

PROGRESS REPORT

Title: High-performance Liquid Chromatography of human hemoglobins on a weak cation-exchanger (1-83-0008).

Principle Investigator: Ching-Nan Ou, Ph.D.

Date: January 13, 1986

An optimal chromatographic condition combining the pH and ionic strength gradient was developed for the separation of 14 common hemoglobins as described in the previous progress report. The chromatographic condition is continuously used for the separation of other hemoglobins and for the clinical study of hemoglobinopathy. As a result, we now are able to differentiate hemoglobin N Baltimore from other fast migrating hemoglobins such as hemoglobin I, J, Camden and Hope. The identification of hemoglobin Lepore was also made by the method. Since there are more than 300 different hemoglobin variants which have been identified, it is impossible to resolve all of them by the condition. Nevertheless, only less than 20 of those hemoglobins are commonly encountered. I am in the process of trying to develop a standard chromatogram for all these common hemoglobin variants. It may require a period of time to collect all these variants. Therefore, I am requesting that the support for this project be continued until the end of the year.

The investigation supported by this grant has resulted in two more publications (one published, and one in progress) and also one abstract. The published article and abstract are attached.

Ching-Nan Ou

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High-performance Liquid Chromatography in the Diagnosis of Hemoglobinopathies and Thalassemias

Report of Three Cases

BEVERLY BARTON ROGERS, M.D., ROBERT A. WESSELS, M.D., CHING-NAN OU, PH.D.,
AND GREGORY J. BUFFONE, PH.D.

High-performance liquid chromatography is a technic that has recently been applied to the diagnosis of hemoglobinopathies and thalassemias. Its advantages over other methods include increased sensitivity, resolution and simplicity, as well as speed. In this report, the authors present an HPLC procedure that uses a weak cation exchange column and a gradient elution system for the diagnosis of hemoglobinopathies and thalassemias. The authors illustrate the utility of this procedure by reporting three cases in which the technic enabled them to make the correct diagnosis, which by traditional methods would have been missed or equivocal. (Key words: Chromatography; Electrophoresis; Hemoglobinopathy; Sickle disease; SC disease; Thalassemia) *Am J Clin Pathol* 1985; 84: 671-674

HIGH-PERFORMANCE liquid chromatography (HPLC) is a sensitive and precise method for detecting hemoglobin abnormalities.^{6,7} For routine diagnostic pur-

*Department of Pathology, Baylor College of Medicine and
Department of Pathology, Texas Children's Hospital,
Houston, Texas*

poses, hemoglobin electrophoresis using cellulose acetate at an alkaline pH and citrate agar at an acid pH have been used in most pathology laboratories. If confirmation was required, it could be achieved by the use of low-pressure, macro-column chromatography, a labor-intensive procedure.⁵ Low-pressure chromatography, using a weak cation exchange material such as CM-cellulose or an anion exchange material such as DEAE-cellulose, takes two to three days for a complete separation of hemoglobin variants. Microchromatographic technics decrease the chromatographic time but sacrifice resolution. Isoelectric focusing also has been used to define hemoglobin variants and has provided excellent resolution.¹ However, the hemoglobin is not easily quantitated by this technic nor is

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Address reprint requests to Dr. Buffone: The Department of Pathology, Texas Children's Hospital, P.O. Box 20269, Houston, Texas 77030.

