PROGRESS REPORT

TITLE:

High-performance Liquid Chromatography of Human Hemoglobins on a Weak Cation-Exchanger (1-83-0008).

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Due to the change of procedure for making the 5 um silica particles by the manufacture, the characteristics of the coated weak ion exchanger was slightly different from the previous lot of material. Therefore, a modification of the chromatographic condition was made to resolve all the 14 commonly encountered hemoglobins. Because the new silica was not very durable (it only lasted for 60 injections), search for a durable and consistent silica particles is still in progress. Nevertheless, the standard chromatogram for 19 hemoglobins was established. The identifications of a few rare hemoglobins such as lepore, candem, sealy, N-Baltimore and G Philadelphia were made since the implementation of HPLC for hemoglobin analysis at TCH Clinical Chemistry laboratory two years ago.

The investigation supported by this grant has resulted in one more publication. The standard chromatogram and the published article are attached.

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Liquid Chromatography Used in Diagnosis of a Rare Hemoglobin Combination: Hemoglobin S/Lepore$_{Boston}$

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"High-performance" liquid chromatography (HPLC), applied to hemoglobin analysis, is decidedly more sensitive and gives better resolution than do routine electrophoretic methods. Here we present a case with a rare double heterozygote hemoglobin S/Lepore$_{Boston}$, originally diagnosed as homozygous hemoglobin S by routine electrophoretic methods. Using a gradient elution weak cation-exchange HPLC technique, we could separate hemoglobin S and hemoglobin Lepore$_{Boston}$ and make the correct diagnosis. This case demonstrates how HPLC can be a useful adjunct to routine electrophoresis.

Additional Keyphrases: *cellulose acetate electrophoresis compared · hemoglobin variants · sickle cell trait*

The double heterozygote hemoglobin Hb S/Lepore$_{Boston}$ is rarely diagnosed. There are only eight previously reported cases (*1–5*), only one of whom was a patient in the United States (*5*). Routine electrophoresis on cellulose acetate will usually incorrectly label such patients as homozygous Hb S. Hb Lepore migrates only slightly anodally to Hb S. If Hb S is present, the two bands may appear as one. Moreover, because Hb Lepore is present only in small proportions, the Hb S solubility tests produce a homozygous positive pattern that appears to confirm the electrophoretic findings. One may suspect the diagnosis of Hb S/Lepore$_{Boston}$ in patients with apparently homozygous Hb S by electrophoresis but with hypochromatic, microcytic erythrocyte indices and no sickled cells showing on peripheral smear. However, either concomitant iron-deficiency anemia or α-thalassemia in a patient with homozygous Hb S or Hb S/β-thalassemia may result in a similar picture. The correct diagnosis of Hb S/Lepore$_{Boston}$ has necessitated complex and labor-intensive methods (*2, 4*).

For routine diagnostic purposes, electrophoresis of hemoglobin on cellulose acetate at alkaline pH and on citrate agar at acid pH is used in most clinical laboratories. Chromatographic confirmation of unusual variants has involved low-pressure, macro-column chromatography, a labor-intensive and time-consuming procedure (*6*). Low-pressure chromatography with a weak cation exchange material such as carboxymethyl-cellulose or an anion-exchange material such as diethylaminoethyl-cellulose takes two to three days for complete separation of hemoglobin variants. Use of micro-chromatographic techniques decreases the chromatographic time, but sacrifices resolution. Isoelectric focusing has also been used to define hemoglobin variants, with excellent resolution (*7*); however, it does not lend itself to easy quantification of the hemoglobin nor is it easily performed by a routine laboratory, and thus its use has been confined primarily to research laboratories.

