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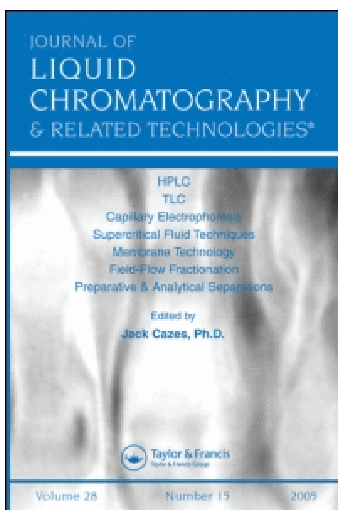

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
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To cite this Article Shelton, Joan B. , Shelton, J. Roger and Schroeder, W. A.(1984) 'High Performance Liquid Cbromatographic Separation of Globin Chains on a Large-Pore C_4 Column', Journal of Liquid Chromatography & Related Technologies, 7: 10, 1969 — 1977

To link to this Article: DOI: 10.1080/01483918408068849
URL: http://dx.doi.org/10.1080/01483918408068849

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JOURNAL OF LIQUID CHROMATOGRAPHY, 7(10), 1969-1977 (1984)

HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF GLOBIN CHAINS ON A LARGE-PORE C_4 COLUMN

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ABSTRACT

Excellent resolution of human and baboon globin chains may be obtained by HPLC on a Vydac large-pore C_4 column. The procedure is rapid and uses a gradient between aqueous trifluoroacetic acid and trifluoroacetic acid in acetonitrile. The common human γ chains are easily separable from each other as are some α- and β-chain variants from the normal chains and from each other.

INTRODUCTION

Because only a small sample of hemoglobin is necessary to separate globin chains rapidly by HPLC, this methodology affords an effective and sensitive way of examining hemoglobin samples for abnormalities. Three HPLC procedures which have been described for the separation of globin chains use a Waters μBondapak C_{18} column but differ in the eluting solvents and gradients. Congote and Kendall (1) develop with a gradient between TFA-water-acetonitrile and water-acetonitrile. Huisman *et al*. (2) employ initially an isocratic development with a phosphate-methanol-acetonitrile mixture and complete the chromatogram with a gradient between two solutions with different ratios of the compounds. Shelton *et al*. (3) use a gradient between different mixtures of perchlorate solution, methanol, acetonitrile, phosphoric acid, and nonylamine. Although the procedures differ markedly in the time

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required for completion of the chromatogram, they all succeed in separating globin chains with very small differences in sequence. Thus, the $^{G}\gamma$ and $^{A}\gamma$ chains which differ only by a methylene group are separable. The above references are those of the most recent description of methodology by the three groups and cite prior publications. Shelton et al. (4) have also reported experiments with packings other than that in a Waters μBondapak C_{18} column.

Recently, large pore HPLC columns with shorter hydrocarbon chains have become available commercially. The use of such a column and its advantages in separating globin chains are reported here. An application of these methods to the separation of baboon globin chains has been described (5).

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MATERIALS AND METHODS

Equipment consisted of an Altex system previously described (4) or one with two Waters 6000 A solvent delivery systems, a Waters U6K universal injector, an Altex/Hitachi Model 155-10 UV-Vis Variable Wavelength Detector, an Axxiom Model 711 HPLC System Controller (Cole Scientific, Calabasas, CA), and a Watanabe Model SR6252 Single Pen Chart Recorder.

The chromatographic column which is manufactured by The Separations Group, Hesperia, CA 92345 was a Vydac large-pore (330 Å) C_4 column (Cat. #214TP54) (4.6 x 250 mm).

The developers for the chromatograms were linear gradients between mixtures of 0.1% aqueous TFA and 0.1% TFA in acetonitrile. Mixture A had 80% of aqueous TFA and Mixture B had 40%. When the Waters system was employed, the developers were degassed with helium. Mixtures may be prepared and used over a period of several days to a week.

Hemoglobin solutions were prepared as previously described (4) or were samples that had been isolated by chromatography. If it was necessary to concentrate a solution after isolation, this was done with Millipore Immersible CX Filtration Units (Millipore Corp., Bedford, MA), and the concentrated solution was dialyzed salt free.

The sample size was 0.1 to 0.2 mg. All chromatograms were made at ambient temperature. Specific conditions for development are given in the text or figure legends. Purging is not necessary after each run; after return in 2 min to the desired percentage of B for the start of the next gradient, reequilibration is done with 25 ml. At the end of 5 or 6 runs, purging is done with a 2-min gradient to 100% B and 15 min of isocratic development with 100% B. The column was stored in 100% acetonitrile.