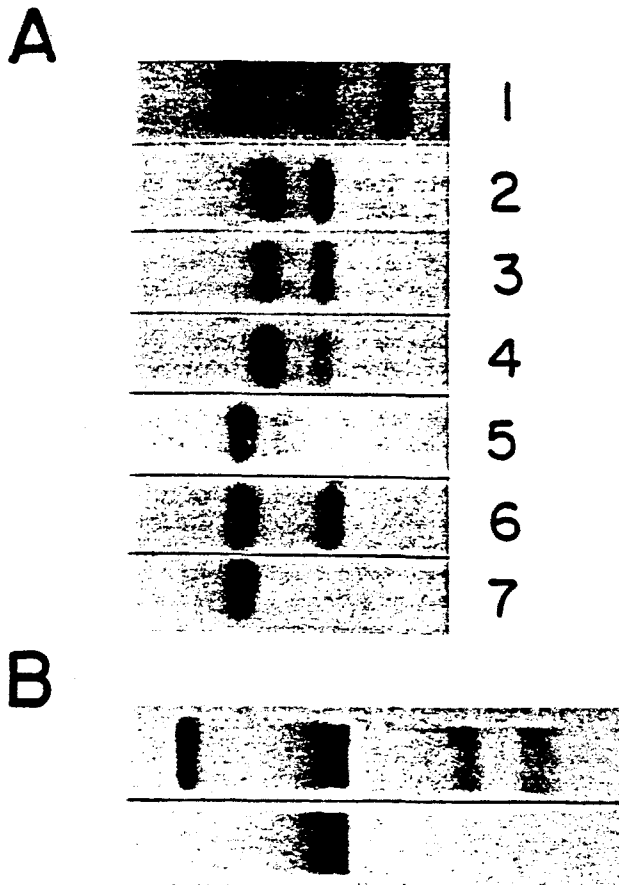


FIG. 1A. Cellulose acetate electrophoresis membrane. (1) Control, bands from left to right are hemoglobins A, F, S, and C. (2) Patient 1. (3) Patient 2. (4) Sibling, case 2. (5) Mother, case 2. (6) Father, case 2. (7) Patient 3. B. Citrate agar electrophoresis membrane. Top: Control, bands from left to right are hemoglobins F, A, S, and C. Bottom: Patient 3.

it typically performed by a routine pathology department, and, for these reasons, it has been confined primarily to research laboratories.

Both anion and cation exchange packing materials have been prepared for HPLC application. Anion exchange chromatography of hemoglobins using HPLC has been able to resolve some of the major hemoglobins,⁶ with improved separation over electrophoresis of HbA and HbF in the neonate.² However, it lacks the ability to resolve the rare hemoglobin variants. A cation exchanger developed by Wilson and associates¹⁰ provided higher resolution in comparison with the anionic exchanger but still lacked the resolving power for certain hemoglobins. Also, the time to elute all hemoglobin fractions was relatively long, being 90 minutes. Ou and colleagues have reported a high-performance liquid chromatographic technique that uses a poly-aspartic acid linked to silica as a packing material. This weak cation exchange packing material provides for a significant improvement in resolution and sen-

sitivity and a reduction in their chromatographic time to 30 minutes.⁷ We present here a brief description of the method and three cases from our laboratory to illustrate the power of HPLC for hemoglobin study.

Methods

High-performance liquid chromatography was performed by the method of Ou and colleagues, as previously described.⁷ In brief, hemolysate was injected into a column packed with a weak cation exchanger prepared by coating silica with poly-aspartic acid (Poly CAT A, Custom LC, Houston, TX). The chromatographic separation was achieved using gradient elution. Mobile phase A contained 40 mM Bis-TRIS and 4 mM KCN (pH 6.5). Mobile phase B contained 40 mM Bis-TRIS, 4 mM KCN and 0.2 M NaCl (pH 6.8). Using a flow rate of 1 mL/minute, the column was equilibrated with mobile phase of 22% B, 78% A. Phase B was increased linearly to 56% and 100% at 16 and 22 minutes, respectively, and then decreased to

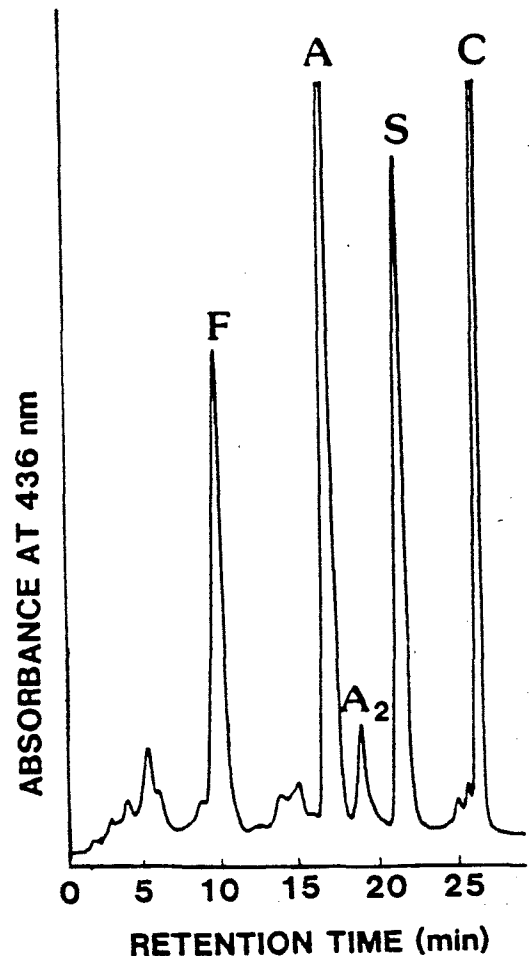
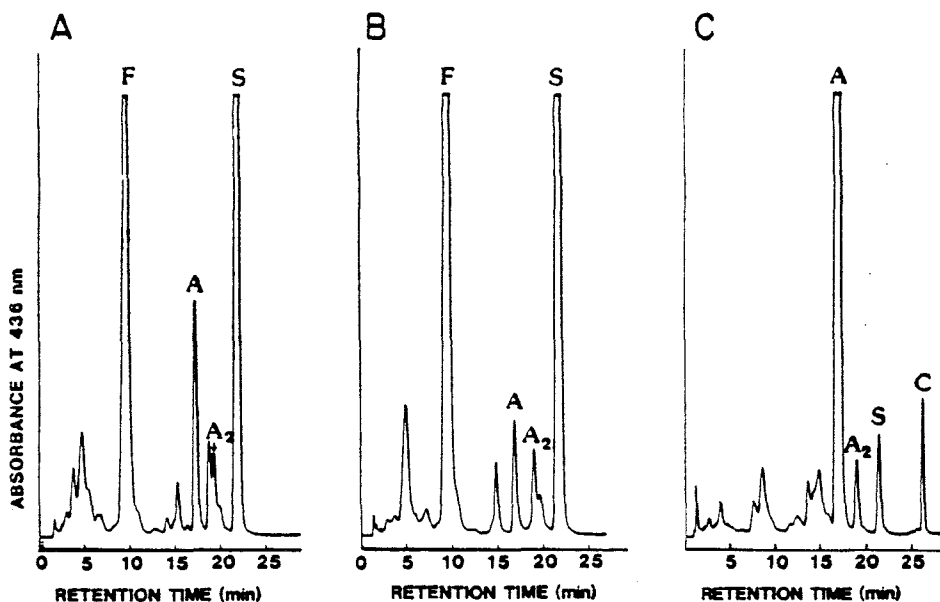


