

The Detection and Diagnosis of Hemoglobin A₂' by High-Performance Liquid Chromatography

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Abstract

Hemoglobin (Hb) A₂' is a hematologically silent variant of HbA₂ that is detected easily by high-performance liquid chromatography (HPLC), where it elutes in the S window. Our purposes were to define diagnostic criteria for the HbA₂' trait using the Variant II (Bio-Rad, Hercules, CA) and to determine the prevalence of HbA₂' in a metropolitan patient population. All Hb screens (N = 5,862) performed during a 26-month period were reviewed for new hemoglobinopathies. We identified 57 cases of HbA₂' trait, making it the fourth most prevalent Hb variant detected in this population after HbS, HbC, and β -thalassemia minor. For HbA₂' trait cases, the mean HbA₂ level was 1.7% (SD, 0.17%), and the mean HbA₂' level was 1.3% (SD, 0.18%). Six possible HbA₂'/ β -thalassemia double heterozygotes were identified, for whom the sum of the HbA₂ and HbA₂' exceeded 4% of total Hb. Hb variants that might interfere with detection of HbA₂' include HbS, glycosylated HbC, and HbG₂. Diagnostic criteria proposed for the HbA₂' trait by HPLC are HbA₂ of 2% or less, S window peak of 1% to 2%, no previous diagnosis of HbS, and absence of HbG and HbC.

Hemoglobin (Hb) A₂' (also called HbB2) is a hematologically silent variant of HbA₂ that results from the substitution of arginine for glycine at the 16th amino acid position of the δ -globin chain.¹⁻⁷ HbA₂' is the most common of the known HbA₂ variants and has been reported to occur in 1% to 2% of African Americans.⁴ HbA₂' has been detected in heterozygous and homozygous states and in combination with other Hb variants and thalassemia.^{5,8-10} The major clinical significance of HbA₂' is that failure to detect it might lead to underestimation of the total HbA₂ and failure to recognize β -thalassemia minor. For the diagnosis or exclusion of β -thalassemia minor, the sum of the HbA₂ and the HbA₂' levels must be considered.⁴

The substitution of arginine for glycine confers a net positive charge gain on the δ -globin chain, which accounts for its mobility cathodal to HbA₂ on alkaline electrophoresis. Because HbA₂' accounts for such a small percentage of the total Hb in heterozygotes, it is difficult to detect by this method. HbA₂' is easier to detect by isoelectric focusing (IEF); however, this method is not widely used as a primary screening method beyond the neonatal period, when HbA₂ levels normally are low. HbA₂' coelutes with HbA₂ by microcolumn chromatography, and, thus, the total HbA₂ level measured by this method would be expected to be accurate, although the HbA₂' component would not be identified. In recent years, high-performance liquid chromatography (HPLC) has replaced alkaline electrophoresis as the primary screening method for hemoglobinopathies in many laboratories.¹¹ HPLC offers the advantages of decreased manual labor, lower cost, and direct quantification of even minor Hb components, including HbA₂. HbA₂' is perhaps detected most easily by HPLC, in which it produces a minor peak in the S window.^{4,12}

In 2001, the University Hospitals of Cleveland Core Laboratory, Cleveland, OH, switched from alkaline electrophoresis to HPLC as the primary screening method for hemoglobin identification. Shortly afterward, we observed that some samples had small, unexplained peaks in the S window. We suspected that these cases might represent HbA₂', which prompted us to review all of our HPLC tracings retrospectively and prospectively to determine the prevalence of HbA₂' in our patient population and to better define the diagnostic criteria for HbA₂'.

Materials and Methods

The study was approved by the institutional review board of University Hospitals of Cleveland. All HPLC tracings from November 1, 2001, to December 31, 2003, were reviewed, and all new hemoglobinopathy and thalassemia diagnoses were identified. Samples with a peak in the S window accounting for less than 3% of the total Hb were selected for further examination as possible cases of HbA₂', after excluding samples with known HbS, HbC, or HbG-Philadelphia.

