Journal of Chromatography, 174 (1979) 385–392

CHROM. 11,814

SEPARATION OF PEPTIDES BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY FOR THE IDENTIFICATION OF A HEMOGLOBIN VARIANT

W. A. SCHROEDER, JOAN B. SHELTON and J. ROGER SHELTON

Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, Calif. 91125 (U.S.A.)*

and

DARLEEN POWARS

Department of Pediatrics, University of Southern California School of Medicine, Los Angeles, Calif. 90033 (U.S.A.)

(Received February 23rd, 1979)

SUMMARY

High-pressure liquid chromatography on a reversed-phase column has been used to separate the tryptic peptides of a human hemoglobin variant which was then identified as hemoglobin E.

INTRODUCTION

High-pressure liquid chromatography (HPLC) is a procedure that has become increasingly important in all phases of chromatographic work, whether in routine analysis or in research. Because of the amphoteric character of peptides, either cation- or anion-exchange materials have had widespread use in their separation. However, the availability of reversed-phase HPLC packings provides a different mode of separation for peptides, which is dependent more on hydrophobicity and, hence, may be somewhat more akin to the mechanisms that obtain in peptide separations by paper or thin-layer chromatography. The increasing literature on the separation of peptides by HPLC[1–10] has been devoted largely to devising methodology and to the separation of artificial mixtures of peptides. This report describes the application of reversed-phase HPLC to the separation of the aberrant peptide in a complex mixture of tryptic peptides from a hemoglobin (Hb) variant that was detected during screening of cord blood for such variants.

MATERIALS AND METHODS

General methods

Blood samples were collected in Vacutainers, with EDTA as anticoagulant. Hematological data were obtained with a Coulter Counter (Model S).

* Contribution No. 5978.

Electrophoresis was carried out on cellulose acetate (Helena Labs., Beaumont, Texas, U.S.A.) at pH 8.4 or on starch gel at pH 9.0.

Cord-blood screening for Hb variants involved use of the method of Schroeder *et al.*[11]. The Hb composition of samples was determined by micro-chromatographic procedures[12] or use of larger-scale analytical columns[13,14]. A scaled-up version of a micro-chromatographic method to be described below was used for isolation of the Hb variant.

Osmotic fragility was determined by the procedure that is described by Huisman and Jonxis[15].

For amino acid analysis, samples were hydrolyzed *in vacuo* in redistilled 6 *M* hydrochloric acid at 110° for 24 h. Analysis was done with a Beckman 120 B amino acid analyzer that had been modified to take 12-mm flow-cells (MER Chromatographic, Mountain View, Calif., U.S.A.) and a single column (43 × 0.6 cm) of Beckman AA-15 resin with buffer flow-rate of 40 ml h^{-1}. The integrator was an Infotronics CRS 309 (International Technical Instruments, Boulder, Colo.,U.S.A.).

Isolation of the Hb variant

The erythrocytes were washed three times with normal saline and hemolyzed for 20 min at room temperature with 1.5 times their volume of water and 0.4 volume of carbon tetrachloride. After centrifugation to remove cell debris, the solution was dialyzed overnight against water at 4°.

The chromatographic separation was made on a column (22.5 × 2.5 cm) of DEAE-cellulose DE-52 (Whatman, Clifton, N.J., U.S.A.) that had been equilibrated in 0.2 *M* glycine–0.01% potassium cyanide solution to pH 7.19 as described by Schroeder *et al.*[12]. The sample was 650–800 mg of Hb in 10–14 ml of water. Each chromatogram was developed with 0.2 *M* glycine–0.015 *M* sodium chloride–0.01% potassium cyanide at a flow-rate of 80 ml h^{-1}. Under these conditions, the Hb variant moved rapidly through the column. Before it began to pass into the eluate, the chromatogram was stopped and the upper part of the column with the other Hb types was removed. The variant was eluted from the remaining column with 2% potassium cyanide solution, dialyzed and concentrated on CM-Sephadex[16] before removal of the heme with acid acetone at −20°. Under these conditions, the variant is mixed with HbA_2. A total of 480 mg of globin was available.

