A Method of HbF Determination for Potential Use in Underdeveloped Countries

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ABSTRACT

The objective of this study was to develop a simple, cost-effective method of HbF determination potentially useable in underdeveloped countries to determine sickle cell patient response to hydroxyurea treatment. Normal adult blood (HbA), cord blood (HbF), and a 50:50 mixture (HbA+F) were the three sample types used in procedure development. Normal blood samples were collected from the research team, and de-identified cord blood samples were provided by Cardinal Glennon Pediatric Research Institute, St. Louis, MO. The hematocrit of all blood samples was standardized to 35%. The method, based on the Kleihauer-Betke (K-B) test principle, used a citrate solution to selectively elute HbA from RBCs while HbF remained intracellular, and spectrophotometric absorbance of the eluate was the primary outcome measure. A procedure was developed and optimized utilizing a 395 nm wavelength, 30 sec centrifugation time, 6 min incubation time, 20 μL blood volume, and 0.07 M sodium citrate in a 0.06 M sodium phosphate buffer solution. Reproducibility was demonstrated (N = 39) with a mean HbA absorbance of 1.285 (SD 0.069), mean HbA+F absorbance of 0.690 (SD 0.050), and mean HbF absorbance of 0.035 (SD 0.005), also exhibiting linearity (r^2 = 0.99). This simple, cost-effective method of HbF determination shows potential as a basis for determining sickle cell patient response to hydroxyurea treatment in underdeveloped countries.

ABBREVIATIONS

HbS-sickle hemoglobin, HbA-normal hemoglobin, SCD-sickle cell disease, SCT-sickle cell trait, RBCs-red blood cells, HPLC-high performance liquid chromatography, HbF-fetal hemoglobin, K-B-Kleihauer-Betke.

INDEX TERMS

Developing countries, hemoglobin F, hydroxyurea, sickle cell anemia, spectrophotometry.

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INTRODUCTION

Sickle cell anemia is one of the most commonly inherited genetic diseases, annually affecting approximately 275,000 births worldwide.[1] The disease is most prevalent in African countries and countries in which a large proportion of the population is of African descent, such as the Caribbean nation of Haiti. In Western Africa, 1.7% of children are born with the disease, and in the Caribbean, the percentage is 0.4%.[1] In particular, the prevalence of sickle hemoglobin (HbS) in Haiti, representing both homozygotes and heterozygotes, is 15.2%.[2]

Sickle cell syndromes result from a single point mutation causing the substitution of valine for glutamic acid at position six of the β-globin chains, producing HbS ($\alpha_2\beta_2^s$) instead of normal hemoglobin, HbA ($\alpha_2\beta_2$).[3,4,5,6] The sickle cell β-globin gene must be inherited in the homozygous state for this mutation to result in sickle cell disease (SCD). The heterozygous state produces sickle cell trait (SCT) and few, if any, complications. Other clinical conditions involving the sickle cell gene can occur in individuals inheriting one sickle cell allele with a β-thalassemia allele or another β-chain hemoglobinopathy (compound heterozygotes).[7]

The clinical symptoms of SCD stem from a change in the intracellular polymerization of HbS when in the deoxyhemoglobin form.[8] This altered polymerization

results in sickle-shaped red blood cells (RBCs), which become trapped in the microvasculature and cause hypoxia, tissue death, and the severe pain associated with sickle cell crises. In addition, extravascular and intravascular hemolysis causes hemolytic anemia. A host of other complications are also present that, together, will lead to early death if left untreated.[7]

Mortality from SCD is high in underdeveloped countries due to inadequacies in both diagnostic testing and medical intervention. In developed countries, laboratory testing for the diagnosis of sickle cell conditions involves observing sickle cells on a blood smear, obtaining a positive hemoglobin solubility test, and determining the zygosity of the patient (HbSS or HbAS) by hemoglobin electrophoresis or high performance liquid chromatography (HPLC). The hemoglobin solubility test, hemoglobin electrophoresis, and HPLC are rarely available in underdeveloped countries such as Haiti due to cost, method complexity, and limited access to stable electricity. In the absence of laboratory testing to diagnose SCD early in life, treatment for patients in underdeveloped countries is either delayed until symptoms are recognized, or never delivered, both resulting in early death.