HPLC was performed on the Variant II (Bio-Rad, Hercules, CA) using the Beta-Thal Short Program (Bio-Rad). Briefly, EDTA-anticoagulated blood samples undergo hemolysis and dilution in the Variant II and then are injected into an assay-specific analytic cartridge, to which a buffer gradient of increasing ionic strength is delivered. Hb fractions are eluted from the cartridge based on their ionic interaction with the cartridge material. The separated Hb fractions pass through a flow cell, where absorbance is measured at 415 nm. A report is produced that includes a chromatogram and a relative quantification of each hemoglobin component. To aid in the interpretation of results, "windows" (ie, time ranges) have been established for the most frequently occurring Hb types based on their characteristic retention times. The manufacturer defines the HbA₂ window as 3.3 to 3.9 minutes and the HbS window as 4.3 to 4.7 minutes. The reference range for the proportion of HbA₂ by HPLC in our laboratory is 2.0% to 3.5% of the total Hb.

All patient samples with newly detected Hb variants by HPLC were submitted for confirmatory alkaline electrophoresis at pH 9.2. Alkaline electrophoresis was not done for all cases of HbA₂' because many of these cases were identified only retrospectively. Among prospectively identified suspected cases of HbA₂', representative samples were submitted for alkaline electrophoresis, and 4 samples were sent to Mayo Medical Laboratories, Rochester, MN, for verification or exclusion of HbA₂' by IEF. CBC count results were obtained for patients with HbA₂' if they were available in the laboratory information system.

Results

A total of 5,862 HPLC Hb screens were reviewed, and 1,350 new hemoglobinopathy diagnoses were made during

this 26-month period. We identified 57 cases of the HbA₂' trait, making it the fourth most common hemoglobinopathy condition diagnosed in our patient population after HbS trait (n = 587), β-thalassemia minor (n = 183), and HbC trait (n = 163) **Figure 1**. All patients with HbA₂' for whom race data were available were African American (42/42), and most were female (54/57). Many samples with HbA₂' were from women of childbearing age, which reflects the local practice of screening all new pregnant mothers for hemoglobinopathies.

The HbA₂' trait was considered present when the HPLC results showed a minor peak in the S window, there was no known history of HbS, and other interfering Hb variants could be excluded. For heterozygotes with the HbA₂' trait, the HbA₂' levels ranged from 1.2% to 2.0% (mean, 1.7%; SD, 0.17%), and the HbA₂' levels ranged from 1.0% to 2.0% (mean, 1.3%; SD, 0.18%). In 55 of 57 cases, the proportion of HbA₂' was slightly less than the proportion of HbA₂. The retention times for HbA₂' ranged from 4.55 to 4.62 minutes (mean, 4.59 minutes; SD, 0.02 minute). These results are summarized in **Table 1**. A representative chromatogram of the HbA₂' trait is shown in **Figure 2A**. HbA₂' was confirmed in 3 representative cases by IEF. We were unable to detect HbA₂' by alkaline electrophoresis in any of our patient samples on which it was attempted. No cases of homozygous HbA₂' were identified during this period. CBC count results were available for 20 patients with the HbA₂' trait diagnosed after January 1, 2003. Of these patients, 14 had microcytosis, anemia, or both. Iron studies were not done, or results were not available for these patients.

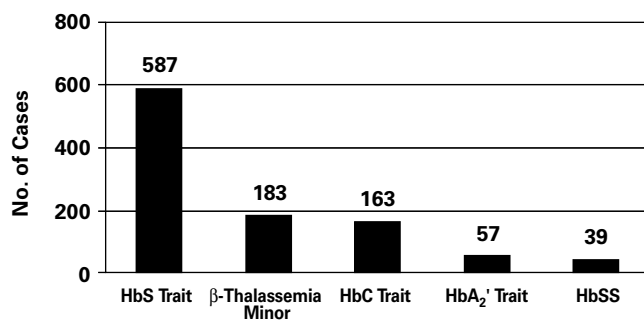


Figure 1 New hemoglobinopathy diagnoses, November 2001 to December 2003. Hb, hemoglobin.

Table 1 Clinical and High-Performance Liquid Chromatography Features of 57 Patients With the Hemoglobin (Hb) A₂' Trait

Feature	Result
Sex	54/57 female
Race	42/42 African American*
Age range	1-88 y
HbA ₂ ' (%)	1.7 (SD, 0.17)
HbA ₂ ' (%)	1.3 (SD, 0.18)

* Race data were available for only 42 patients.