Anion-exchange "high-performance" liquid chromatography (HPLC) can resolve some of the major hemoglobins (*8*). It better separates Hb A and Hb F in the neonate than does electrophoresis (*9*), for instance, but not many of the rare hemoglobin variants. The cation exchanger developed by Wilson et al. (*10*) provided better resolution in comparison with the anion exchanger, but still lacked resolving power for certain hemoglobins, and the time to elute all hemoglobin fractions was relatively long, 90 min. Ou et al. (*11*) developed an HPLC technique involving a polyaspartic acid linked to silica as a packing material. With this weak cation-exchange material resolution and sensitivity are significantly improved over routine electrophoretic methods, and chromatography requires 30 min. Here we report a case of a rare hemoglobin combination, and demonstrate the ability of this weak cation-exchange column to resolve these hemoglobin variants.

Materials and Methods

HPLC was performed as previously described (*11*). In brief, after a 60-fold dilution of packed cells, we injected 20 μL of hemolysate into a 20 × 0.5 cm HPLC column packed with a weak cation exchanger, polyaspartic acid (Poly CAT A; Custom LC, Houston, TX 77072). The chromatographic separation was achieved by gradient elution of the following mobile phases. Phase A contained, per liter, 40 mmol of 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol ("Bis-Tris") and 4 mmol of KCN (pH 6.5); phase B contained the same as A but at pH 6.8 and also included 0.2 mmol of NaCl per liter. Using a flow rate of 1 mL/min, we equilibrated the column with a mixture of 220 mL of B and 780 mL of A per liter. Phase B was increased linearly to 560 mL/L and 1000 mL/L at 16 and 22 min, respectively, then decreased to 220 mL/L at 24 min. We monitored the effluent at 436 nm and measured peak areas.

We electrophoresed hemoglobin on cellulose acetate and citrate agar by standard methods (*12*); peak areas on the cellulose acetate membranes were quantified with a densi-

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tometer (Model R-110, Microzone; Beckman Instruments, Fullerton, CA). For the Hb S differential solubility tests we used a qualitative biphasic system ("Sickle Quick"; General Diagnostics, Morris Plains, NJ). Hemoglobin concentrations and erythrocyte indices were determined with routine automated equipment (Coulter Counter Model S-Plus; Coulter Electronics, Hialeah, FL).

Case Report

The patient, a nine-year-old black girl, had been diagnosed as having sickle cell disease four years earlier when she presented elsewhere with swollen palms. Since then she had had 12 hospitalizations for "crises"—defined by swollen, painful extremities and frequently associated with fever. Her last four hospitalizations were at our institution (Table 1). A mild hypochromic, microcytic anemia was present at all times, but no sickled cells were seen on peripheral smear. Treatment included medication for pain, hydration, and Fluidotherapy® (consult reference *13* for details). Her spleen was never detectable by palpation, nor was she transfused.

During the present hospitalization she was admitted with fever and ankle pain. Her physical examination was remarkable for obesity and soft-tissue swelling of the right ankle. Again, she was mildly anemic (Table 1). She was treated for five days with intravenous ampicillin and received one dose of chloramphenicol. Blood cultures showed no pathogen growth. On day 3, she became jaundiced, with a serum total bilirubin concentration of 88 mg/L (57 mg/L direct reacting) and a hemoglobin concentration of 76 g/L. Values for liver enzymes were within normal limits and an abdominal examination by ultrasound showed a spleen of normal size and no biliary stones or obstruction. By day eight her hemoglobin concentration had stabilized and total serum bilirubin had also dropped to 24.0 mg/L. She was discharged.

Electrophoresis of her hemoglobin on cellulose acetate at our institution when she was seven years old showed a broad band in the S position, accounting for 83.3% of the total hemoglobin (other hemoglobins were Hb F 13.5%, Hb A_2 3.2%). Results of a differential solubility test were consistent with a homozygous Hb S pattern. On this admission, electrophoresis of her hemoglobin on cellulose acetate appeared to be homozygous Hb S with high Hb F (Figure 1*a*, Table 2), while on citrate agar prominent bands were found at the S and F position and a faint band at the A position (Figure 1*b*). Using our HPLC technique, we identified a peak between the Hb A and the Hb A_2 position, which was at the Hb $Lepore_{Boston}$ position (Figure 2), the migration of the $Lepore_{Boston}$ hemoglobin having been previously defined by use of this method. A presumptive diagnosis of double