Recently, our laboratory developed a simple and cost-effective method (Sickle Confirm) to test for HbS and determine the zygosity of patients, thereby diagnosing SCD without the need for electrophoresis or HPLC. Field testing has shown high sensitivity (100%) and specificity (100%) in distinguishing normal subjects from sickle cell heterozygotes and in differentiating heterozygotes from homozygotes (sensitivity 100% and specificity 95%).[9] Now that diagnosis of SCD in underdeveloped countries is possible, focus must be directed toward treatment of the disease.

Established treatments for SCD involve infection prophylaxis, pain management, and blood transfusion with iron chelation.[10] A more recent approach to treatment involves the drug hydroxyurea to increase expression of fetal hemoglobin (HbF), which proportionately reduces the percentage of HbS. HbF ($\alpha_2\gamma_2$) interrupts HbS polymerization, preventing formation of sickle cells and reducing frequency of vaso-occlusive events.[11] Hydroxyurea is relatively inexpensive, administered orally, proven effective by clinical trials, and is the only HbF-inducing drug currently approved by the U.S. Food and Drug Administration for treatment of SCD.[12,13,14] However, the drug also presents challenges. Sickle cell patients exhibit a wide range of baseline HbF levels; and when treated with hydroxyurea, the magnitude of their HbF response also varies widely.[12,15] It is estimated that 10-20% of patients show no increase in HbF levels.[7] Since hydroxyurea is mildly carcinogenic, HbF levels must be monitored during treatment to determine the minimum effective dosage or to stop the drug in non-responders.[10, 16]

In developed countries, HbF levels are monitored by electrophoresis or HPLC, but these methods are impractical in underdeveloped countries. Spectrophotometric methods of HbF measurement based upon the resistance of HbF to alkali denaturation have been described, but these methods are limited by method complexity and the need for transport, handling, and storage of a caustic base (NaOH or KOH) and potassium cyanide (KCN).[17,18,19,20] Therefore, a simple and cost-effective method of quantitative HbF measurement using non-hazardous reagents is needed for laboratories in underdeveloped countries that are involved in sickle cell testing and hydroxyurea treatment. In response to this need, this study has developed a method of HbF determination based on the Kleihauer-Betke (K-B) test principle by introducing whole blood into a weak citric acid buffer solution and spectrophotometrically measuring the amount of HbA, eluted from the RBCs. From this measurement, the percentage of HbF in the blood can be calculated based on the inverse relationship of HbF to HbA. If HbS undergoes acid elution from RBCs in a similar manner as HbA, the method could become the basis of a procedure for determining HbF response of sickle cell patients receiving hydroxyurea treatment in underdeveloped countries.

MATERIALS AND METHODS
Materials and Equipment
Citrate Phosphate Buffer Solution (0.07 M sodium citrate and 0.06 M sodium phosphate) from Fetal Hemoglobin kit (Sigma-Aldrich, St. Louis, MO)

Junior Model 35S spectrophotometer (Perkin-Elmer, Waltham, MA)

QuickGel Alkaline hemoglobin electrophoresis system (Helena, Beaumont, TX)

Specimens

Normal whole blood samples (source of HbA) were collected by standard venipuncture technique from members of the research team. De-identified whole cord blood samples (source of HbF) were obtained from Cardinal Glennon Pediatric Research Institute, St. Louis, MO. Specimens were obtained weekly, and only non-hemolyzed specimens were accepted. To standardize the amount of hemoglobin, hematocrits of all test samples were adjusted to 35%. When normal and cord blood samples were to be mixed, the cord blood ABO type was determined, and a normal sample was collected from a compatible member of the research team. Due to de-identification of samples, the study was determined to be non-human research by the Saint Louis University Institutional Review Board and, therefore, IRB waived.

