
HPLC is a sensitive and rapid method for separating and quantifying hemoglobin (Hb) variants [1–11]. Accurate quantification of Hb S is an essential component in the discrimination of uncomplicated Hb S trait from other conditions characterized by the presence of Hb S. This is particularly important at our institution where the initial screening of new Air Force recruits for potentially disqualifying hematologic conditions is essential before the inception of basic training activities. The Bio-Rad Variant (Bio-Rad Labs.) is an automated cation-exchange HPLC method technically well suited for the rapid and accurate quantification of Hb A2 and Hb F [8]. Chromatography of each sample is completed in 6 min. The system also resolves and allows presumptive identification of common Hb variants (Hb S and C) [9]. Confirmation of the Hb variants detected by the system must be performed by using an alternative method.

We evaluated the analytical performance of the Bio-Rad Variant HPLC System in the resolution and quantification of Hb S. Reference ranges for Hb S and Hb A2 were established for individuals with uncomplicated Hb S trait. Our reference laboratory (Brooks Epidemiology Lab) receives 250 to 300 requests for Hb electrophoresis per month. Referring facilities submit an EDTA-anticoagulated whole-blood specimen and are asked to provide complete blood count (CBC) results, a stained peripheral smear, recent transfusion history, pertinent patient history, ethnic background, age, and serum ferritin results, if available. A Hb electrophoresis panel consisting of a screen for sickle Hb, quantification of Hb fractions (normally Hb A, Hb A2, and Hb F), alkaline agarose gel electrophoresis, and acid agarose gel electrophoresis (as required for confirmation of the identity of abnormal Hbs) is performed. Specimens from 319 patients identified as containing Hb S were used to evaluate the analytical performance of the Bio-Rad Variant and establish reference ranges. Clinical specimens were used in accordance with the policies of the Clinical Investigation Division of Wilford Hall Medical Center, Lackland Air Force Base, TX. Of these cases, 174 were diagnosed as uncomplicated S trait. Criteria used for this diagnosis included normal Hb concentration (females ≥120 g/L, males ≥140 g/L), mean cell volume (MCV) ≥84 fL, and Hb A/S phenotype (Hb S <50% and >35%). Twenty-two cases were diagnosed as presumptive Hb S–α thalassemia traits (Hb S <35%, MCV <80%, and normal serum ferritin) and 13 specimens from nine patients with apparent homozygous Hb S were evaluated. Five patients were identified with probable Hb S–β+ thalassemia and one neonate was identified with probable Hb S–β– thalassemia. The remaining cases consisted of Hb S trait with an abnormal Hb concentration or MCV, Hb S trait with iron-deficiency anemia, infants with Hb S trait, or cases for which CBC results were not available.

Sickle screening was accomplished with a commercially prepared solubility test kit, Columbia Calibre Sickle Cell Reagent Set (Columbia Diagnostics).

Hb S in one neonatal sample was confirmed with a monoclonal antibody assay, HemoCard (Isolab). Alkaline Hb electrophoresis on agarose gel was performed on EDTA-anticoagulated whole-blood samples with the Paragon Hemoglobin Electrophoresis Kit (Beckman Instruments) in the Paragon Accessory System (Beckman). Acid Hb electrophoresis on agarose gel was performed to confirm the identity of the Hbs resolved by alkaline electrophoresis with the Paragon Acid Hemoglobin (Acid Hb) Kit (Beckman). The percentage of each Hb resolved by alkaline electrophoresis was measured by densitometry with the Appraise Densitometer (Beckman).

Quantification of Hb A2 and Hb F and screening for the presence of Hb variants were accomplished by cation-exchange HPLC with the β-thalassemia Short Program in the VARIANT (Bio-Rad Labs.). Hb A and Hb S concentrations were corrected for small unknown peaks (glycosylated Hbs and degradation peaks) with the following formula: Hb S \(\text{corrected} = [100 - (\text{Hb A2 + Hb F})]\(\text{Hb S}/(\text{Hb A + Hb S})\), where 100 – (Hb A2 + Hb F) is the Hb percentage remaining after quantification of Hbs A2 and F by HPLC, and Hb S/(Hb A + Hb S) is the proportion of the remaining Hb that is Hb S relative to the total of Hb A and Hb S measured by HPLC or alkaline electrophoresis. Hb A2 \(\text{corrected} may also be calculated by multiplying the relative proportion of Hb A2 (Hb A2/Hb A + Hb S) times the remaining percentage of Hb \(100 - (\text{Hb A2 + Hb F})\), or, more simply, Hb A2 \(\text{corrected} = 100 - (\text{Hb A2 + Hb F}) - \text{Hb S}\) corrected.

