Hemoglobin Variant Detection from Dried Blood Specimens by High Performance Liquid Chromatography*

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Abstract

The convenience of dried blood filter paper specimens for genetic screening programs has prompted us to test the stability of these specimens for hemoglobin identification by cation exchange high performance liquid chromatography. This report shows that identification of Hb AA, Hb AF, Hb AS, Hb FAS, Hb AJ, Hb FJ, Hb EF, and Hb SS can be achieved by high performance liquid chromatography even after six weeks of storage at room temperature. Also, accurate hemoglobin quantitation can be obtained from the same samples within three weeks of storage at room temperature.

The combination of dried blood samples and high performance liquid chromatography provides an accurate system to screen for hemoglobinopathies, even after long periods of sample storage at ambient conditions.

Introduction

Blood samples collected on filter paper are utilized for genetic newborn screening programs, including sickle cell disease. Conventional electrophoretic methodologies (cellulose acetate and citrate agar electrophoresis) are widely used to screen dried blood samples for hemoglobinopathies.2,8,10 However, if the analysis is done a few days after the collection time, poor resolution of hemoglobin (Hb) bands may occur and interfere with accurate identification.9 Limitations of the conventional techniques have prompted the use of more sensitive procedures, such as isoelectric focusing electrophoresis (IEF) and high performance liquid chromatography (HPLC).

Detection of hemoglobin types from dried blood using IEF has been discouraged because of the appearance of fuzzy bands which makes difficult the identification of common hemoglobin variants.5 On the other hand, HPLC has been successfully employed for the identification of hemoglobinopathies from liquid samples.1,4,6,7,11,12 Moreover, it has been shown that for fetal hemoglobin quanti-
tation, HPLC is more accurate and provides better separation patterns than IEF.\textsuperscript{3}

The present study addresses the need of using sensitive techniques, such as HPLC, for the correct identification and quantitation of hemoglobin variants from dried blood. Our results showed that all the hemoglobin variants tested (Hb AA, Hb AF, Hb AS, Hb FAS, Hb AJ, Hb FJ, Hb EF, and Hb SS) could be correctly identified even after six weeks and accurately quantitated after three weeks of storage time at ambient conditions.

Methods

Newborn and adult blood specimens from individuals screened at the Comprehensive Sickle Cell Center, Meharry Medical College, Nashville, Tennessee, were collected in heparinized capillary tubes or ethylenediaminetetraacetic acid (EDTA) tubes, spotted on A & S 903 filter paper discs, and stored in the dark at room temperature (RT) for up to 10 weeks. Every seven days for 10 consecutive weeks each dried blood sample was transferred into a 1.5 ml Eppendorff tube and eluted with hemolysate reagent,\textsuperscript{*} centrifuged at 12,000 rpm for 15 minutes at 4°C, and filtered through a 0.45 μm filter unit into another 1.5 ml tube. These hemolysate samples could be analyzed immediately or stored at -70°C for up to one year. Hemolysates from anticoagulated blood samples to be used as controls were prepared in a similar manner. Hemolysates (10 μl) from dried blood filter paper and liquid blood samples were transferred into H-style Nalgene vials for hemoglobin variant detection and quantitation using an HPLC program\textsuperscript{5} and a PolyCat A 300, stainless steel column packed with hydrophilic cationic polymer.\textsuperscript{†} A gradient made up of mobile phase A, containing 40 mM bis-tris and 4 mM KCN at pH 6.5, and mobile phase B, containing 40 mM bis-tris, 4 mM KCN and 0.2 M NaCl at pH 6.8, was set with a 2.0 ml per minute flow rate. The equipment used consisted of a 600 E multisolvent delivery system with a 700 WISP, 484 tunable UV/VIS detector interfaced with a Baseline 810 chromatography work station program.\textsuperscript{‡} At the end of each run, qualitative and quantitative peak integration results were automatically performed by the incorporated Water’s HPLC computer system.

Results

HPLC VALIDATION TEST

Anticoagulated blood from 157 adults with normal Hb AA patterns, was spotted on filter paper, dried, and eluted weekly for 10 consecutive weeks. The HPLC quantitative evaluations are shown in figure 1. Each point of analysis represents the results of six to 22 samples. By carefully monitoring peak chromatograms, it was possible to establish relative percentage (base 100) values. These findings demonstrated a decrease in the Hb A component from 100 percent (reference liquid sample) at 0 weeks (time zero) to 78 percent at 10 weeks, i.e., dried blood filter paper samples showed an increase of degradation products and background to 22 percent after 10 weeks.

QUALITATIVE AND QUANTITATIVE ANALYSIS

In figure 2 are shown representative chromatograms for hemoglobins AF, AS, and SS during a 10 week period. The starting Hb F component of Hb AF is five percent in the liquid control, and both can be identified after up to six weeks of storage at ambient conditions.

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storage time at room temperature. The HPLC quantitative analysis is expressed in relative area percentages. The hemoglobin variant percentage trends for all blood specimens tested (Hb AA, Hb AF, Hb AS, Hb FAS, Hb AJ, Hb FJ, Hb EF, and Hb SS) showed that under the conditions reported, each hemoglobin type exhibits particular forms of degradation (figures 3 and 4). These findings demonstrated that relative percentages losses of Hb AA after nine weeks are 22 percent or a decrease to 78 percent (figure 3, panel A), which is in agreement with percent-

**FIGURE 1.** HPLC validation test: High performance liquid chromatography percentage trends (base 100) of normal adult Hb AA samples obtained from dried blood filter paper eluates. Slope indicates Hb AA component losses are probably due to degradation processes.