Basic Procedure

A basic tube procedure was written based on the K-B slide procedure and then optimized to demonstrate HbA elution in solution and produce a reliable method. Citrate Phosphate Buffer Solution from the Fetal Hemoglobin kit (Sigma-Aldrich), which is a version of the K-B test, was prepared weekly as directed by the package insert.[21] To adapt the K-B test procedure to a spectrophotometric method, 4 mL of buffer solution was pipetted into three labeled 12 x 75 mm test tubes. These were placed into a dry heat block incubator for 15 min at 37 °C, and then three specimens: 1. normal blood (HbA), 2. a 50:50 mixture of normal and cord blood (HbA+F), and 3. cord blood (HbF), were added to the pre-warmed aliquots. After a carefully timed incubation, the tubes were centrifuged at 3100 rpm to pellet the RBCs. Approximately 3 mL of supernatant was transferred to clean tubes, avoiding the RBC pellet, and measured spectrophotometrically, using Citrate Phosphate Buffer Solution as the blank.

Analysis was also performed on six control tubes with each experiment: three controls containing HbA, HbA+F, or HbF in 4 mL saline (0% hemolysis), and three controls as above in 4 mL de-ionized water (100% hemolysis).

Research Design

Optimization of Wavelength: The wavelength used in the method was determined by spectral scan using the eluate of a HbA sample.

Optimization of Centrifugation Time: Minimum centrifugation time was determined by adding each of the three sample types to 4 mL of Citrate Phosphate Buffer Solution, incubating, and testing centrifugation times of 5 min, 1 min, 30 s, and 15 s. The RBC pellets that formed at each centrifugation time were observed during transfer of supernatant, and the minimum centrifugation time required for the pellets to remain solid was determined.

Optimization of Incubation Time: To determine optimal incubation time, the three sample types were incubated in 4 mL of Citrate Phosphate Buffer Solution in one minute intervals from 4 to 10 min. An incubation time was chosen that maximized elution of HbA, minimized elution of HbF, gave distinctly separate absorbance values for the three sample types, and produced linear results.

Optimization of Sample Volume: Optimal sample volume was determined by testing different volumes of HbA blood, from 10 to 30 µL in increments of 5 µL, in the acid elution procedure until a maximum absorbance value of approximately 1.3 was obtained (upper end of the spectrophotometer's linear range).

Optimization of Citrate Phosphate Buffer Concentration: When prepared as indicated in the Sigma-Aldrich Fetal Hemoglobin kit, Citrate Phosphate Buffer Solution contains 0.07 M sodium citrate and 0.06 M sodium phosphate. To determine if this concentration was appropriate for this adapted method of HbF determination, the buffer solution was serially diluted three times, and the three sample types were tested using each dilution.

Evaluation of Reproducibility: To demonstrate reproducibility, a total of 39 runs of the three sample types were performed across two days using the optimized procedure. The same samples and the same batch of buffer solution were used for all runs.

Evaluation of Linearity: Linearity of the optimized procedure was determined by testing different HbA: HbF mixtures in 10% increments, ranging from a ratio of 100:0 to 0:100. This experiment was performed twice, keeping all variables the same. Hemoglobin electrophoresis was performed on the various mixtures to confirm that linearity displayed in the experiment

corresponded to the HbA:HbF ratios in the mixtures. Densitometry was not available to determine actual HbA and HbF concentrations.

Data Analysis
Using the statistical software SPSS 17.0, reproducibility was determined by analyzing mean absorbance values and standard deviations of the HbA, HbA+F, and HbF samples tested. In addition, a one-way ANOVA test and Tukey's HSD post-hoc test were used to show significance of the differences between absorbance values of each sample type. To analyze linearity, Microsoft Excel 2003 was used to average the absorbance values of each HbA:HbF ratio and calculate the coefficient of determination (r^2).

RESULTS
Experiments testing a series of variables to optimize the procedure resulted in a wavelength of 395 nm, centrifugation time of 30 sec, incubation time of 6 min, blood volume of 20 µL, and Citrate Phosphate Buffer concentration of 0.07 M sodium citrate and 0.06 M sodium phosphate as stated in the Fetal Hemoglobin kit.

