in patients with HbA. These problems are primarily due to the software of the agarose electrophoresis method, which does not take into account the changes in peak shape in the presence of HbS. Furthermore, agarose electrophoresis does not detect HbF levels <2%. HbF <10% is correctly identified by the HPLC method and quantified more accurately than by electrophoresis. In other words, as long as HbF is identified by the HPLC instrument, the quantitation of this hemoglobin is accurate.

When HbS and HbF are both identified in a blood sample, % HbA can be calculated by

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<th>Electrophoresis II</th>
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subtraction of these two fractions from 97.5% (with 2.5 % accounting for the average level of HbA2). In this case, the indirect quantitation of HbA by HPLC correlates well with the results obtained by agarose electrophoresis (Fig. 5).

The decrease of HbS in sickle cell patients before and after transfusion is similar by both the HPLC and electrophoretic methods (Fig. 6), with minor differences in some patients probably attributable to the difference in integration of HbF (and consequently in HbS) by electrophoresis, as noted previously.

The HPLC instrument was calibrated daily for HbA1c quantification. Two controls (low and high) were run to monitor analytical precision for HbA1c, (CV = 2.2% , mean level = 9.72%, n = 34; and CV = 2.3%, mean level = 5.75%, n = 34). The respective CVs of HbS and HbF assays by HPLC (n = 16) were 0.9% (mean level = 43%), and 4.3% (mean level = 1.35%). The CV for HbS assay was 2.9% by agarose electrophoresis (mean level = 17%). The reproducibility (CV) of retention times by HPLC for HbF and HbS (n = 16) were 0.57% and 0.47%, respectively. Based on mixing blood samples (five-point linearity) with different HbS concentrations, HbS analysis by HPLC was linear (r² = 0.989) between 5 and 95%. However, levels of HbA below 7% are reported as <7%.

Sample carryover for the HPLC instrument averaged 4.7%. This carry-over is not important in HbA1c analysis due to the narrow range of observed values, but it is more important in quantitation of hemoglobin variants because of their wider ranges of values. For blood samples with <6% HbS (or other Hb variant), the HPLC analysis should be repeated after assay of a normal sample.

**Discussion**

HbS can be quantified by the Variant II Turbo instrument provided the blood sample meets specific conditions. Here we show that this HPLC instrument with the fast gradient for HbA1c analysis can accurately separate, identify, and quantify HbS if certain precautions and quality assurance practices are observed. There are 3 major problems in quantitation of HbS by the HPLC technique: First, hemoglobin C co-migrates with HbS. Second, HbF sometimes is not detected because it is too low (<0.2%) or too high (>10%). When the HbF level is high, it co-migrates with the labile fraction and is identified by the instrument as labile fraction. Third, HbA is detected indirectly because the instrument is designed to separate HbA into several fractions in order to quantify HbA1c.

HbC is detected and identified by the HPLC instrument as HbC when present by itself or in combination with HbA. Blood samples with both HbS and HbC are identified by the HPLC software as HbS. Therefore, it is important not to use this instrument for quantification of unknown patients but to restrict it to those who are definitely known to have HbS. Many patients with sickle cell disease require repeated or chronic transfusions, such as in the management of stroke or risk for stroke. It is for these patients who have been previously identified as having HbS and who are regularly transfused that the described procedure may be of greatest benefit. For patients with HbS and HbC, one alternative is to report the data as a sum of Hb (S+C) especially since the change in %HbA (pre- and post-transfusion) is most meaningful.

When HbF is identified by the HPLC instrument, the quantification of this hemoglobin is more accurate by HPLC than by agarose electrophoresis. Usually this identification occurs when the HbF level is 0.2 to 10%. Outside this range, the HPLC software does not recognize HbF and the sample
HPLC quantitation of HbS

either has to be analyzed by another technique or the chromatogram examined carefully. HbA level is quantified indirectly by subtraction (97.5 - (% HbS + HbF)), as indicated above.

Previously, Higgins et al [19,20] and Thomas et al [21] showed that HPLC techniques for HbA1c, with different gradients, can be used for rapid screening for Hb variants. Here we demonstrate that the BioRad Variant II Turbo HPLC method for HbA1c, without modification, can be used to detect HbS and quantify it accurately in ~90 sec, fully automated, and without any sample treatment. However, the quantitation presents a different type of challenge compared to the screening. Unless they are carefully addressed, several problems can lead to unreliable results as we pointed out.

In addition to speed and automation, the HPLC technique offers several other advantages in quantifying HbS. Agarose electrophoresis is designed for analysis of multiple samples simultaneously (batching). Running single samples by agarose electrophoresis is expensive for labor and materials. On the other hand, these expenses in HPLC are not affected by running single samples. For the consumables alone, electrophoresis in HPLC are not affected by running single samples. For the consumables alone, electrophoresis is ~4 times more expensive than HPLC. Finally, the HPLC method entails less sample manipulation, which reduces the chance for errors.

References