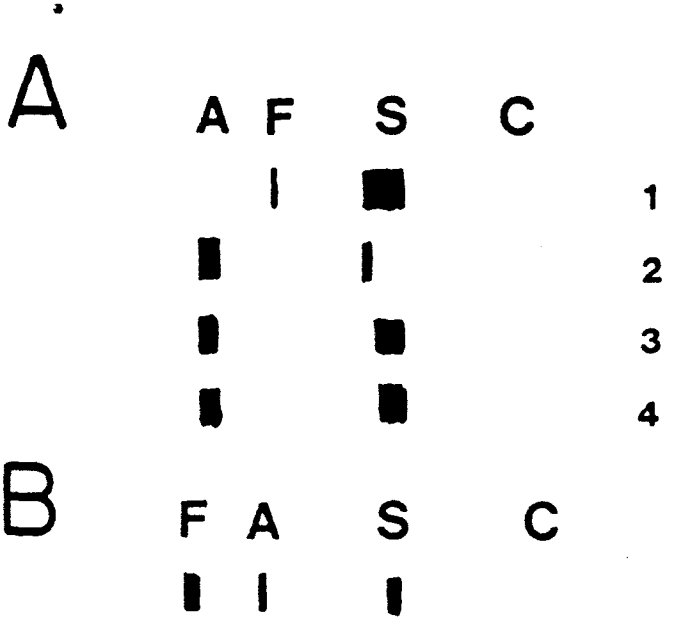

Fig. 1. *A*, diagram of cellulose acetate electrophoretogram membrane: (*1*) patient, (*2*) mother, (*3*) father, (*4*) brother; *B*, diagram of patient's citrate agar electrophoretogram membrane

heterozygous Hb S/$Lepore_{Boston}$ was made. Subsequently, we analyzed samples from family members (Figures 1 and 2, Table 2). The father and brother had sickle trait patterns. We detected the Hb $Lepore_{Boston}$ peak in the mother's HPLC pattern and her cellulose acetate electrophoresis pattern showed a faint band just anodal to the S position (the Hb $Lepore_{Boston}$). Samples were sent for confirmation to Dr. T. H. J. Huisman, Laboratory of Protein Chemistry, Department of Cell and Molecular Biology, Medical College of Georgia, Augusta, Georgia. His laboratory performed the following studies:

The Hb variant was isolated by chromatography on diethylaminoethyl-cellulose, and the α and non-α chains were separated on a column of carboxymethyl-cellulose. Peptides in a tryptic digest of the aminoethylated derivative of the non-α chain were separated by reversed-phase HPLC, and their composition was determined by automated analysis for amino acids. Because peptides T-2, T-3, T-5, and T-10 had the amino acid composition expected for the corresponding fragments of the δ chain, while the compositions of T-12 and T-13 were those of β chain peptides, it was concluded that the non-α chain of this variant was the $\delta\beta$-hybrid chain of Hb $Lepore_{Boston}$, which is characterized by a crossover between residues δ-87 and β-116.

Summary

The prognosis of patients with Hb S/$Lepore_{Boston}$ is unpredictable. Reports of such patients have varied from an

Table 1. Summary of Pertinent Patient Data

Age, yr/months	Symptoms	Hgb, g/L	MCV/MCH/MCHC[a]	Reticulocytes, %	Hospital stay
8/5	right ankle pain, fever	113	68/22/320	5.6	3 days
8/7	left hip and leg pain	109	67/22/320	3.9	3 days
8/9	left ankle and leg pain	101	68/22/330	2.3	2 days
9/1	left knee pain fever	105	69/22/330	3.7	2 days
9/5	right ankle pain fever	100	68/22/320	1.7	8 days

[a] Erythrocyte indices: mean cell volume (MCV), fL; mean cell hemoglobin (MCH), pg; and mean cell hemoglobin concentration (MCHC), g/L.