During the reproducibility experiment, it was noted that supernatants of the HbA tubes were darkest in color and HbF tubes were lightest in color, the latter often appearing colorless. In addition, the RBC pellets in the HbA tubes were 3 mm in diameter and brown in color, the pellets in the HbA+F tubes were 4 mm and red-brown in color, and the HbF tube pellets were 5 mm and red. The three sample types gave distinct mean absorbance values and low standard deviations within and across both days of reproducibility testing, as shown by the low p-values ($< 1 \times 10^{-6}$) of the ANOVA and Tukey's HSD post-hoc tests. (Table 1) An ANOVA statistic was selected to compare mean values of the three absorbance readings because it would not increase the risk of type I error, whereas performing three separate T-tests would artificially increase the alpha producing type I error. The p-value was lower than the α ($< 1 \times 10^{-6}$) indicating that a statistically significant difference exists between at least one mean value pair. A post-hoc test such as Tukey's HSD must be performed following a statistically significant ANOVA to determine which mean value pairs are statistically different. In our case, each mean absorbance value was statistically different from the other two mean

absorbance values.

In the linearity experiment, supernatant color decreased proportional to decreasing HbA and increasing HbF concentrations in the HbA:HbF ratio tubes ranging from 100:0 to 0:100. This linear decrease of HbA in the supernatant was visually obvious, and the average absorbance values from the two consecutive experiments produced an r^2 value of 0.99. (Figure 1) Visual inspection of the electrophoresis gel showed that the HbA:HbF mixtures demonstrated expected gradual decrease in HbA banding intensity and proportional increase in HbF banding intensity. (Figure 2)

Table 1. Reproducibility Data Within Days and Across Days

Mean and Standard Deviation Within Day 1 (N = 20)

	HbA	HbA+F	HbF
Mean	1.260	0.681	0.034
SD	0.072	0.048	0.006
Saline control	0.019	0.021	0.017
Water control	1.349	1.286	1.281

Mean and Standard Deviation Within Day 2 (N = 19)

	HbA	HbA+F	HbF
Mean	1.311	0.699	0.035
SD	0.056	0.050	0.005
Saline control	0.014	0.015	0.019
Water control	1.361	1.396	1.429

Overall Mean and Standard Deviation Across Days (N = 39)

	HbA	HbA+F	HbF
Mean	1.285	0.690	0.035
SD	0.069	0.050	0.005

Comparison of Means

One-Way ANOVA	p-value $< 1 \times 10^{-6}$
Tukey's HSD Post-Hoc	
HbA vs HbA+F	p-value $< 1 \times 10^{-6}$
HbA vs HbF	p-value $< 1 \times 10^{-6}$
HbA+F vs HbF	p-value $< 1 \times 10^{-6}$

DISCUSSION
A simple and cost-effective method of measuring HbF is necessary for facilities participating in SCD diagnosis and hydroxyurea treatment in underdeveloped countries. This research developed and evaluated a method to selectively elute HbA from intact RBCs using a citrate buffer solution, while retaining HbF intracellularly. Absorbance of eluted HbA was measured spectrophotometrically and was shown to be proportional to the amount of HbA in the original blood sample. Using the assumption that HbA_2, the only other normal adult hemoglobin present in RBCs,
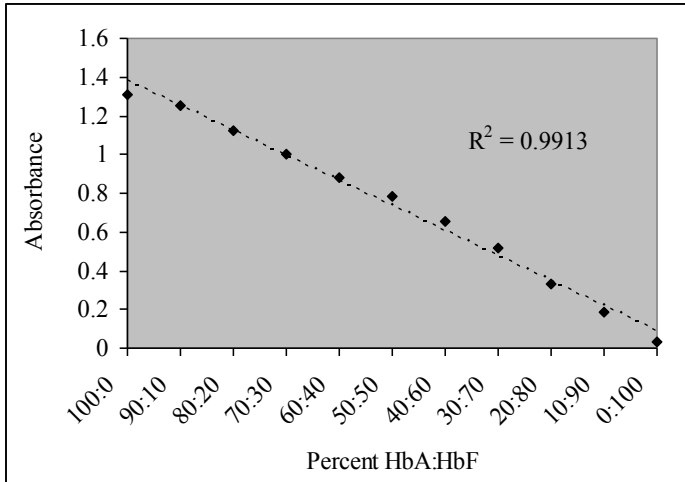
HbA:HbF	1st run	2nd run	Average
100:0	1.327	1.293	1.310
90:10	1.276	1.227	1.252
80:20	1.110	1.139	1.125
70:30	0.996	1.012	1.004
60:40	0.857	0.902	0.880
50:50	0.771	0.803	0.787
40:60	0.669	0.634	0.652
30:70	0.536	0.491	0.514
20:80	0.370	0.300	0.335
10:90	0.197	0.168	0.183
0:100	0.027	0.037	0.032
Controls	HbA	HbA+F	HbF
saline	0.037	0.014	0.015
D-I water	1.386	1.313	1.347

D-I = de-ionized

Figure 1. Absorbance Values of Samples with Decreasing HbA Percentage and Increasing HbF Percentage

is too low in concentration to affect the results, HbF can be calculated as total hemoglobin minus measured HbA.