FIG. 2. Cation-exchange high-performance liquid chromatography pattern of control containing hemoglobins F, A, A₂, S, and C.

FIG. 3. Cation-exchange chromatography patterns. A. Patient 1. B. Patient 2. C. Patient 3.



22% at 24 minutes. The effluent was monitored at 436 nm, and peak areas were used for quantitation.

Hemoglobin electrophoresis was done on cellulose acetate and citrate agar using standard methods.⁸ Cellulose acetate membranes were scanned using the Beckman Microzone Densitometer[®] (Model R-110). Hemoglobin A₂ was quantitated using DEAE-cellulose micro-columns (Isolab, Akron, OH) according to the method of Abraham and co-workers as modified by Huisman and colleagues.⁴

Report of Three Cases

Case 1

This five-month-old black female was born to a mother with previously diagnosed sickle cell trait. At three months of age, the child was diagnosed as "probable sickle cell disease" on the basis of results from the State Laboratory newborn screening program. The results of cellulose acetate and acid citrate electrophoreses were HbF = 76%, HbS = 24%. The patient was admitted to the hospital at five months of age with otitis. She had a hemoglobin level of 11.1 g/dL, and no sickled cells were seen on the peripheral smear. Because there was a lack of symptoms or signs compatible with sickle cell disease, a hemoglobin electrophoresis was requested. Hemoglobin analysis by cellulose acetate electrophoresis (Fig. 1A) showed HbA = 0%, HbF = 50.3%, HbS = 47.1%, and HbA₂ = 2.6%. The HbA region was indistinct due to overlap by HbF, so that no definite band was seen. Hemoglobin analysis by HPLC (Figs. 2 and 3A) showed HbA = 8.8%, HbF = 41.7%, HbS = 44.5%, HbA₂ = 5.0%. Based on these data, a diagnosis of S/β⁺ thalassemia was made.

Case 2

The patient was a two-year-old black female in good health who was referred for hemoglobin electrophoresis as part of a family study. Her two and a half-month-old brother was found to have only HbF and HbS on a newborn screen done by the State Laboratory at one week of age. A hemoglobin analysis of the patient's blood specimen by cellulose acetate electrophoresis showed HbA = 0%, HbF = 54.8%, HbS = 42.6%, and

HbA₂ = 2.6% (Fig. 1A). As in case 1, the HbA region was indistinct. On examination of the peripheral smear, no sickled cells were seen. HPLC quantitation (Fig. 3B) showed HbA = 3.6%, HbF = 50.2%, HbS = 43.4%, and HbA₂ = 2.8%. A diagnosis of S/β⁺ thalassemia was made. The immediate family was studied (Table 1 and Fig. 1A) with quantitation of hemoglobin fractions by cellulose acetate, HPLC, and, when appropriate, Hb A₂ by low-pressure ion exchange column chromatography. Because of the unusually high HbF in the mother, a Kleihauer-Betke stain was performed and showed a heterogeneous pattern. On the basis of an elevated HbF with a heterogeneous pattern and an elevated HbA₂, the mother appears to have β⁺ thalassemia trait with high HbF (a rare variant of β⁺ thalassemia) rather than β⁺ thalassemia trait and hereditary persistence of HbF.⁹ The father was diagnosed as having sickle cell trait.

