Vol. 89, No. 3, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 13, 1979 Pages 851-858

RAPID SEPARATION OF THE α,β, $^{G}\gamma$ and $^{A}\gamma$ HUMAN GLOBIN CHAINS BY REVERSED-PHASE HIGH PRESSURE LIQUID CHROMATOGRAPHY*

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Received June 22,1979

SUMMARY

The main human globin chains present in cord blood hemoglobins, α,β,$^{G}\gamma$ and $^{A}\gamma$, can be separated in 45 minutes by reversed-phase high pressure liquid chromatography. The chains were identified by carboxymethyl cellulose chromatography and partial amino acid analysis of the cyanogen bromide fragments of the two γ chains. The purification of cyanogen bromide fragments and the separation and quantitation of their dansylated amino acids were accomplished using a similar system to that used for the separation of the globin chains. These results show the potential of this type of chromatography for the analytical and semi-preparative analysis of globin chains and the large advantages over conventional chromatographic techniques.

INTRODUCTION

At present the best method for the detection of hemoglobinopathies involving globin chain synthesis is the separation and quantitation of the labeled globin chains on carboxymethyl-cellulose columns (CMC) in the presence of urea (1,2). This method is particularly important in the instances where globin chain synthesis has to be analyzed in blood samples with low reticulo-

Abbreviations: CNBr-3, Cyanogen bromide fragment 3; CMC, carboxymethyl cellulose; HPLC, high pressure liquid chromatography; TFA, trifluoroacetic acid

* Supported by Grant MA-6072 of the Medical Research Council of Canada (MRC-C) and also in part by grants MT-1658 of the MRC-C and grant HDO-4365 of the U.S. Public Health Service. L.F. Congote is a Scholar of the MRC-C.

0006-291X/79/150851-08$01.00/0

cyte counts. These samples are mixtures of large amounts of unlabeled hemoglobins and very small amounts of labeled globins and therefore their analysis requires the separation of milligram amounts of globin chains. Unfortunately, the CMC does not separate the two γ chains $^{G}\gamma$ and $^{A}\gamma$. The measurement of the ratio $^{G}\gamma/^{A}\gamma$ requires the use of a time consuming and complex methodology involving the amino acid analysis of cyanogen bromide fragments (2). These $^{G}\gamma/^{A}\gamma$ ratios are important for the classification of various inherited hemoglobinopathies (3). Very recently it has become possible to separate and quantitate the $^{G}\gamma$ and $^{A}\gamma$ chains at the microgram level by isoelectric focusing in the presence of Nodinet-40 (4). In this communication we present a rapid method which can be applied at both the microgram and milligram level and which represents a valuable alternative to the conventional CMC methodology for the separation and analysis of globin chains.

METHODS

Blood samples were collected in heparinized tubes, and after removal of plasma the cells were washed three times in Krebs-Ringer phosphate buffer (1). For experiments involving protein synthesis the cells were incubated with 200μCi [^{3}H]leucine (58-105 Ci/mmol, Amersham) in 0.5 ml of the incubation mixture described by Alter et al (1) which contains 20 nmoles of 19 amino acids per ml with the exception of leucine. In some experiments the cells were labeled with 1mCi [^{3}H] amino acid mixture from New England Nuclear (NET 250, specific activities 2 to 100 Ci/mmol) using the same system as above, but only with 7 nmoles of all the non-labeled amino acids. The globin chains were precipitated out of red cell lysates with acidified acetone (1) and dissolved in 0.5% trifluoroacetic acid (TFA) using a concentration of 2 mg protein/ml just prior to chromatography.