The accuracy of the HPLC method for quantification of Hb S was evaluated by split-sample comparison with...
alkaline agarose gel electrophoresis. Regression statistics were determined by linear regression analysis.

Linearity was evaluated by using dilutions of Hb S prepared with samples from patients with homozygous Hb S disease (Hb S 92% by densitometry) and a patient with normal adult Hb (no detectable Hb S).

Samples with various concentrations of Hb S were prepared by serial dilution of a blood sample from a patient with Hb S trait with a blood sample from a patient with normal adult Hb. Four replicates of each sample were assayed each day for 5 days [12]. Functional sensitivity was defined as the lowest concentration with a CV of 20% as determined by plotting the precision profile (CV vs concentration).

Reference ranges for Hb S and Hb A2 in uncomplicated Hb S trait were established on the basis of calculation of the 95% nonparametric limits [13].

Comparison of the Variant HPLC method with the established electrophoretic method for 309 samples is shown in Fig. 1. Linear regression analysis comparing uncorrected HPLC Hb S percentages with densitometry yielded the following slope and intercept: 0.958 (95% confidence interval 0.935–0.982) and −0.8% (−1.8–0.1%). The slope and intercept with corrected HPLC Hb S percentages were 0.971 (0.948–0.994) and 0.7% (−0.2–1.6%). The correlation between the two methods was excellent: \( r_{\text{HPLC, uncorrected}} = 0.977 \) and \( r_{\text{HPLC, corrected}} = 0.978 \).

The HPLC method for quantification of Hb S was linear from 0% to 92% Hb S. (No patient specimen containing 100% Hb S was available for analysis.)

Assay imprecision (CV) was <2% at Hb S concentrations ranging from 15% to 66% and was 5% at an Hb S concentration of 10.5%. The functional sensitivity limit for quantitative measurement of Hb S, as estimated from the plot of CV vs Hb S concentration, was <5% Hb S. We found that Hb S is readily detectable by the HPLC method at amounts <5% (as confirmed by an immunologic test for Hb S) and that HPLC was more sensitive than electrophoresis at detecting low amounts of Hb S.

The 95% nonparametric reference ranges for Hb S_{HPLC, corrected} (34.8–42.4%) and Hb A2 (3.3–4.5%) in uncomplicated Hb S trait were established after analysis of the 174 patient samples.

We evaluated the analytical performance of the Variant HPLC in the detection and quantification of Hb S and established reference ranges for Hb S and Hb A2 for patients with uncomplicated Hb S trait. The Variant HPLC method accurately quantified Hb S and correlated well with the standard electrophoretic method \( (r = 0.978) \). On the basis of the linearity study and the estimation of functional sensitivity, the reportable range for quantification of Hb S was determined to be 5.5–92%. The detectable amount of Hb S was <5%, allowing for the reliable detection of Hb S disorders in neonates. The excellent correlation obtained for the HPLC quantification and the densitometric quantification of Hb S is similar to the results obtained by Papadea and Cate for this analyte \( (r > 0.93) \) [9]; our measurement of assay imprecision was quite low \( (CV < 2%) \) and was of similar magnitude to their reported imprecision \( (CV < 3%) \). Increased Hb A2 concentrations were measured by HPLC in patients with uncomplicated Hb S trait \( (95\% \text{nonparametric reference range: } 3.3–4.5\%) \) relative to ranges reported by other conventional methods \( (2.5–3.5%) \) [14], and for Hb A2 as measured by other investigators by HPLC in the absence of Hb S trait \( (mean = 2.4\%, range 1.4–2.9%) \) [8]. The higher Hb A2 range in sickle trait is postulated to be the result of coeluting glycated Hb S or Hb S degradation products [11]. Although the number of cases evaluated was small (five total), Hb A2 concentrations observed in patients with Hb S–β⁺ thalassemia \( (6.5–6.8%) \) did not overlap with this higher reference range for Hb A2 established by HPLC in uncomplicated Hb S trait.

Our results validate the use of the Variant for the quantification of Hb S. An alternative method, however, must be used to confirm the identification of Hb S in patients without previously documented Hb S.

References


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**Fig. 1.** Comparison of the BioRad Variant with electrophoresis.

Hb S concentration quantified by HPLC was corrected for small unknown peaks and compared with Hb S concentration measured by electrophoresis. The regression equation is \( y = 0.971x + 0.7, r = 0.978 \).