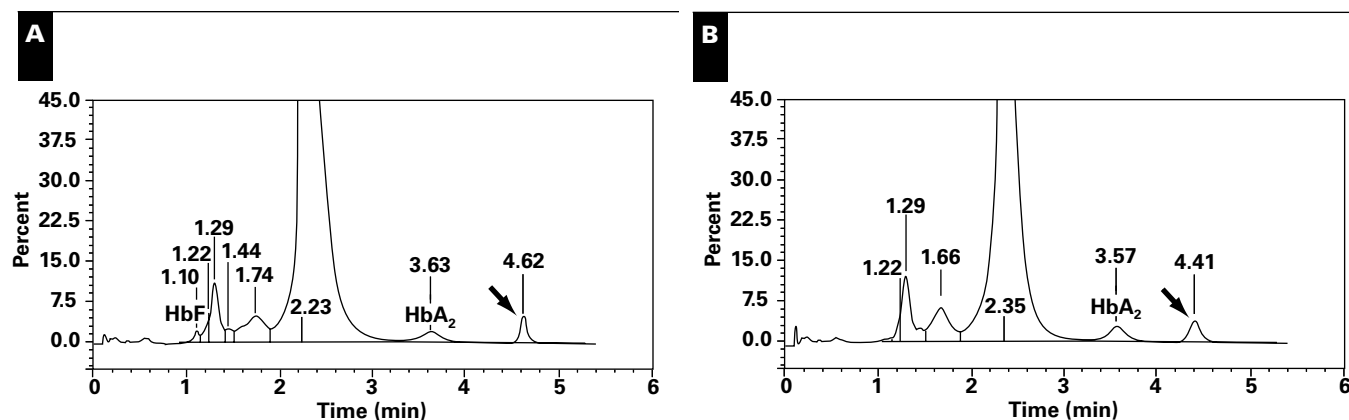


Figure 2 High-performance liquid chromatography results from 2 patients with hemoglobin (Hb) A₂' trait. **A**, HbA₂' trait. **B**, Suspected HbA₂' trait/β-thalassemia minor double heterozygote. The HbA₂' peaks are indicated (arrows).

We identified 6 cases with possible double heterozygosity for HbA₂' and β-thalassemia minor based on HPLC findings. Cases were considered suggestive of HbA₂'/β-thalassemia when the sum of HbA₂ and HbA₂' was greater than 4.0% of the total Hb. Ethnicity information was available for 4 of these cases, and all were African American. The clinical and hematologic data for these patients are shown in **Table 2**. Three of these patients had low or borderline low mean cell volume. Two patients (cases 2 and 6) had normal mean cell volumes and low RBC counts. One of these patients (case 6) was a 49-year-old man with a myelodysplastic syndrome, hepatitis C, and hemochromatosis. Patient 2 was evaluated during pregnancy, but no other clinical information was available. No CBC results were available for case 4.

Three of the patients with possible HbA₂'/β-thalassemia had HbA₂' levels that were equivalent to the levels seen in cases of simple HbA₂' trait, and the other 3 cases had HbA₂' levels exceeding 2% of total Hb. In all 6 cases, the HbA₂ levels were within the normal range. A representative chromatogram is shown in **Figure 2B**.

There were 3 cases in which small peaks appeared in the S window and accounted for 0.6% to 0.8% of the total Hb. HbA₂' trait was considered unlikely in these cases because of the low percentage of Hb in the S window, the normal HbA₂ levels, and race other than African American in 2 of the 3

cases. One of these samples was submitted for IEF, which confirmed the absence of HbA₂'. The explanation for these minor S window peaks remains unknown.

Discussion

Based on this large series of patients, the following criteria for the diagnosis of HbA₂' trait by HPLC are proposed: (1) S window peak of 1.0% to 2.0% of the total Hb, (2) HbA₂ level of 1.0% to 2.0% of the total Hb, (3) no previous diagnosis of HbS, and (4) absence of HbC and HbG. HbA₂' was the fourth most common Hb variant detected in our patient population, with an estimated prevalence of 1.1%. Our HPLC findings and prevalence estimates concur with the results of Joutovsky et al,¹² who analyzed more than 60,000 samples in an ethnically diverse patient population. However, the actual prevalence of HbA₂' in both patient populations probably is higher because double heterozygosity for HbA₂' and HbS or HbC cannot be detected by the current Variant II method. In addition, a selection bias favoring pregnant females and patients undergoing evaluation for anemia skewed our population sample, such that hematologically normal males and nonpregnant females are underrepresented. In our series, HbA₂' was not detected in any persons who were not African

Table 2 Hematologic and High-Performance Liquid Chromatography Findings in Six Patients With Suspected HbA₂'/β-Thalassemia Minor