The apparatus utilized for high pressure liquid chromatography (HPLC) consisted of a Milton Roy Mini-pump Model 196-0066-001 connected to a pressure gauge and two columns. The first column was a guard or precolumn filled with glass beads which had chemically bounded octadecyl (C_{18}) groups (Whatman No. 6561-403). The analytical column was a μBONDAPAK C_{18} (30 cm x 4.6 mm) from

Waters Associates. The proteins eluted from the column were detected with a Perkin Elmer model LC-55 Spectrophotometer set at 280 nm and fractions of 0.7 - 0.8 ml were collected. In radioactive samples the fractions were eluted and were mixed with Instagel (Packard) and counted. The gradient mixer used was a simple device consisting of two laboratory glass beakers of 30 ml and 50 ml (33 and 40 mm i.d., respectively) inter-connected with an inverted U-shaped glass tube of about 2-3 mm diameter. The large beaker was the mixing vessel and had a small magnetic stirrer. The column was made up in acetonitrile (HPLC grade, Fisher) and was equilibrated for at least 15 minutes at a flow rate of 1.5 ml/min with solution A which is a mixture of 45% (v/v) acetonitrile and 0.5% (v/v) TFA in water. The water used in these studies was deionized, distilled and filtered through 0.22μ filters. Then a solution of 0.05 to 4 mg. globin chains prepared as above was applied to the column (the volume of the sample is irrelevant) and the chains were eluted at a rate of 1.5 ml/min with a gradient consisting of 40 ml solution B in the 50 ml Beaker and 28 ml solution C in the 30 ml Beaker. Solution B was a mixture of 45% (v/v) acetonitrile and 0.3% (v/v) TFA in water and solution C consisted of 50% (v/v) acetonitrile in water. After elution the column is readily regenerated by passing through it 80% (v/v) acetonitrile in water for 5-10 min and then solution A for 15 min.

Globin chains were analyzed on CMC columns as described by Alter et al (1). Chains containing radioactivity eluted from the HPLC were evaporated over nitrogen or lyophilized, mixed with adult and fetal globin chain markers and applied to the CMC columns. The chains γ_1 and γ_2 were treated with cyanogen bromide and the CNBr-3 fragment was located by mobility on Sephadex-G50 (2) and purified on HPLC as indicated above, but using a linear gradient at a flow rate of 1.5 ml/min consisting of 30 ml of 0.1% (v/v) TFA in H_2O and 30 ml of a mixture of 45% (v/v) acetonitrile and 0.1% (v/v) TFA both in water. The retention time of CNBr-3 in this system is approximately 18 minutes. The CNBr-3 fragments eluted were taken to dryness, hydrolyzed in 6N HCl and dansylated by the method of Gray (5). The dansylated amino acids were separated using the same HPLC column and a modification of the method described by Wilkinson (6) which allows the isocratic elution of the dansylated derivatives of histidine, arginine, serine, glycine, threonine and alanine (Bennett, Browne, Solomon, in preparation). These are 9 of the 13 amino acids present in CNBr-3 (2).

RESULTS AND DISCUSSION

Fig. 1 shows the separation of human cord blood globin chains by HPLC. In this particular chromatographic run 300μg of globin chains were placed on the column and this has been proven to be a convenient amount of sample to use for the prenatal diagnosis of