This new method is based on the principle of the qualitative K-B slide test, which determines presence or absence of HbF-containing RBCs based on elution of HbA in citric acid solution. Since the Fetal Hemoglobin kit procedure recommends that the Citrate Phosphate Buffer Solution be pre-warmed at 37 °C, we followed this recommendation to achieve maximum elution and tested other variables to optimize the procedure.

Saline and de-ionized water were used in place of Citrate Phosphate Buffer Solution as controls to determine background hemolysis (saline) and maximum hemolysis (water). Saline controls accounted for natural hemolysis occurring in the test system, and de-ionized water controls predicted maximum absorbance should complete elution of all hemoglobin in the RBCs occur. Although the control tubes served the intended purpose, they were not ideal because free hemoglobin in the control tubes produced a red supernatant (although the saline controls were essentially colorless), whereas eluted hemoglobin exposed to acidic buffer in the experiment produced a brown color. Therefore, absorbance values of the controls did not perfectly parallel absorbance values of the experimental tubes because wavelengths used in this study were optimized for brown-colored hemoglobin solutions, not red hemoglobin solutions. This may have specifically affected sensitivity of the saline controls in detecting small amounts of natural hemolysis. Although the

controls were imperfect, they served their purpose of ruling out major errors in technique because the saline controls consistently showed low absorbance values similar to HbF blood samples, and the de-ionized water controls consistently showed high absorbance values similar to HbA blood samples.
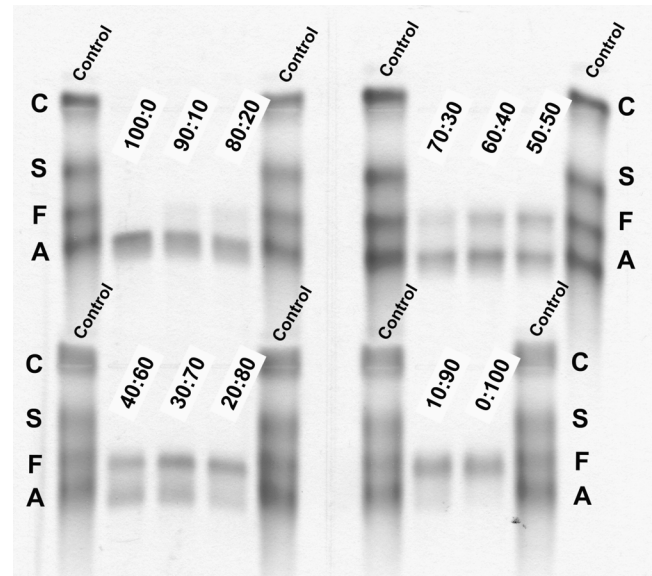

Figure 2. Alkaline Electrophoresis of HbA:HbF Ratio Samples

To optimize the procedure, it was necessary to determine the wavelength of maximum absorbance of the hemoglobin eluted in acidic buffer because it was brown rather than red. The wavelength used to measure absorbance of red hemoglobin solutions in many previously developed procedures was not applicable in this test system.

Optimization of centrifugation time proved to be an essential component of developing the optimized procedure. A 30-second centrifugation time, which was shorter than the centrifugation time used initially, allowed better delineation between the HbA, HbA+F, and HbF samples and better reproducibility of absorbance values. We hypothesize that co-elution of HbF with HbA occurred during longer centrifugation and that longer exposure to centrifugal forces caused additional hemolysis. Both of these phenomena would increase absorbance of HbF and HbA+F samples to a greater degree than HbA samples, causing poor delineation between the samples' absorbance values.