Tryptic hydrolysis and preparation of the sample

A 25-mg sample of the globin was dissolved in 1.9 ml of water, and the pH was adjusted to 8.8 with 0.1 *M* sodium hydroxide. After the sample volume had been adjusted to 2.0 ml, 4.0 mg of ammonium hydrogen carbonate (Mallinckrodt, St. Louis, Mo., U.S.A.) were added to give 0.025 *M* concentration, and the pH was 8.1. Following addition of 0.25 mg of trypsin (Trypsin TCPK; Worthington, Freehold, N.J., U.S.A.) in 0.125 ml of 0.001 *M* hydrochloric acid, the suspension was stirred for 4 h at 37°; when the pH (which was 8.6 at room temperature) was lowered to 2.0, all the precipitate dissolved. This solution was filtered through a 0.5-μm fluoropore filter with an inorganic pre-filter (Millipore, Bedford, Mass., U.S.A.) in a Swinney filter-holder and then freeze-dried. The 26 mg of dry material was dissolved in 0.5 ml of 0.01 *M* ammonium acetate of pH 6.07 (see below), and the solution was filtered as before. A 100-μl sample was injected into the HPLC system.

HPLC of the tryptic digest

The HPLC equipment consisted of a single Waters 6000A solvent delivery system, U6K universal injector, and column (300 × 3.9 mm) of μBondapak C_{18} (10 μm) (Waters Assoc., Milford, Mass., U.S.A.), an Altex/Hitachi Model 155-10 UV–Vis variable wavelength detector (Altex, Berkeley, Calif., U.S.A.), and a single-channel recorder (Linear Instruments Corp., Irvine, Calif., U.S.A.). The linear gradient was formed with a two-vessel system of the type described by Bock and Ling[17]; each vessel had an I.D. of 2.2 cm.

The chemicals used were ammonium acetate (Allied Chemical, Morristown, N.J., U.S.A.; B and A reagent grades), and acetonitrile (gold label 99+%, spectrophotometric grade; Aldrich, Milwaukee, Wisc., U.S.A.), which was filtered through an 0.5-μm FH organic filter (Millipore). Deionized water was filtered through an 0.45-μm HA aqueous filter (Millipore). The following solvents were made up 24 h before use. A solution of 0.01 *M* ammonium acetate was adjusted to pH 6.07 with dilute acetic acid; acetonitrile and 0.01 *M* ammonium acetate were mixed to give 40% (v/v) of acetonitrile, and the pH was adjusted to 6.07. Both solutions were filtered through 0.45-μm HA aqueous filters. De-gassing was done only to the extent that this occurred during filtering under vacuum.

The HPLC column was equilibrated with 40 ml of 0.01 *M* ammonium acetate (pH 6.07) at a flow-rate of 1.5 ml min^{-1} at room temperature. After the flow-rate had been reduced to 1 ml min^{-1}, the sample as prepared above was injected with a Precision Sampling pressure-lok liquid syringe (Series B-110). Development was made with a linear gradient of 50 ml of 0.01 *M* ammonium acetate (pH 6.07) in the mixer and 50 ml of 40% acetonitrile in 0.01 *M* ammonium acetate (pH 6.07) in the reservoir; the pressure was 1500 p.s.i., the absorbance was recorded at 220 nm at 1.0 a.u.f.s. with a chart speed of 40 cm h^{-1}, and 0.5-ml fractions were collected.

A few millilitres before the end of the gradient, 2 ml of acetonitrile were injected and passed through the system with the last of the gradient. There followed then, in sequence, 40 ml of water, a 30-ml linear gradient from water to acetonitrile and, finally, 25 ml of acetonitrile. Before the next usage, equilibration was carried out with 0.01 *M* ammonium acetate as already noted.