Case No./Sex/Age(y)	Race	RBC, × 10 ⁶ /μL	Hb, g/dL	MCV, μm ³	HbA ₂ , %	HbA ₂ ', %	HbF, %
1/M/1	AA	5.01	11.3	81	3.3	1	1.6
2/F/18	AA	3.92	12.6	92	3	1.5	0.3
3/M/75	AA	4.04	10.9	79	2.8	1.2	<1.0
4/M/97	U	NA	NA	NA	2.7	2.3	1
5/M/3	AA	4.89	11.4	70	3.5	2.2	3.6
6/M/49	U	2.83	NA	96	2.9	2.1	1.5

AA, African American; Hb, hemoglobin; MCV, mean cell volume; NA, not available; U, unknown.

American, which is consistent with the published literature on ethnic distribution of HbA₂'. However, it is reasonable to expect that HbA₂' will occur in persons of mixed racial background, and, therefore, race other than African American should not exclude the diagnosis if all other diagnostic criteria are met.

HbA₂' is a hematologically silent Hb variant; however, many of our patients were anemic, microcytic, or both. Iron deficiency might explain the anemia and microcytosis in some of these patients, and if so, this might have biased the mean HbA₂ and mean HbA₂' levels obtained in our series, because iron deficiency causes decreased HbA₂ production. This effect seems unlikely, however, because the mean HbA₂' level of 1.3% in our 57 patients is similar to the mean of 1.2% obtained by Joutovsky et al¹² in 81 cases of HbA₂' trait.

The recognition of HbA₂' is important for the diagnosis and exclusion of β-thalassemia minor. In fact, 5 of 6 cases of suspected HbA₂'/β-thalassemia were missed on initial evaluation and were identified during retrospective review. We were unable to obtain fresh samples to submit for confirmatory testing in any of these cases. Although the RBC indices in some of these cases were not consistent with β-thalassemia minor, coexisting hematologic disorders (such as myelodysplastic syndrome and liver disease in case 6) might account for the observed values. When HPLC results suggest HbA₂'/β-thalassemia and RBC indices are inconsistent with this interpretation, confirmation of the

Table 3
Conditions That Might Interfere With Detection of Hemoglobin (Hb) A₂'

Condition	Reason
HbS trait or HbSS	HbA ₂ ' hidden in the S peak
Transfused HbSS	HbS peak might be very small
HbCC or HbC trait	Glycosylated HbC elutes in S window
HbG	HbG ₂ elutes in S window

diagnosis by alternative methods is recommended, as clinically indicated. We also noted that 3 patients had HbA₂' levels ranging from 1.0% to 1.5%, and 3 patients had higher HbA₂' levels, ranging from 2.1% to 2.3%. Previous family studies of persons with double heterozygosity for these 2 conditions have suggested that the proportion of HbA₂' might be determined by whether the δ-chain mutation is in the *cis*- or *trans*- configuration with the β-thalassemia mutation.^{5,7,9,10}

Several hemoglobinopathy conditions that might interfere with the detection and diagnosis of HbA₂' are listed in **Table 3**. In the HbS trait and HbSS conditions, HbA₂' will be masked by the overwhelming amount of HbS in the S window. In HbC trait and HbCC conditions, the glycosylated fraction of HbC elutes in the S window and also will mask the presence of HbA₂' **Figure 3A**. In our patient population, HbS and