Table 2. Quantitative Hemoglobin Data on Patient and Family Members

				Band, cellulose acetate, %					Component, HPLC, %				
Subject	Hgb	MCV	MCH	F	A	S	A_2	*	F	A	S	A_2	Lepore
Patient	113	68	22	13		84[a]	3		14		71	3	12
Mother	14.4	73	24		87		2	11[b]	7	78		3	12
Father					61	34	5		4	56	36	4	
Brother					61	35	4		3	54	39	4	

Abbreviations as in Table 1. Percent values are percent of total Hb. [a] Broad band in the S position. [b] Band migrating slightly anodal to S position.

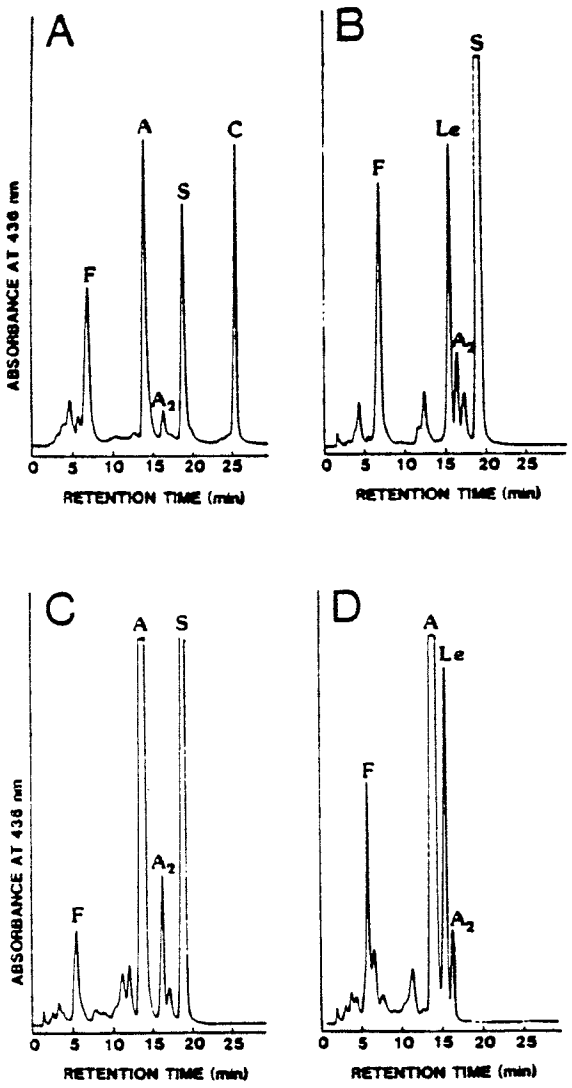

Fig. 2. Cation-exchange HPLC patterns for (*A*) control, (*B*) patient, (*C*) father, (*D*) mother (Le = Hb $Lepore_{Boston}$)

asymptomatic 76-year-old woman who had had several uneventful pregnancies, to patients with moderately severe anemia with hepatosplenomegaly, spontaneous abortions, pneumonia, and pains in the bone (*1–5*). Our patient appears mildly affected. She had had repeated hospitalizations for pain, which was easily treated. Her hemoglobin concentration has been stable and she has required no transfusion. A hemolytic episode was documented in her most recent hospitalization, but its cause was not elucidated. She did not have hepatomegaly or splenomegaly, and her growth has been normal except for obesity. Although an incorrect diagnosis would not have directly affected the treatment of this patient, it would have implications for genetic counseling.