RESULTS

Figure 1 depicts the separation of the globin chains of a normal adult and Fig. 2 that of chains from a newborn infant. On this packing, the peaks are sharp and almost symmetrical. The α peak always shows greater asymmetry than β or γ peaks and sometimes has an inflection on the trailing edge.

Not only are the major chains well separated but, in each instance, a number of minor peaks are evident. These minor peaks are common to all samples of the same type and presumably represent the chains in the minor components that may be observed on all chromatograms of hemolysates on ion exchange columns; their identity is under investigation. Although evidence of such minor peaks may be seen in chromatograms by other procedures (1,2,3), they are more obvious here because of the significantly sharper peaks.

The separation of the three γ peaks is excellent. This sample may be atypical in that, contrary to previous experience (3,6), the percentage of $^{A}\gamma^{T}$ chain is greater than that of the $^{A}\gamma^{I}$ chain. The relationship remains the same even if the minor peaks after the $^{G}\gamma$ and $^{A}\gamma$ peaks are included in the calculations, although the percentages change slightly. Presumably such a minor peak after the $^{A}\gamma^{T}$ chain is under the leading edge of the $^{G}\gamma$ peak.

It is not necessary to purge the column after each chromatogram as is done in other methods (1,2,3). The small

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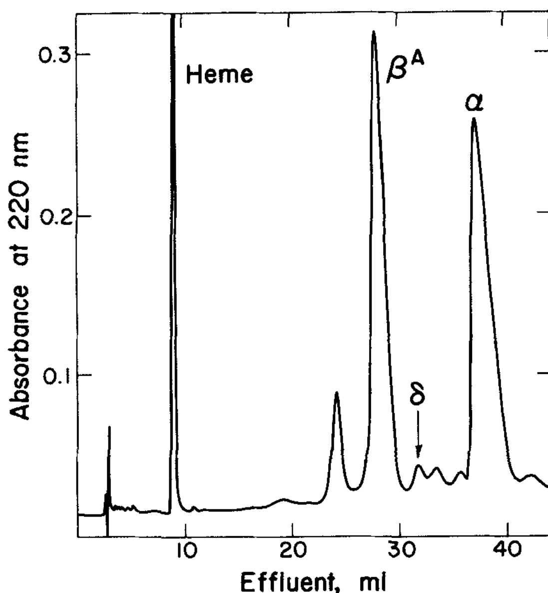

FIGURE 1 Separation of the globin chains of a normal adult. The sample was 0.12 mg, the gradient from 44 to 56.5% of Mixture B in 60 min, and the flow rate 1 ml/min.

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amount of material that is labeled "purge" in Fig. 2 came from five chromatograms and amounted only to 2-3% of the total that was injected. In some experiments in which the material in a peak was isolated for amino acid analysis, approximately quantitative data indicated a recovery of at least 85%.

The procedure also is able to separate other globin chains as depicted in Fig. 3. The δ chain is well separated from the β^A chain (Fig. 3a) and in a position far from β^C, $\beta^{O\text{-Arab}}$, β^E, and probably other β chains which as hemoglobins may not be separable from Hb A_2 on ion exchangers. Thus, Hb A_2 can now be determined chromatographically in the presence of Hb E, and not only by radioimmuneassay (7). Of the examples shown, only β^A and β^S chains do not separate well (nor, or course, δ and β^S), but the difference is adequate to distinguish them. Numerous modifications of procedure did not improve the β^A-β^S separation.

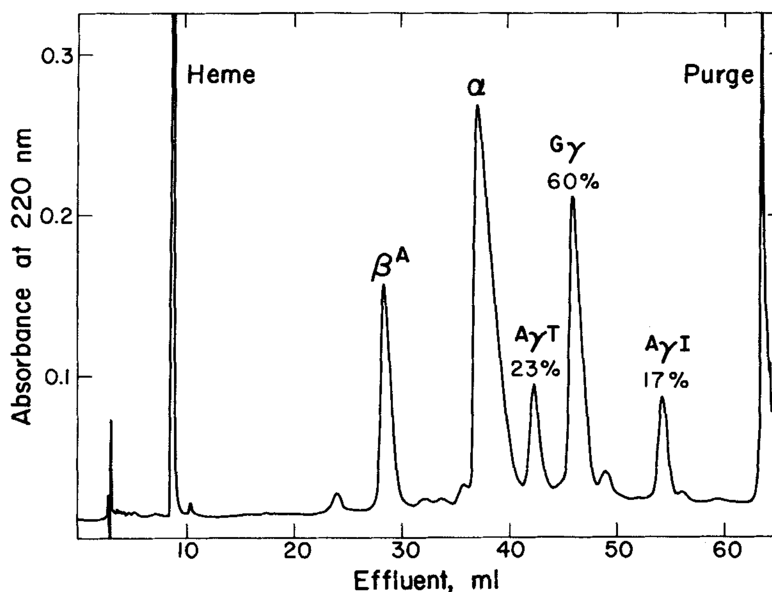

FIGURE 2 Separation of the globin chains in the cord blood of a newborn child. Sample was 0.10 mg. Same conditions as Figure 1.

