Journal of Chromatography, 228 (1982) 298—304
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1139

Note

Rapid separation of human globin chains by high flow-rate chromatography under low pressure using CM-Trisacryl M

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(First received August 6th, 1981; revised manuscript received October 16th, 1981)

The separation of globin chains is often necessary for the characterization of abnormal hemoglobins or of abnormal synthesis of hemoglobin. Usually the separation is achieved by chromatography on carboxymethyl cellulose according to the method of Clegg et al. [1]. Recently, the separation of hemoglobin chains on CM-Sepharose CL-6B has been described. The major advantage of this method is the possibility to use the columns repeatedly [2]. High-performance liquid chromatography on bonded silica has also been used recently, with the advantage of rapidity and with the possibility of separating chains with minor structural differences like the three types of γ chains, a separation which is not possible by ion exchange [3—5].

In this paper we describe an ion-exchange technique using a new cation exchanger, CM-Trisacryl M. This medium is a hydrophilic acrylic copolymer with carboxylic groups. Chain separation is possible on a preparative scale, with good resolution, and within less than 3 h.

MATERIALS AND METHODS

CM-Trisacryl M (bead diameter 40—80 μm) was purchased from Pharm-industrie (Gennevilliers, France) and Sephadex G-25 from Pharmacia (Uppsala, Sweden). All chemicals were reagent-grade products from Merck (Darmstadt, G.F.R.), with the exception of ethanolamine (puriss) which was obtained from Fluka (Buchs, Switzerland).

A Gilson minipuls peristaltic pump and an electronic gradient maker from Gilson Medical Electronics (Villiers le Bel, France) were used throughout this study. We have also occasionally used a simple glass gradient mixer made of two identical cylindrical vessels for the preparation of linear gradients.

0378-4347/82/0000—0000/$02.75

The chromatography columns were either from Wright Scientific Ltd. (Poole, Great Britain), or made in the laboratory. The absorbance of the column effluent was continuously monitored by a Gilson spectrochrom F analyzer connected to a recorder (Linear Instruments, Chicago, IL, U.S.A.). The pump, the column and the effluent analyzer were connected with PTFE tubing using common 1/4"-28 thread fittings purchased from Gilson. The back pressure induced by the column was measured with a Bourdon tube stainless-steel manometer.

Globin samples

The globin samples were obtained by acid—acetone precipitation [6] and lyophilization of either purified Hb-A, or of crude hemoglobin solutions prepared from cord blood or from a patient with sickle cell disease.

Buffers

The starting buffer contained 8 *M* urea, 0.05 *M* β-mercaptoethanol, either 0.01 *M* or 0.04 *M* ethanolamine (see legends of figures), and was adjusted to pH 6.80 with phosphoric acid; the limiting buffer was the same as the starting buffer but with 0.1 *M* ethanolamine.

Packing of the column

CM-Trisacryl M was suspended in an equal volume of limiting buffer (without urea), degassed under vacuum, and packed by gravity in a glass chromatography column (internal diameter 2.2 cm, height 25 cm or 12 cm). The column was washed with several volumes of limiting buffer (without urea), at a flow-rate of 120 ml/h. The dead space above the gel was reduced to a minimum by adding sufficient CM-Trisacryl M to fill the column completely.

Chromatography

Before chromatography, the column was rinsed with starting buffer, at a flow-rate of 120 ml/h, for 30 min. The globin sample (usually 60 mg) dissolved in starting buffer was pumped on top of the gel, and the gradient maker, the absorbance recorder and the fraction collector were started.

The separations were carried out at various flow-rates, ranging from 60 to 240 ml/h. The shapes of the gradients are described in the legends of the figures. At the end of the chromatography, the column was either equilibrated with starting buffer if another chromatogram was to be performed on the same or on the following day, or rinsed with several volumes of limiting buffer without urea and stored for further use.

Recovery of globin chains after chromatography

The fractions containing separated globin chains were pooled together as shown by the horizontal double arrows in Figs. 1—3. They were desalted by gel filtration through Sephadex G-25 columns (50 cm × 3.2) equilibrated with 0.5% formic acid, and lyophilized.