RESULTS

Case report

Although the basic aim of cord-blood screening at the Los Angeles Sickle Cell Center has been the detection at birth of infants with sickle cell disease[11,18], variants other than HbS or HbC are also detectable. Thus, in addition to HbF and HbA, the blood of a newborn black infant contained a third component with properties different from those of HbS or HbC. This component had the electrophoretic behavior of HbC at alkaline pH on cellulose acetate or starch gel. The chromatographic behavior was that of HbC on DEAE-cellulose[14] and approximated that of HbS on CM-cellulose[13]; this is the behavior of HbE and probably also of HbC_{Harlem}. The Hb composition at birth and at 10 weeks of age is listed in Table I. When the family (which consisted of the parents and two siblings) was examined, the variant was present in the mother and both siblings; quantitative data are in Table I.

TABLE I

HEMOGLOBIN (Hb) COMPOSITION* AND HEMATOLOGICAL DATA ON THE FAMILY

Hb type or other parameter	*Propositus*		*Father*	*Mother*	*Sib I (9 years)*	*Sib II (3 years)*
	Birth	*10 weeks*				
HbX + HbA_2 (%)	4.1	19.8	2.1	32.1	30.8	30.0
HbA (%)	12.5	43.2	86.6	59.6	61.3	60.6
HbF (%)	66.7	26.0	3.5*	2.3*	2.3*	3.6*
HbA_1 + F_1 (%)	16.7	11.0	7.8	6.0	5.6	5.8
RBC ($\times 10^{12}$ l^{-1})		4.51	5.22	4.68	5.57	4.51
Hb (g dl^{-1})		12.6	15.6	12.7	12.9	11.6
PCV(ll^{-1})		0.37	0.46	0.38	0.39	0.34
MCV (fl)		83	86	83	73	76
MCH (pg)		27.8	29.6	27.1	24.0	25.6
MCHC (g dl^{-1})		33.6	33.9	33.1	32.9	34.0

* The Hb percentages were determined by microchromatography[12]. In this method, the tail of HbA carries over into the position of HbF. These percentages of HbF are in the normal range of this method.

HPLC separation of peptides: Identity of the variant

Fig. 1 depicts the chromatographic separation of the peptides. All zones were pooled, blown dry with air, hydrolyzed, and analyzed for amino acid composition. Because each zone had either one or two peptides, it was possible to identify the

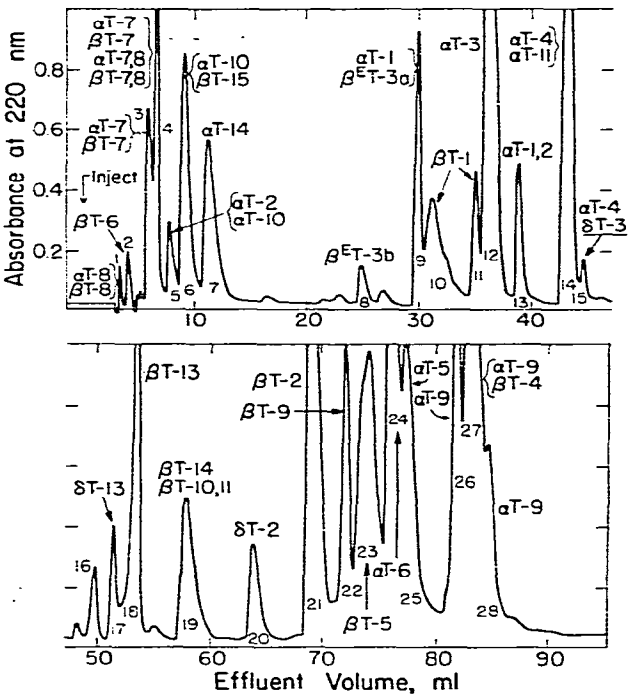

Fig. 1. Separation of the tryptic peptides of the α- and β-chains of HbE on a reversed-phase column as described in the text.

components, as the composition of all tryptic peptides from the α- and β-chains is well-known. The identity of the peptide(s) in each zone is given in Fig. 1. Thus, it was apparent from the analyses in Table II that parts of the aberrant peptide were present in Zones 8 and 9. Zone 8 had the amino acid composition of residues 27–30 of the β-chain, and Zone 9 contained αT-1 and residues 18–26 of the β-chain, with the substitution of lysine for glutamic acid. The Hb variant, therefore, is HbE.