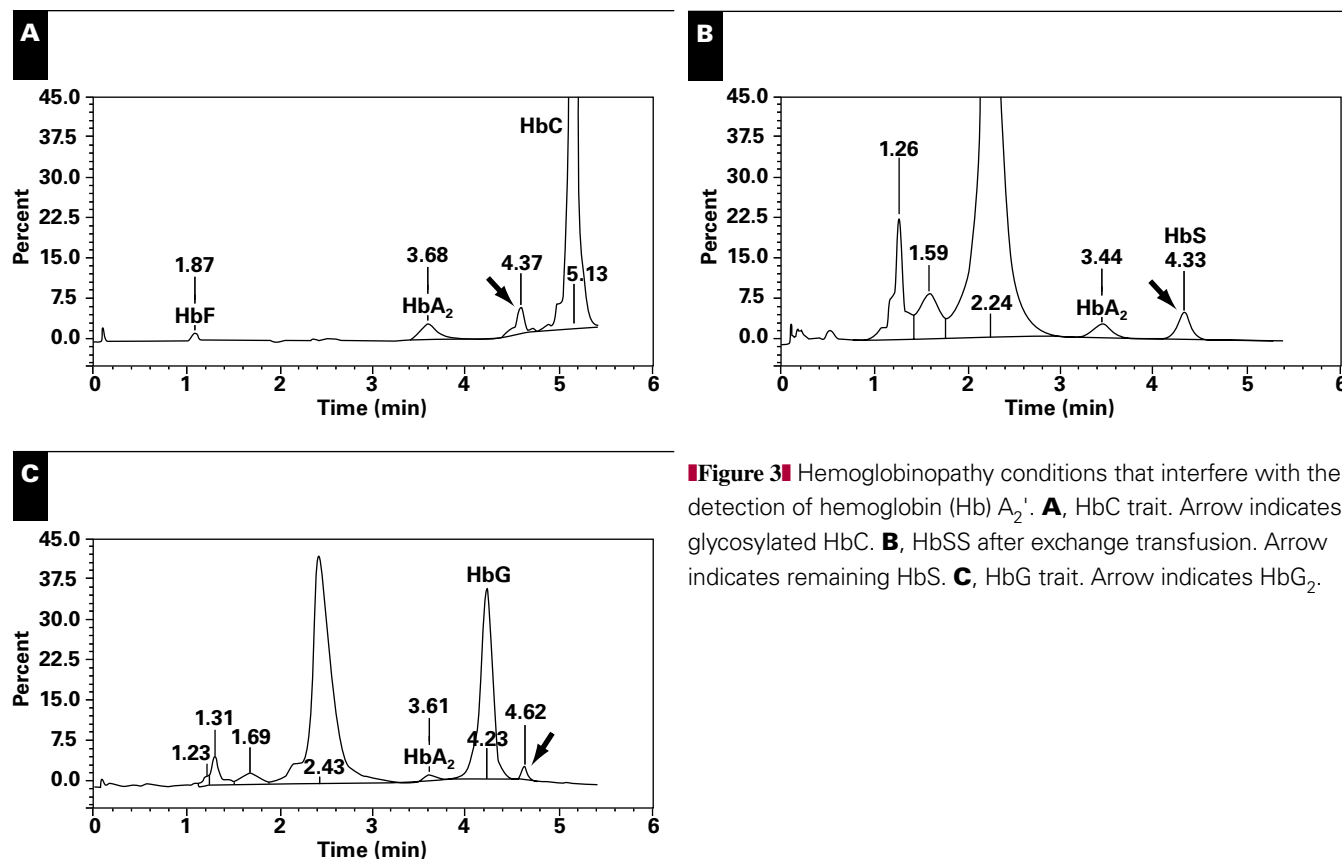


Figure 3 Hemoglobinopathy conditions that interfere with the detection of hemoglobin (Hb) A₂'. **A**, HbC trait. Arrow indicates glycosylated HbC. **B**, HbSS after exchange transfusion. Arrow indicates remaining HbS. **C**, HbG trait. Arrow indicates HbG₂.

HbC are the most common β -chain Hb variants detected, and, therefore, the prevalence of HbA₂' in our patient population probably is underestimated.

On the other hand, some hemoglobinopathy conditions produce HPLC results that could be misinterpreted as HbA₂'. Patients with HbSS who have undergone exchange transfusion might have extremely low levels of residual HbS. We have observed HbS levels as low as 2.6% in such cases **Figure 3B**. If the laboratory is unaware of the history of sickle cell anemia and exchange transfusion, the HPLC results could be misinterpreted as HbA₂'. Another possibility is that a patient who has received a transfusion from a donor with HbS trait also could have a very low level of HbS. HbG-Philadelphia, which is an α -chain variant, elutes in the D window, and the HbG₂ component, which consists of normal δ -chains combined with α G chains, also elutes in the S window and could be confused with HbA₂' **Figure 3C**. Hb Manitoba and Hb Montgomery have retention times identical to HbA₂' but account for a significantly greater proportion of the total Hb and, therefore, should not be confused with HbA₂'.¹²

HbA₂' is a δ -chain variant readily detectable by HPLC. As HPLC becomes more widely used for hemoglobinopathy screening, familiarity with the diagnostic criteria for HbA₂' will lead to more frequent recognition of this clinically harmless Hb variant.

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