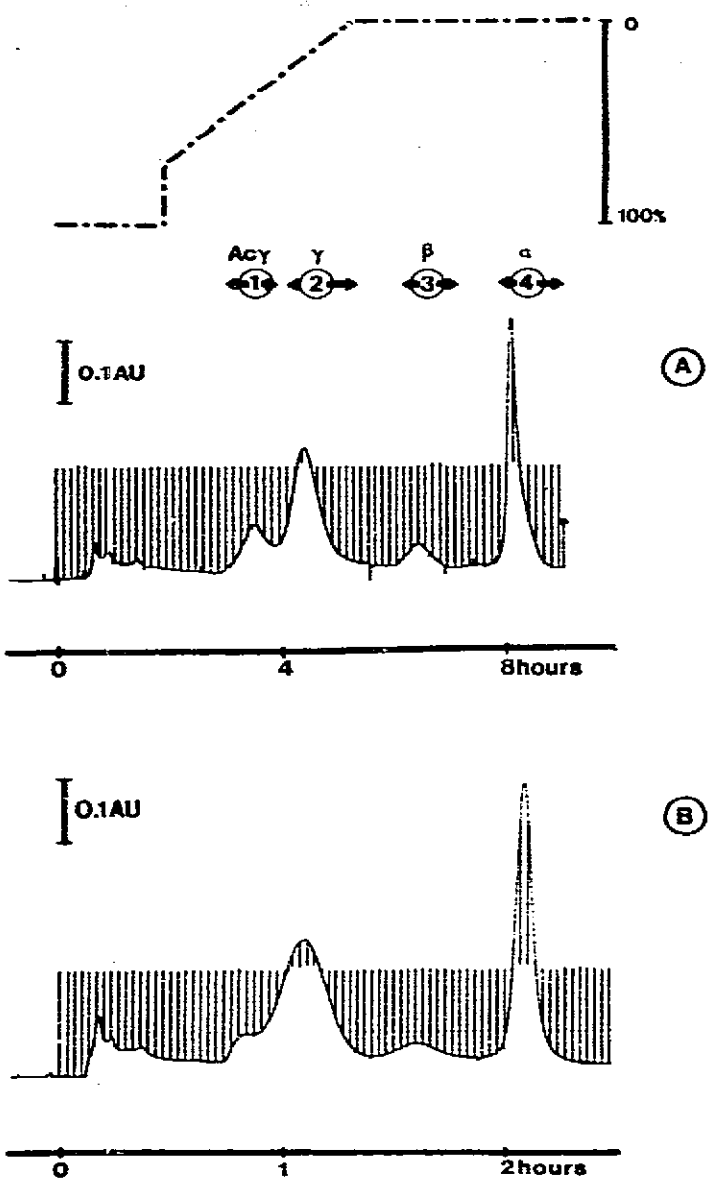

Fig. 1. Separation on CM-Trisacryl M of chains of whole globin prepared from cord blood. Photograph of the automatically recorded absorbance profile. Aliquots of 60 mg were loaded on the column (length 25 cm). The flow-rates were 60 ml/h (A) and 240 ml/h (B). The chart paper speeds were 1 cm/h (A) and 4 cm/h (B). The fractions were collected every 8 min (A), or 2 min (B) (the vertical lines intersecting the recorder tracing are made by the event marker of the collector). The composition of the buffer pumped on the column after loading the sample (depicted by the dotted curve), was as follows: 100% of initial buffer (0.01 *M* ethanolamine) for 120 min (A) or 30 min (B) followed by a step down to 70%, a linear variation from 70% to 0% in 4 h (A), or in 1 h (B), and pure final buffer.

RESULTS AND DISCUSSION

Representative chromatograms are shown in Figs. 1—3. The peaks were identified and the homogeneity of the pooled fractions was checked by cellulose acetate electrophoresis [7] (see Fig. 4).

A satisfactory resolution of the globin components of cord blood is shown in Fig. 1A. Peaks 1—4 were identified as acetyl γ, γ, β and α chains, respectively. Fig. 1B shows that a four-fold increase in flow-rate does not change the resolution appreciably, thus allowing an adequate separation of cord blood globin chains in less than 2.5 h. Fig. 2 shows that by using high flow-rates, it is likewise possible to separate β, β_S, and α chains in less than 2.5 h. The fractions, pooled as indicated in Fig. 2, were shown to be homogeneous and to contain, in order of increasing elution volumes, normal β, β_S and α chains. Fig. 3 shows that, in the case of easier separations like the separation of α and β chains from purified Hb-A, the time required to separate the two components completely can be shortened to 60 min. It has to be stressed, however, that with the gradient system described in Fig. 3 no adequate resolution can be obtained either of globin components of cord blood or of β_A and β_S.