TABLE II

AMINO ACID COMPOSITIONS OF PEPTIDES IN ZONES 8 AND 9

Amino acid	*Zone 8*			*Zone 9**				
	nmoles	*β27–30*		*nmoles**	*αT-1*		*β-18–26*	
		*Found***	*Expected***		*Found***	*Expected***	*Found***	*Expected***
Lys				60.3	1.00	1	0.94	0
Arg	41.4	0.96	1					
Asp				106.8	1.00	1	2.09	2
Ser				21.5	0.95	1		
Glu				43.6			1.08	2
Pro				20.4	0.91	1		
Gly	44.1	1.02	1	85.9			2.12	2
Ala	43.1	1.00	1	26.1	1.16	1		
Val				134.3	1.00	1	2.77	3
Leu	43.5	1.01	1	22.7	1.01	1		

* In these calculations, 22.5 nmoles of the appropriate amino acid is assumed to be part of αT-1; the remainder is associated with β18–26.

** In residues.

On this chromatogram, β^{A}T-3 would be expected between Zones 13 and 14.

DISCUSSION

Although HbE is not common in the black race, its detection in another black family is of minor consequence; this example is presented to show the power of HPLC for the separation of peptides. Parenthetically, it may be noted that the hematological data of the carriers for HbE (Table I) in this black family, with the possible exception of Sib I, agree with accepted age-related values[19,20]. In contrast, Fairbanks *et al.*[21] report microcytosis in HbE carriers from Southeast Asia and Northern Europe.

The 5-mg sample for the chromatogram is of the order commonly applied in fingerprinting methods and could be reduced if more sensitive amino acid analysis were used. If material were required for sequencing a peptide, it could be isolated by pooling the appropriate zone from a succession of chromatograms or, if the desired peptide was well separated, by increasing the load.

The size of the peak is unrelated to the quantity of material and is determined by the number of residues (hence, the absorbance related to the C=O bond) and the presence of an aromatic group. Thus, βT-15 (Tyr-His; the major peptide of Zone 6) appears to be greater in quantity than many others; likewise, Zone 20 with δT-2 is exaggerated because of the tryptophyl residue.

As mentioned, this preparation contained HbA_2. Despite the complexity of the chromatogram, three δ-chain peptides are evident in Zones 20, 15, and 17.

Peptides αT-12, αT-13, βT-10, βT-11, and βT-12 were not detected on this chromatogram, presumably because the mercapto groups were not blocked in this globin. On the other hand, all peptides were detected when a tryptic hydrolysate of an aminoethylated β-chain was chromatographed.

Because of the different mechanism of the reversed-phase column as compared with ion-exchange columns, the order of emergence of peptides is very different by the two methods. For example, peptides αT-1 and αT-11 (which are similar in length and composition) separate with some difficulty on cation-exchange columns: here they are far apart (zones 9 and 14). The reverse is also true: peptides well separated by ion-exchange chromatography may be coincident on this column. Peptides, es-