The combination Hb S/$Lepore_{Boston}$ is infrequently reported. Previous reports include two Greek (*4*), two Italian (*2*), and three Jamaican patients (*3, 4*), as well as one black patient from the United States (*5*). In American blacks the Lepore gene is rare. However, sickle heterozygosity is estimated at 8% (*14*). We believe that, although unusual, the double heterozygote Hb S/Lepore should occasionally be seen, and that the rarity of reported cases is ascribable to the difficulty in diagnosis by routine clinical methods. This case and previous case reports demonstrate that use of routine cellulose acetate electrophoresis and sickle-cell solubility tests leads to the incorrect diagnosis of Hb S homozygosity in these patients. In our case, the citrate agar electrophoresis showed a faint band in the A position. This could be interpreted as sickle cell disease with Hb A_2 being the faint band or a double heterozygote Hb S/β^+-thalassemia with low Hb A. We previously demonstrated the utility of our HPLC technique in detecting low concentrations of Hb A in the double heterozygote Hb S/β^+-thalassemia (*15*). Our HPLC method also quantifies the concentration of Hb $Lepore_{Boston}$ accurately and quickly. We do not have examples of the other types of Hb Lepore at hand and so cannot report how they migrate in our HPLC technique.

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References

1. Stamatoyannopoulos G, Fessas P. Observation on hemoglobin "Pylos": the hemoglobin Pylos–hemoglobin S combination. J Lab Clin Med 1963;62:193–200.

2. Silvestroni E, Bianco I, Baglioni C. Interaction of hemoglobin Lepore with sickle cell trait and microcythemia (thalassemia) in a southern Italian family. Blood 1965;25:457–469.

3. Ahern EJ, Ahern VN, Aarons GH, Jones RT, Brimhall B. Hemoglobin $Lepore_{Washington}$ in two Jamaican families: interaction with beta chain variants. Blood 1972;40:246–56.

4. Stevens MCG, Lehmann H, Mason KP, Serjeant BE, Serjeant GR. Sickle cell-Hb $Lepore_{Boston}$ syndrome. Am J Dis Child 1982;136:19–22.

5. Headings VE, Winter W, Reindor C, Castro O. Sickle Lepore hemoglobin identified in a black American infant. Am J Pediatr Hematol Oncol 1983;5:259–64.

6. Huisman THJ, Jonxis JHP. The hemoglobinopathies, techniques of identification, New York: Marcel Dekker, Inc., 1977:124–62.

7. Basset P, Braconnier F, Rosa J. An update on electrophoretic and chromatographic methods in the diagnosis of hemoglobinopathies. J Chromatogr 1982;227:267–340.

8. Huisman THJ, Gardiner MB, Wilson JB. Experience with the quantitation of hemoglobin types by high pressure liquid chromatography. In: Hanash SM, Brewer GJ, eds. Advances in hemoglobin analysis, vol 60. New York: Alan R. Liss, Inc., 1980:69–82.

9. Gardiner MB, Carver J, Abraham BL, Wilson JB, Huisman THJ. Further studies on the quantitation of the hemoglobins A, S, C, and F in newborn babies with different hemoglobinopathies using high pressure liquid chromatography. Hemoglobin 1982;6:1–13.

10. Wilson JB, Headlee ME, Huisman THJ. A new high-performance liquid chromatographic procedure for the separation and quantitation of various hemoglobin variants in adults and newborn babies. J Lab Clin Med 1983;102:174–186.