Using the 30-second centrifugation time, incubation intervals from 4 to 10 min were tested. The 4-, 5-, and 5.5-minute incubation times gave lower HbA sample absorbance values than longer incubation times, suggesting that elution of HbA was incomplete. Absorbance values of the HbA samples quickly leveled off after 6 min of incubation. Compared to shorter incubation times, 7-minute incubation showed a slightly higher absorbance in the HbF tubes, and HbF sample absorbance rose dramatically at 8 min, suggesting that HbF was co-eluted during longer incubation times. Therefore, 6 min was the optimal incubation time because it represented nearly complete elution of HbA, minimal elution of HbF, and excellent delineation and good linearity of the HbA, HbA+F, and HbF samples based on absorbance values.

The volume of blood used in the test system was optimized to prevent absorbance values from being above linearity of the spectrophotometer while remaining high enough to give maximum separation of absorbance values for the three sample types. Sample volume of 20 µL gave HbA sample absorbance values of approximately 1.3, which accomplished both goals.

When Citrate Phosphate Buffer Solution was serially diluted three times and each dilution tested to determine the optimal buffer concentration, it was found that absorbance values of the three sample types were not clearly delineated using the diluted solutions. The original concentration, however, produced high HbA absorbance values, mid-range HbA+F absorbance values, and low HbF absorbance values, each being easily distinguishable from the others. Therefore, buffer concentration of 0.07 M sodium citrate and 0.06 M sodium phosphate, as stated in the Fetal Hemoglobin kit, was used in the optimized procedure.

The optimized procedure was performed multiple times both on a single day (intra-run variability) and across different days (inter-run variability) to verify reproducibility, which was confirmed by low standard deviations for each sample type both within and across days. The distinct differences in absorbance values of HbA, HbA+F, and HbF samples were shown to be statically significant by low p-values of the ANOVA and Tukey's HSD. Testing a series of HbA:HbF blood mixtures in 10% increments showed a linear relationship between absorbance value and HbA level and an inverse linear relationship between absorbance value and HbF level. The coefficient of determination (r^2) of 0.99 indicates that 99% of variability in absorbance values could be accounted for by variation of the HbA:HbF ratios. Visual inspection of the electrophoresis gel supported the expected relative concentrations of HbA and HbF in the mixtures tested, but densitometry was not available for confirmation of the electrophoresis results.

Throughout the study, visual observations of supernatant color supported the absorbance value readings. The size and color of the RBC pellets produced after centrifugation also followed the same logic. The smaller size and lack of red color of the 3-mm pellet in the HbA tubes, along with dark brown supernatant, represented nearly complete elution of hemoglobin from the RBCs. Conversely, the larger size and red color of the 5-mm pellet and the pale to colorless supernatant in the HbF tubes represented minimal HbA elution. As expected, the red-brown 4-mm pellet and medium-brown supernatant color in the 50:50 HbA+F tubes fell between the pellet size and supernatant color of the HbA and HbF tubes.

This study tested HbA samples because the principle of the K-B test is based on HbA. Since this adapted method was successful at eluting HbA and retaining HbF, further investigations will be performed to determine if HbS elutes from RBCs in a manner similar to HbA. These studies will use sickle cell samples in place of HbA samples, establishing whether or not the method can be used to measure HbF levels in sickle cell patients. Future studies are also needed to create a table relating the hematocrit of a patient sample to the

volume of blood inoculum required in the test, rather than standardizing all samples to a hematocrit of 35%. Additionally, investigations are needed to determine how to calculate HbF levels from absorbance values read on the spectrophotometer.

Since diagnosis of SCD in underdeveloped countries is now possible with the development of the Sickle Confirm method, patients are more likely to receive treatment. The HbF-inducing drug hydroxyurea is planned as a part of future treatment programs for SCD in Haiti. Not every sickle cell patient benefits from hydroxyurea treatment, so HbF levels must be monitored and non-responders taken off the drug to avoid possible carcinogenic side effects. In addition, since clinics and patients in underdeveloped countries cannot absorb the costs of ineffective treatments, redistribution of the drug from non-responders to responders should be done to improve stewardship of limited resources. This research successfully developed a simple and cost-effective spectrophotometric method of HbF determination in the context of non-sickle cell samples (HbA), and shows potential as the basis for a test to measure response to hydroxyurea treatment in sickle cell patients.

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