The present method is faster than the conventional carboxymethyl-cellulose chromatography [1] and the recently described procedure using CM Sepharose CL-6B [2]. This is due to the high flow-rates, which cannot be used in carboxy-

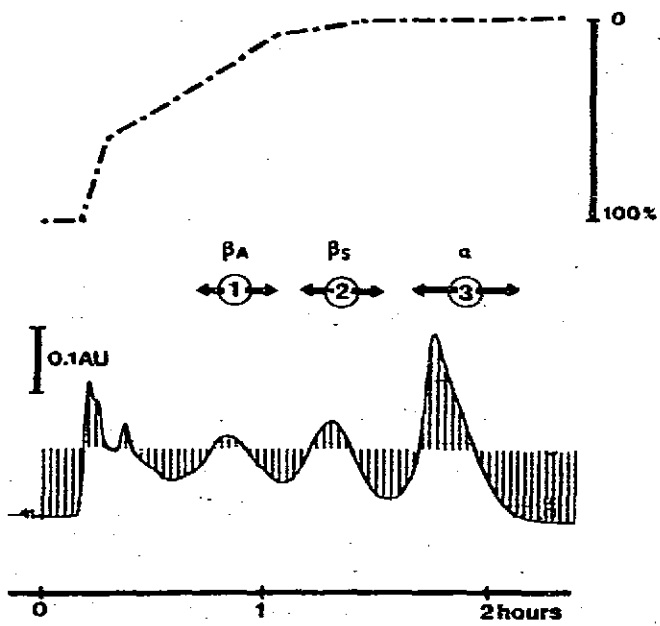

Fig. 2. Separation on CM-Trisacryl M of chains of whole globin obtained from a patient with sickle cell disease. An aliquot of 60 mg was loaded on the column (length 25 cm). The flow-rate was 240 ml/h. The chart paper speed was 4 cm/h. The fractions were collected every 2 min. The composition of the developer pumped on the column after loading the sample (depicted by the dotted curve) was as follows: 100% of initial buffer (0.04 *M* ethanolamine) for 12 min, followed by linear variations from 100% to 57% in 6 min, from 57% to 5% in 45 min and from 5% to 0% in 24 min, ending with pure final buffer.

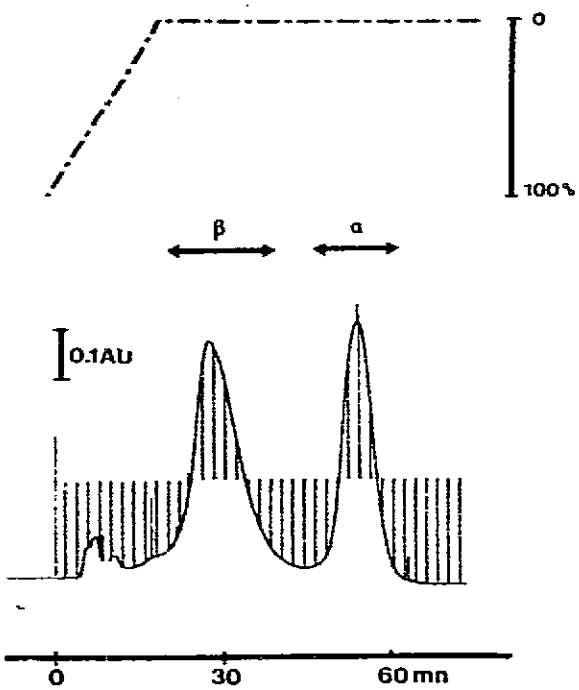

Fig. 3. Separation on CM-Trisacryl M of chains of globin prepared from purified Hb-A. An aliquot of 60 mg was loaded on the column (length 12 cm). The flow-rate was 240 ml/h. The chart paper speed was 6 cm/h. The fractions were collected every 2 min. The composition of the buffer pumped on the column varied linearly from 100% of initial buffer (0.01 *M* ethanolamine) to 0% in 15 min, followed by pure limiting buffer (see the dotted curve).

methyl-cellulose chromatography because of the rapid clogging of the column. The pressure induced by the high flow-rate was never higher than 1.8 bar, thus well below the safe limit of the 3 bars recommended by the manufacturer. A chromatographic separation of globin chains faster than the present method can be obtained only by reversed-phase chromatography, but in the latter case only small amounts of chains can be loaded on the columns at once [3—5]. We have chosen ethanolamine-containing buffers, instead of the usual sodium phosphate buffers, in order to introduce a scavenger of cyanate ions, which are potentially present because of the isomeric transformation of urea into ammonium cyanate [8]. Stark et al. [9] have shown that the cyanate present in aqueous urea can lead to covalent modifications of the proteins in solution. This elegant way to solve the potential problem by adding a scavenger of cyanate in aqueous urea has been described by Henschen and Edman [10], who used it for the preparative separation of human fibrinogen chains. The CM-Trisacryl M columns have been used repeatedly. We think that the main advantage of the repeated use of the columns is not to save money but rather to help in making chain separation faster: one does not have to pack a new column before each chromatographic run.