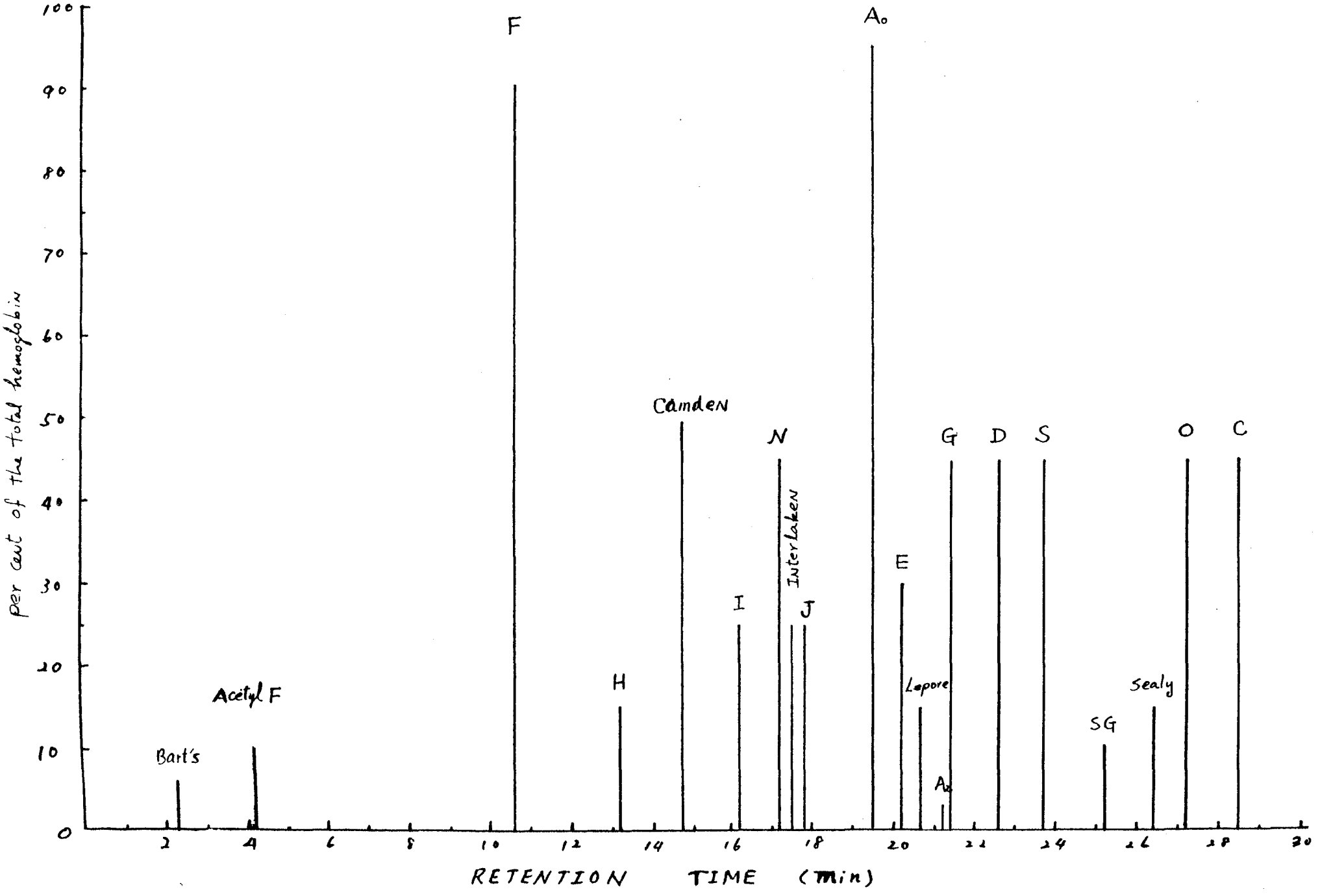
11. Ou CN, Buffone GJ, Reimer GL, Alpert AJ. High-performance liquid chromatography of human hemoglobins on a new cation exchanger. J Chromatogr 1983;266:197–205.

12. Schmidt RM, Brosious EF. Basic laboratory methods of hemoglobinopathy detection, 6th ed. Atlanta, GA: U.S. Dept. Health, Education, and Welfare, Center for Disease Control, HEW Publ. No (CDC) 77-8266, 1976.

13. Alcorn R, Bowser B, Henley EJ, Holloway V. Fluidotherapy® and exercise in the management of sickle cell anemia. J Am Phys Ther Assoc 1984;64:1520–22.

14. Motulsky AG. Frequency of sickling disorders in U.S. blacks. N Engl J Med 1973;288:31–3.

15. Rogers BB, Wessels RA, Ou CN, Buffone GJ. High performance liquid chromatography in the diagnosis of hemoglobinopathies and thalassemias: report of three cases. Am J Clin Pathol 1985;84:671–


per cent of the total hemoglobin
100
90
80
70
60
50
40
30
20
10
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
RETENTION TIME (min)
Bart's
Acetyl F
F
H
Camden
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