Table 1. Quantitation of Hemoglobin Fractions in Case 2 by Cellulose Acetate, HPLC, and DEAE-Cellulose Column

Patient	HB Fractions	Cellulose Acetate	HPLC	DEAE-Cellulose Column
2-year-old girl	Hb A	0	3.6%	
	Hb F	54.8%	50.2%	
	Hb S	42.6%	43.4%	
	Hb A ₂	2.6%	2.8%	
2½-month-old boy	Hb A	0	0	
	Hb F	80.2%	74.6%	
	Hb S	19.8%	24.7%	
	Hb A ₂	0	0.7%	
Mother	Hb A	84.2%	82.4%	
	Hb F	11.0%	11.9%	
	Hb S	0	0	
	Hb A ₂	4.8%	5.7%	5.2%
Father	Hb A	54.8%	53.5%	
	Hb F	0	0	
	Hb S	42.0%	42.1%	
	Hb A ₂	3.2%	4.4%	

Although the HPLC method did not detect any HbA in the two and a half-month-old sibling, a diagnosis of S/β^+ thalassemia was made based on the family study.

Case 3

The patient was a seven-year-old black female who was brought to the emergency room unresponsive and in asystole. Her hemoglobin level was 2.4 g/dL. Resuscitation attempts, including 2 units of O-negative blood, failed. The child's history was significant for repair of an atrial septal defect at age three years, as well as a vague "blood disease." Blood cultures taken at the time of admission grew *Streptococcus pneumoniae*. The patient died shortly thereafter. The autopsy findings, including acutely hemorrhagic adrenals, were consistent with sepsis. Irreversibly sickled cells were apparent in the histologic sections of the spleen. Hemoglobin electrophoresis on cellulose acetate was performed on post-transfusional blood obtained at autopsy, and the values were HbA = 89.2%, HbS = 3.7%, and 7.1% of the total hemoglobin in the HbA₂ region (Fig. 1A). Hemoglobin C was not detectable by citrate agar electrophoresis (Fig. 1B). Analysis by HPLC showed the following results: HbA = 81.8%, HbF = 3.1%, HbS = 4.2%, HbA₂ = 2.6%, HbC = 3.4% (Fig. 2). From this data, Hb SC disease with recent transfusion was diagnosed. Old records were obtained, and it was documented that Hb SC disease was diagnosed four years previously by routine methods.

Discussion

The high-performance liquid chromatographic technic described here can easily be used in the reference and routine clinical laboratory for evaluation of hemoglobinopathies and thalassemia. The procedure is fast (30 minutes per sample), sensitive, and precise. The excellent resolving power of this column has been demonstrated previously.⁷ The three cases presented emphasize the value of the increased sensitivity achieved by this technic. Cases 1 and 2 demonstrate the ability to detect HbA when routine methods fail. In the absence of family studies (case 1), this can be important.

Because of its marked sensitivity, HPLC would be useful as a confirmatory test of sickle cell anemia in newborn screening programs. False positive diagnoses of sickle cell anemia are unavoidable in newborn screening programs using routine electrophoretic methods of hemoglobin analysis.³ Emphasized by cases 1 and 2, HPLC can detect very small quantities of HbA in the S/β^+ thalassemia double heterozygote, who, without Hb A, would be given the diagnosis of sickle cell anemia.

However, HPLC does have limitations, as demonstrated by Case 2. This family appears to have a rare vari-

ant of β^+ -thalassemia, with exceptionally low levels of Hb A in the double heterozygote S/β^+ thalassemia. In the two and a half-month-old sibling, this HPLC technic was unable to detect HbA. We would predict that, by HPLC, HbA will be detectable in this child as he ages, as it was in the two-year-old sibling. Case 3 illustrates the increased sensitivity of HPLC in the diagnosis of hemoglobinopathy in a transfused patient. HbC was not seen on citrate agar electrophoresis, but was easily detectable by HPLC. All of these cases demonstrate the effectiveness of HPLC to detect small quantities of hemoglobin fractions not seen by routine methods and highlight its usefulness to diagnose hemoglobinopathies and thalassemia.