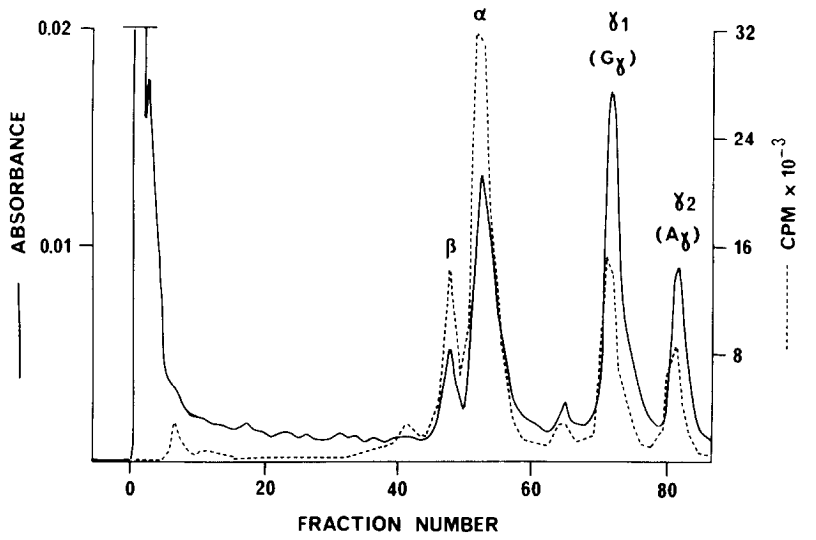

Fig. 1. High Pressure Liquid Chromatography (HPLC) of 300 µg globin chains from cord blood labeled with [^{3}H]leucine as indicated in the methods. There is a delay of 20 seconds between the absorbance at 280 nm (0.6 cm path length) and the fraction collector.

thalassemias (Congote et al, in preparation). The four chains can be readily separated with column loadings of 1 mg but in amounts above 4 mg the separation of α and β chains is incomplete while there is still a good resolution of the two γ chains. The gradient described here for the separation of globin chains combines a decreasing amount of TFA (0.3% to 0) and an increasing concentration of acetonitrile (45% to 50%). The TFA gradient is necessary for the separation of the α and β chains, whereas the acetonitrile gradient separates the α, $^{G}\gamma$ and $^{A}\gamma$ chains. Further experiments have shown that the chains α, β and β^{S} can be separated using solution B in an isocratic mode (results not shown) but the resolution was not as good as that obtained on CMC columns and the peaks were not as well separated as those in the gradient described in Fig. 1. A comparison of Figs. 1 and 2 indicates the

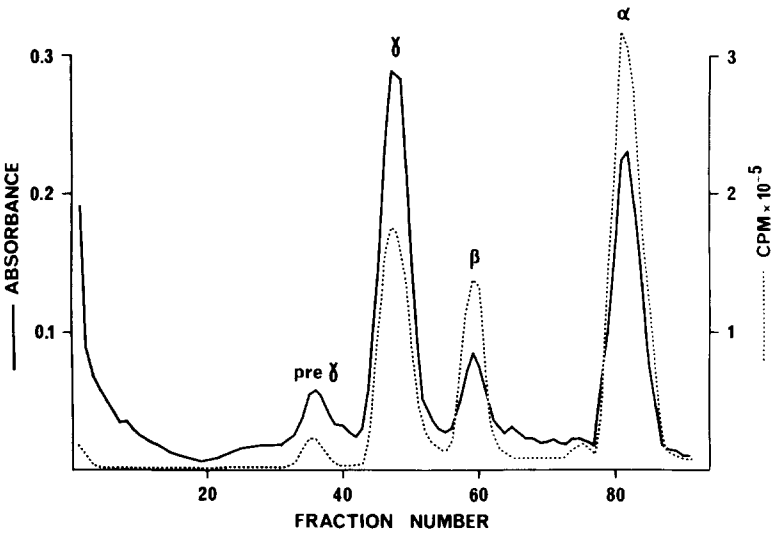

Fig. 2. Carboxymethyl cellulose (CMC) chromatography of 20 mg of the same sample of cord blood shown in Fig. 1. The absorbance was measured at 280 nm and 1/5 aliquots (1 ml) of the fractions were used for counting.

main differences between the two methods described. The relative amounts of globins are almost the same, but the HPLC has the advantage of giving sharper peaks in 1/10 of the time and of separating the 2 γ chains. In order to identify each of the HPLC peaks as observed on classical CMC columns, the labeled globins were analyzed together with α,β and γ chains added as markers (Fig. 3). The peaks correspond very well with the added markers. In the example shown in Fig. 3c a portion of the γ_1 chains was eluted in the pre-γ region of CMC columns. The acetylated γ chain of the fetal hemoglobin F_1 usually emerges in this region (Congote et al, in preparation), but the difference in elution of γ_1 and γ_2 on HPLC is not due to acetylation because the γ chains of hemoglobin F_1 elute in both the γ_1 and γ_2 peaks (results not shown). For this reason we decided to analyze for the other possible identity