**FIGURE 2.** Representative high performance liquid chromatography chromatograms of Hb AF, Hb AS and Hb SS dried blood specimens: Dried blood filter paper eluates were prepared after 0, 1, 6 and 10 weeks of specimens storage at room temperature. Hemoglobin variants were separated by cationic exchange HPLC. Quantitation of the Hemoglobin variants (relative percentage) was performed by integrating the respective peak areas.
Figure 3. Percentage trends of Hb AA, Hb AF, Hb AS and Hb FAS dried blood specimens by high performance liquid chromatography: Absolute percentages for each hemoglobin variant are given at each time point. Blood eluates were prepared from dried blood filter papers stored at room temperature for up to 10 weeks.

Age trend losses observed for Hb AA in the HPLC validation test (figure 1). For Hb AF, the relative percentage values for both Hb A and Hb F after nine weeks is 57 percent (figure 3, panel B), which if compared to the results obtained for Hb A alone (78 percent) accounts for an additional loss of 21 percent. In this particular case, the presence of Hb F may have accelerated the Hb A degradation process. The Hb AS showed comparable relative percentage values at zero weeks (A = 91 percent, S = 89 percent), and the apparent relative stability of the components account for small losses observed (A = 14 percent, S = 24 percent), equiva-

Figure 4. Percentage trends of dried blood filter paper eluates for Hb AJ, Hb FJ, Hb EF and Hb SS by high performance liquid chromatography: Absolute percentages for each hemoglobin variant are given at each time point. Blood eluates were prepared from dried blood specimens stored at room temperature for up to 10 weeks.
lent to Hb A values of 86 percent and Hb S values of 76 percent after 10 weeks of storage (figure 3, panel C). When F is present, i.e., Hb FAS, the percentage of loss for Hb A and Hb S is faster and larger, that is A = 36 percent and S = 37 percent at one week, going to a relative percentage total loss after six weeks of 47 percent for both Hb A and Hb S, while the loss for the Hb F component was 44 percent.

Quantitative determination of the abnormal Hb AJ depicts a rather stable Hb A against a more liable Hb J component at the storage conditions reported. In figure 4, panel A, degradation is shown for Hb A = 3 percent while the Hb J component reached a significant 36 percent loss after six weeks. Similar evaluations for Hb FJ, figure 4, panel B, indicated important percentage trend reductions for Hb F (34 percent) and greater losses for the Hb J component (83 percent) after six weeks of storage time. Although a rapid degradation pattern for the Hb J component is observed in both of these samples (Hb AJ and Hb FJ), it seems that the Hb F component in Hb FJ has a contributing effect in the rapid disappearance of Hb J. For Hb SS after a loss of 15 percent at week one, it diminished to 12 percent after a 10 week period, equivalent to a total loss of 27 percent (figure 4, panel C). It appears that the homozygous condition of the hemoglobin has a positive effect on the stability pattern observed. A similar situation is seen with Hb EF, figure 4, panel D. The Hb E, with an initial percentage of 91 percent, went down 21 percent after nine weeks, but significant losses for Hb F (45 percent) were observed. In this particular case, the apparent stability of Hb E is probably due to its higher starting percentage, whereas Hb F, as previously shown, is more susceptible to degradation. In figures 3 and 4, the quantitation of each hemoglobin variant is given as absolute percentage and not relative percentage, in order to detect the percentage of hemoglobin variant that degrades over elapsed time.

Discussion

VALIDATION TEST BY HPLC

This validation test corroborates reports of hemoglobin losses and the appearance of unknown factors which interfere with interpretation of results when analyzed by conventional methods. In this particular case, analysis of HPLC has recognized the losses and has identified the sample (Hb AA) tested even after a 10 week period storage time at room temperature.

QUALITATIVE AND QUANTITATIVE ANALYSIS BY HPLC

Identification of hemoglobin components from dried blood filter paper eluates is possible by the use of HPLC even after several weeks of storage at ambient conditions. This may represent a good advantage in the blood screening process for hemoglobin disorders such as sickle cell, thalassemia, and other hemoglobinopathies. The detection of small amounts of Hb AA is of particular importance to distinguish between a baby with FAS or FSA. Analysis by HPLC for eight different whole blood specimens has shown that dried blood filter paper eluates stored at room temperature for several weeks suffered various degrees of degradation of their hemoglobin components, but the sensitivity of this method has permitted the qualitative and quantitative evaluation of these same samples. However, there are no published studies that estimate the actual sensitivity and specificity of HPLC for hemoglobinopathies screening.

The aging of the dried blood or the hemolysate can cause an increase of minor peaks in the region that elutes
from two to five minutes and overall background; however, resolution of major peaks is not affected by time, up to six weeks. The stability of the dried blood spot and sensitivity of HPLC provide an advantage to the hemoglobinopathy detection laboratory that receives dried blood samples several weeks after they have been collected (i.e., samples from foreign countries). Because of the several advantages of dried blood over capillary and test tube samples for screening of hemoglobinopathies (easy mailing and handling, transportation, decreased risk of infection from glass breakage and spilling), continuous improvements of detection methodologies are necessary, as well as procedures to minimize the oxidation and degradation processes of hemoglobin that occur during storage at room temperature.

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References