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When globin chains are isolated in this way, the amino acid analyses are in excellent agreement with the expected values. With an amino acid analyzer of appropriate sensitivity, the 50-100 μg of individual chain from a single chromatogram is more than adequate for an analysis.

DISCUSSION

The Vydac large-pore C_4 column which has been used in these experiments not unexpectedly has properties significantly different from those of the small-pore Waters μBondapak C_{18} column. For example, the complex developer with perchlorate-phosphate-methanol-acetonitrile-nonylamine (3) which is effective on the μBondapak column moves the $^G\gamma$ chain ahead of the α chain on the Vydac column but does not adequately separate the two. The

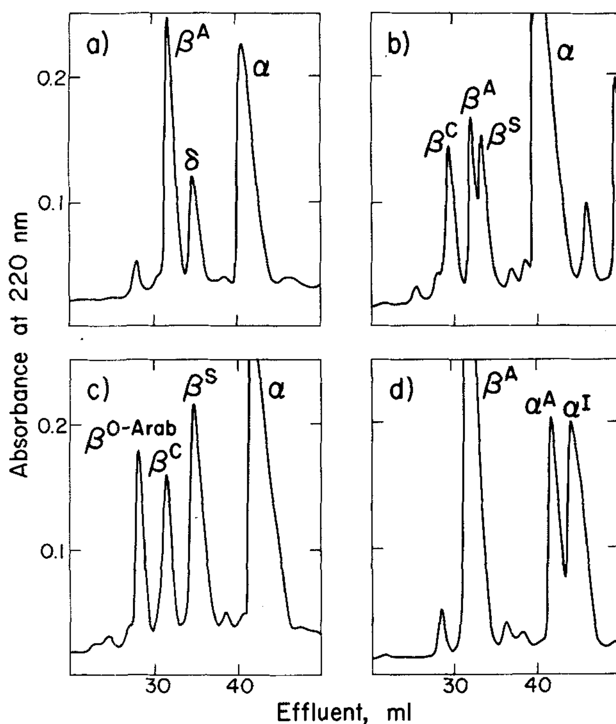

FIGURE 3 Separation of certain other globin chains. a) A 2:1 mixture of chromatographically isolated Hb A and Hb A_2; b) Mixture of hemolysates of cord blood (Figure 2) and of an SC patient; c) Mixture of hemolysates of blood from SC and SO-Arab patients; d) Hemolysate of an AI individual. Same conditions as Figure 1.

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TFA-water-acetonitrile mixtures which are excellent with the Vydac column are similar to those of Congote and Kendall (1). They have the advantage not only of simplicity but also (as frequently pointed out in the literature) of volatility so that subsequent operations such as amino acid analysis, sequencing, etc. may be done after evaporation of the solvent. Because of the excellent separations which were achieved under the stated conditions, no study has been made of variations such as the concentration of TFA, a TFA gradient, flow rate, etc.

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A distinct advantage of the Vydac column is the elimination of the need to purge the column after each chromatogram. After one column had been used approximately 250 times and separations had worsened, reversing the flow returned separations and peak sharpness to the original behavior.

When the experimental conditions for the separations in Figs. 1-3 are applied, there is no gradient of TFA and the actual concentration of acetonitrile changes only from 37.6 to 42.6% Because of the slight gradients if 0.1% aqueous TFA and 0.1% TFA in acetonitrile are the limiting solvents, mixtures have been used to produce a steeper and more reproducible gradient.