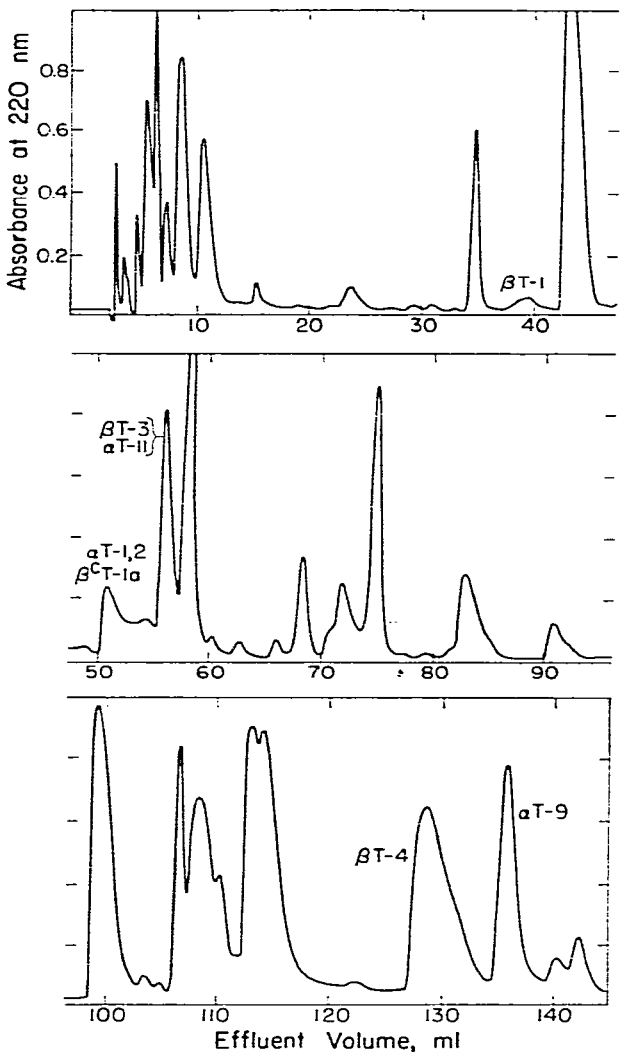

Fig. 2. Separation of the tryptic peptides of the α- and β-chains of HbC on a reversed-phase column under modified conditions as described in the text.

pecially hydrophobic ones (such as βT-4), are easily apparent in good quantity in contrast to their poor yield from ion-exchanger materials.

The reason why βT-1 is present in two zones (10 and 11) is unknown and has not been observed in other chromatograms. This peptide forms a surprisingly broad zone. In a few other instances, for reasons that are not understood, there has been some evidence that a peptide may partition between two zones.

This chromatogram, which was meant to be a pilot run in the study of this abnormal Hb, quickly provided the identification of a well-known variant. Consequently, as an example, this chromatogram by no means shows all that can be achieved by HPLC. Especially at the end of the chromatogram, the zones are close together and this development is only marginally satisfactory. By changing the slope of the gradient or by using isocratic development during part of the chromatogram, marked improvement in separation can be achieved. Fig. 2 is a chromatogram of a 24-h tryptic hydrolysate of the globin of HbC($+A_2$), which was developed with the following sequence: (i) a linear gradient initially between 50 ml of 0.01 *M* ammonium acetate at pH 6.07 in the mixer and 50 ml of 25% acetonitrile in 0.01 *M* ammonium acetate at pH 6.07 in the reservoir, (ii) 20 ml of 25% acetonitrile in 0.01 *M* ammonium acetate isocratically, and (iii) a linear gradient between 15 ml of 25% and 15 ml of 35% acetonitrile in 0.01 *M* ammonium acetate; the chromatogram required 2.5 h for completion. The positions of βT-1 (= δT-1) from the δ-chain of HbA_2, of β^CT-1a from the β-chain of HbC, and of several other peptides are marked. By comparison with Fig. 1, some separations are clearly improved (that of βT-4 from αT-9, for example), whereas, in other instances, separation may be somewhat decreased.