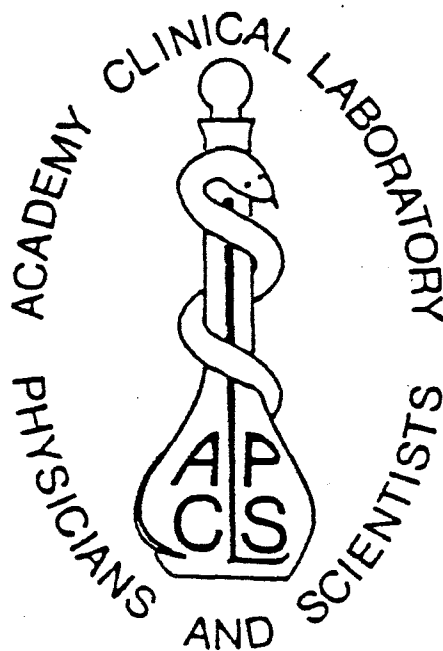
Acknowledgment. The authors thank Rebecca Grove for technical assistance.

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Calvin Sigman

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ANNUAL MEETING

June 1-3, 1983

University of Rochester Medical Center

and

The Genesee Hospital

Rochester, New York

9:30 AM - Hematology-Immunohematology Session

SPEAKER: Gregory L. Reimer (Resident)

SPONSOR: Ching-Nan Ou

5 ANALYSIS OF HUMAN HEMOGLOBINS BY A CATION-EXCHANGE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY. Gregory L. Reimer, Ching-Nan Ou, and Gregory J. Buffone, Pathol. Dept., Texas Children's Hosp. and Baylor Coll. of Med., Houston, TX. 77030

Cellulose acetate and citrate agar electrophoresis and DEAE-cellulose column chromatography are the most widely used methods for hemoglobin analysis. Nevertheless, these methods are limited by the poor resolution of some common hemoglobin variants such as E and O or D and G in the case of electrophoresis and C, E, S and A₂ in DEAE-cellulose column chromatography. We have developed an HPLC method for the analysis of human hemoglobins which is not subject to these limitations. The method uses a combined pH and ionic strength gradient on a poly (aspartic acid) cation exchange column (Poly CAT A). Hemoglobins Bart's, F, A₀, A₂, S, C, D, E, O, SG and some α -chain variants such as G, Q, Sealy, and Winnipeg can be completely separated by the gradient program within 30 min. Comparison of HPLC measurement of hemoglobin A₂ with DEAE-cellulose column chromatography yields the following results: y (HPLC method) = $1.04 x - 0.29$, $r = 0.984$, $N=39$ and $Sy.x=0.27$. The presence of hemoglobin S, C, or E were found to produce a significant positive bias in the DEAE-cellulose column method, while on HPLC these variants were easily resolved. The excellent sensitivity of the method makes it possible to screen newborn cord blood for hemoglobinopathies.

The high resolution of the system and the sensitivity of the method plus the potential for automation make this procedure useful for screening and diagnosis of common hemoglobin disorders in both a research and clinical laboratory environment.

9:45 AM - Hematology-Immunohematology Session

SPEAKER: Thomas R. Fritsche (Guest)

SPONSOR: James Fine

6 SEVERE THROMBOCYTOPENIA IN MALIGNANT TERTIAN MALARIA WITHOUT INTRAVASCULAR COAGULATION. T.R. Fritsche, M.D. Dept. of Lab Medicine, University of Washington, Seattle, Washington.

Pathogenesis of the complications resulting from infection with *Plasmodium falciparum* remain poorly understood, but intravascular coagulation is thought to be an important intermediary mechanism. Thrombocytopenia commonly occurs with both *falciparum* and *vivax* malaria, but rarely leads to clinical hemorrhage despite extremely low platelet counts (down to 18,000 and 20,000 reported for these species, respectively).

Recently a patient was treated in our hospital for *falciparum* malaria acquired in the Sudan. Although the platelet count fell at one point to 11,000, only a few scattered petechiae developed over the lower extremities. Parasitemia peaked at 18% and responded to the administration of chloroquine. Difficulty was experienced by the technologist performing chamber counts in differentiating platelets from malarial trophozoites because of similar size and refractility. Interestingly PT, PTT, TT, and fibrinogen remained within reference ranges, although fibrinogen degradation products were present at greater than 40 ug/ml. At discharge parasitemia had disappeared and platelets had climbed to 57,000.

The causes of malarial thrombocytopenia may result from several processes including disseminated intravascular coagulation excessive splenic pooling, decreased platelet survival, and direct platelet invasion by malarial merozoites with subsequent destruction. Lack of evidence for intravascular coagulation in this case suggests that certain of these other mechanisms may be responsible.