It should be noticed that a rapid separation of hemoglobin chains is particularly important when a large number of samples are to be processed as in the case of antenatal diagnosis of thalassemia or sickle cell disease.

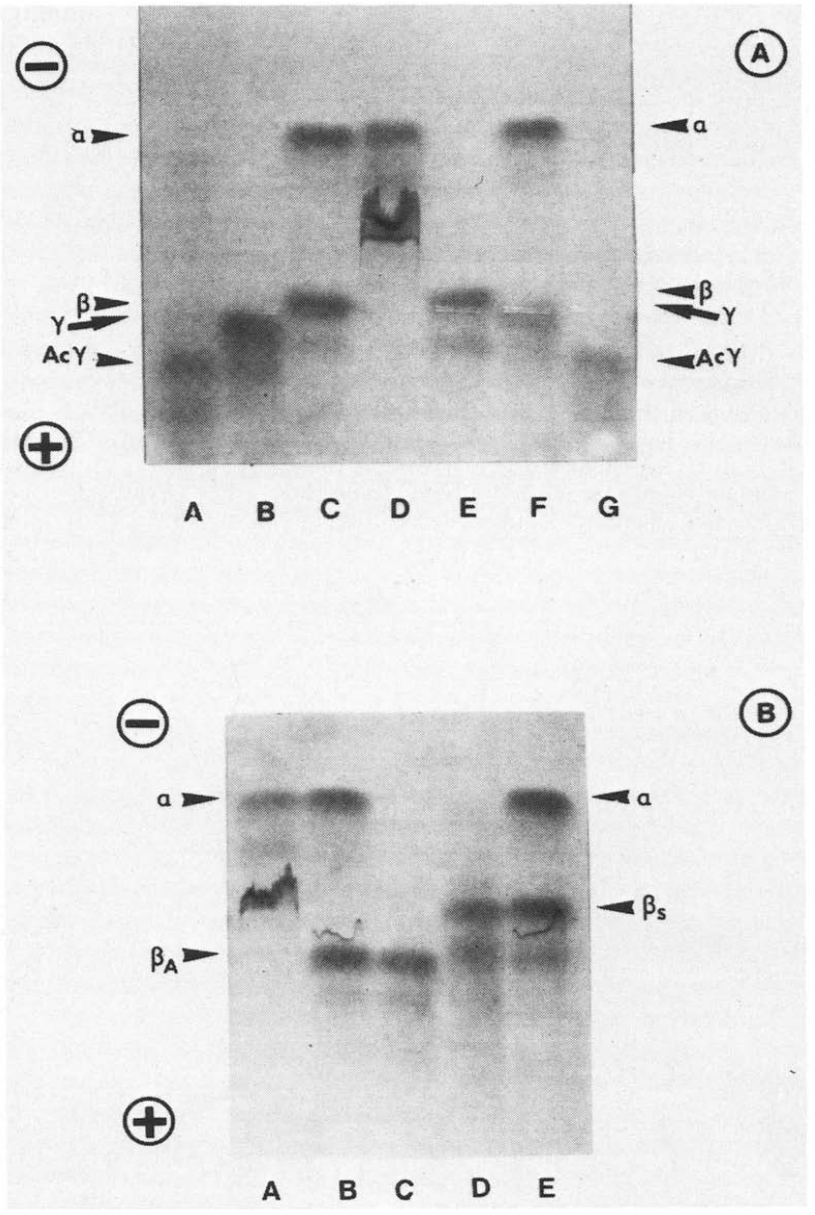

Fig. 4. Cellulose-acetate electrophoresis [7] of separated chains. (A) Chains separated in the chromatographic run shown in Fig. 1A. Lanes A and G = peak 1, labe B = peak 2, lane D = peak 4, lane E = peak 3, in lanes C and F were deposited globins of purified Hb-A and purified Hb-F, respectively. (B) Chains separated in the run shown in Fig. 2. Lane A = peak 3, lane C = peak 1, lane D = peak 2, in lanes B and F were deposited globins of purified Hb-A and purified Hb-s, respectively.

ACKNOWLEDGEMENTS

We wish to thank R. Kernemp (INSERM U129, Paris) for skillfully making the glass parts used in this study. We are also indebted to the Paris VII University (UER Xavier Bichat) for financial support.

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