We are currently making a detailed examination of chromatographic conditions not only for the separation of the peptides from pairs of chains, but also of peptides from the individual chains in order to devise good conditions for separations and to learn more exactly the parameters that control them. By a study of known abnormalities, we hope to learn the influence of sequence and composition on a peptide's behaviour. Modifications such as aminoethylation or carboxymethylation of mercapto groups, or oxidation of mercapto groups and methionyl residues, may produce useful characteristics in the modified peptides. The speed with which chromatography can be accomplished and the small scale of operations means that a pilot run can be made to detect the difference in a variant Hb. With the knowledge so gained, a more sophisticated protocol of development can be devised if necessary.

ACKNOWLEDGEMENTS

We thank Colleen Warford for isolating the hemoglobins. These studies were supported in part by grants HL-02558 and HL-15162 from the National Institutes of Health, U.S. Public Health Service.

REFERENCES

1 W. S. Hancock, C. A. Bishop and M. T. W. Hearn, *FEBS Lett.*, 72 (1976) 139.
2 A. N. Radhakrishnan, S. Stein, A. Licht, K. A. Gruber and S. Udenfriend, *J. Chromatogr.*, 132 (1977) 552.

3 J. J. Hansen, T. Greibrokk, B. L. Currie, K. N.-G. Johansson and K. Folkers, *J. Chromatogr.*, 135 (1977) 155.
4 G. W.-K. Fong and E. Grushka, *J. Chromatogr.*, 142 (1977) 299.
5 I. Molnar and C. Horvath, *J. Chromatogr.*, 142 (1977) 623.
6 E. Lundanes and T. Greibrokk, *J. Chromatogr.*, 149 (1978) 241.
7 W. S. Hancock, C. A. Bishop, R. L. Prestidge, D. R. K. Harding and M. T. W. Hearn, *J. Chromatogr.*, 153 (1978) 391.
8 C. E. Dunlap, III, S. Gentleman and L. I. Lowney, *J. Chromatogr.*, 160 (1978) 191.
9 W. S. Hancock, C. A. Bishop, L. J. Meyer, D. R. K. Harding and M. T. W. Hearn, *J. Chromatogr.*, 161 (1978) 291.
10 G. D. Efremov, J. B. Wilson and T. H. J. Huisman, *Biochim. Biophys. Acta*, in press.
11 W. A. Schroeder, T. H. J. Huisman, D. Powars, L. Evans, E. C. Abraham and H. Lam, *J. Lab. Clin. Med.*, 86 (1975) 528.
12 W. A. Schroeder, L. A. Pace and T. H. J. Huisman, *J. Chromatogr.*, 145 (1978) 203.
13 W. A. Schroeder, L. A. Pace and T. H. J. Huisman, *J. Chromatogr.*, 118 (1976) 295.
14 E. C. Abraham, A. Reese, M. Stallings and T. H. J. Huisman, *Hemoglobin*, 1 (1976) 27.
15 T. H. J. Huisman and J. H. P. Jonxis, *The Hemoglobinopathies: Techniques of Identification*, Marcel Dekker, New York, 1977, p. 80.
16 W. A. Schroeder, T. H. J. Huisman, J. R. Shelton and J. B. Wilson, *Anal. Biochem.*, 35 (1970) 235.
17 R. M. Bock and N.-S. Ling, *Anal. Chem.*, 26 (1954) 1543.
18 D. Powars, W. A. Schroeder and L. White, *Pediatrics*, 55 (1975) 630.
19 M. M. Wintrobe, G. R. Lee, D. R. Boggs, T. C. Bithell, J. W. Athens and J. Foerster, *Clinical Hematology*, Lea and Febiger, Philadelphia, Pa., 7th ed., 1974, p. 1797.
20 J. Hows, S. Hussein, A. V. Hoffbrand and S. N. Wickramasinghe, *J. Clin. Pathol.*, 30 (1977) 181.
21 V. F. Fairbanks, G. S. Gilchrist, B. Brimhall, J. A. Jareb and E. C. Goldston, *Blood*, 53 (1979) 109.