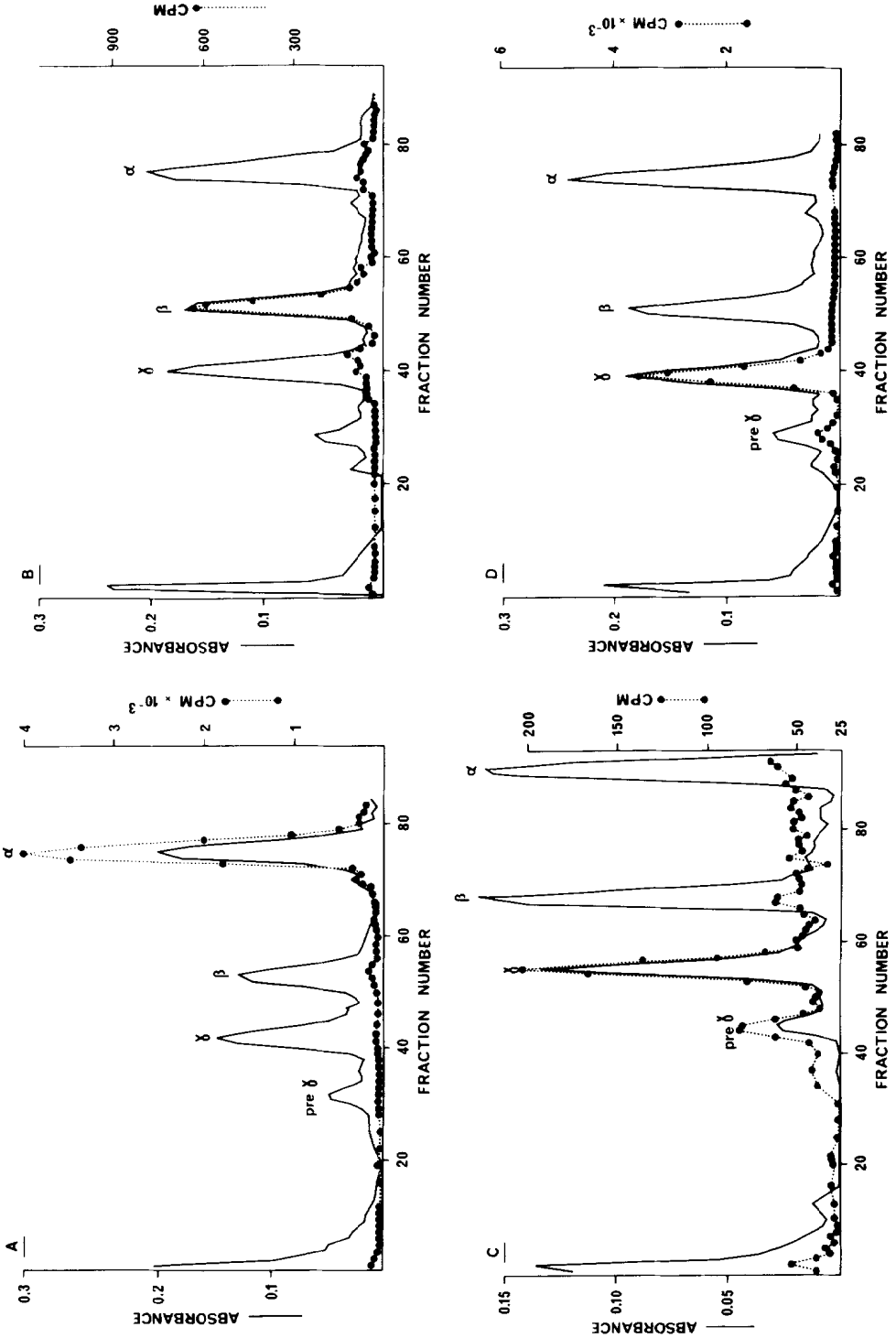

Fig. 3. Analysis of radioactive chains separated on CMC columns which had previously been purified on HPLC. The absorbance at 280 nm indicates the positions of the α, β and γ chains added as markers. The separate chromatograms A,B,C, and D correspond to re-chromatography of the α, β, γ_1 and γ_2 chains from Fig. 1.