The separations are sensitive to changes in slope of gradient and/or to the initial percentage at which the gradient is started. This is illustrated in Fig. 4 in which the same sample was chromatographed under different conditions. In Fig. 4b (same as Fig. 2) the gradient from 44 to 56.5% B changed at 0.21% per ml. If the same slope is used but the gradient is from 47 to 59.5% B, the pattern (not shown) is virtually unchanged but translated along the abscissa so that each peak emerges about 10 ml sooner. Apparently, most of the separation occurs in the initial part of the column, and adequate results probably could be obtained with a shorter column. The chromatogram in Fig. 4a resulted from a higher initial percentage of B (47 instead of 44) and a steeper gradient (0.30 instead of 0.21% per ml). As anticipated, peaks emerge more slowly if the gradient is started at 44% but the change is only 0.15% per ml (Fig. 4c). The separations improve with little loss of quality in peak shape. Despite the marked changes in pattern, the quantitative agreement is good despite the varied experimental conditions; the differences reflect mainly the inclusion or exclusion of minor components. These samples illustrate the versatility with which conditions may be tailored to fit the requirements of a particular separation.

The large-pore packing is also excellent for the resolution of baboon globin chains, although a different gradient is

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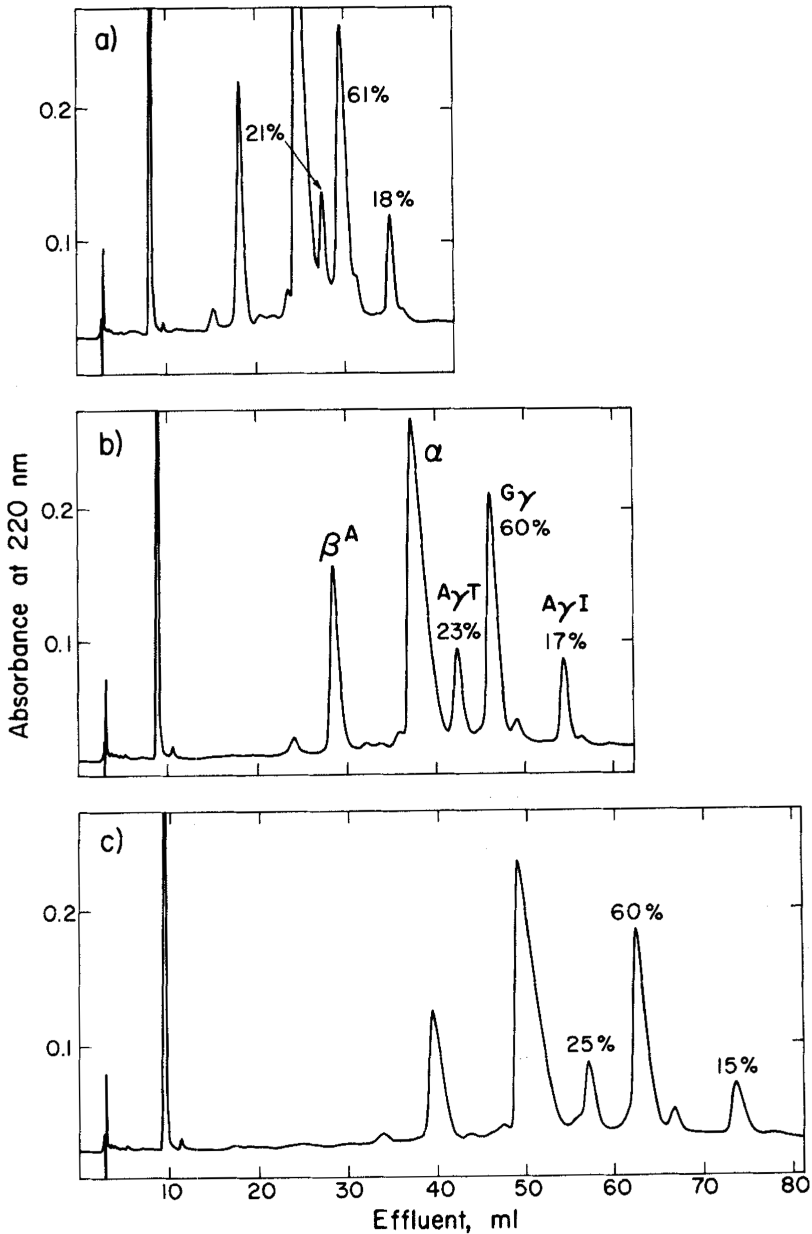

FIGURE 4 The effect of gradient changes on the separation of the components in identical samples. Figure 4b duplicates Figure 2. In these examples, a 60-ml gradient was used at 1 ml/min, and the gradients were as follows: a) 47 to 65% B, b) 44 to 56.5% B, and c) 44 to 53% B.

necessary. Illustrations of its effectiveness have been given (5). The method should be capable of separating hemoglobin chains of other species as well.

ACKNOWLEDGMENTS

This work was supported in part by grant HL-02558 from the National Institutes of Health, U.S. Public Health Service.

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