TABLE 1

Quantitation of the dansylated amino acids Arg, Ser, Gly, Thr, and Ala from the CNBr-3 fragments of the chains γ_1 and γ_2 after separation by HPLC

	Residues per CNBr-3*			
Dansyl derivatives of	γ_1	Expected Values for $^{G}\gamma$	γ_2	Expected Values for $^{A}\gamma$
Arg	0.84	1	1.07	1
Ser	2.80	3	2.74	3
Gly	1.60	1	0.46	0
Thr	0.49	1	0.49	1
Ala	2.31	2	3.23	3

*The amounts of the single amino acids were calculated from the areas of the peaks. The area corresponding to a single amino acid in the CNBr-3 was taken as the total area of Arg, Ser, Gly, Thr and Ala divided by 8. Both CNBr-3 (γ_1 and γ_2) may be contaminated with a small amount of another peptide because of the presence of small amounts of Asp (0.27/CNBr-3, γ_1; 0.51/CNBr-3 γ_2) and Glu (0.26/CNBr-3, γ_1; 0.42/CNBr-3, γ_2). This may also explain the slightly high values of Gly in both γ_1 and γ_2.

of the two chains, namely their relation to the $^{G}\gamma$ and $^{A}\gamma$ chains. The chains γ_1 and γ_2 were treated with cyanogen bromide. The CNBr-3 fragments were purified on HPLC as described in the methods, then hydrolyzed, dansylated and the dansylated amino acids of Arg, Ser, Gly, Thr and Ala were quantitated after elution from HPLC columns (Table 1). Histidine was not included in the calculations because of the formation of mono- and di-dansylated products. Table 1 shows that the chains $^{G}\gamma$ correspond most closely to the γ_1 chain and that $^{A}\gamma$ corresponds to the γ_2 chain.

These results were also confirmed after analysis of globin chains labeled with an amino acid mixture. The ratios of the labeled alanine of the CNBr-3 γ_2/γ_1 was 1.42 (theoretical value for $^{A}\gamma/^{G}\gamma = 1.5$) and the labeled glycine of CNBr-3 γ_2/γ_1 was 0.21 (theoretical value for $^{A}\gamma/^{G}\gamma = 0$).

A potential application of these methods is for analysis of globin chain synthesis and we are now successfully applying it in the analysis of Fetoscopy samples (Congote et al, in preparation). In addition, the method has considerable versatility because with very simple equipment it is possible to do peptide analysis and even amino acid analysis of globin chains, normally a very time consuming process requiring very expensive equipment.

ACKNOWLEDGEMENTS

We are grateful to Dr. C.A. Browne for his very helpful advice during this investigation and to Ms J. Charbonneau and M.T. Sarophim for their help in some of the experiments.

REFERENCES

1. Alter, B.P., Modell, C.B., Fairweather, D., Hobbins, J.C., Mohoney, M.J., Frigoletto, F.D., Sherman, A.S. and Nathan, D.G. (1976) New England J. Med. 295, 1437-1443.
2. Huisman, T.H.J., and Jonxis J.H.P. (1977) The Hemoglobinopathies, Techniques of Identification, Marcel Dekker, Inc., New York.
3. Huisman, T.H.J., Schroëder, W.A., Efremor, G.D., Duma, H., Mladenovski, B., Hyman, C.B., Rachmilewitz, E.A., Bouver, N., Miller, A., Brodie, A.R., Shelton, J.R. and Appel, G. (1974) Ann. N.Y. Acad. Sci 232, 107-124.
4. Comi, P., Giglioni, B., Ottolenghi, S., Gianni, A.M., Ricco, G., Mazza, U., Saglio, G., Camaschella, C., Pich, P.G., Gianazza, E., and Righetti, P.G. (1979) Biochem. Biophys. Res. Commun. 87, 1-8.
5. Wilkinson, J.M. (1978) J. Chromatogr. Sci. 16, 547-552.
6. Gray, W.R. (1972), Methods Enzymol. 25B, 121-137.