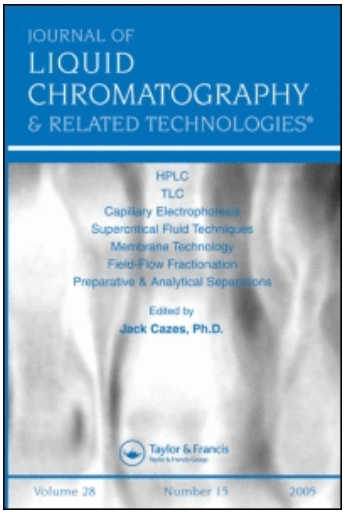

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Further Experiments in the Separation of Globin Chains by High Performance Liquid Chromatography

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FURTHER EXPERIMENTS IN THE SEPARATION OF GLOBIN CHAINS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

The applicability of various column packings, solvents, and gradients for the separation of globin chains by high performance liquid chromatography has been examined. Although numerous combinations may be applied to advantage in special situations, a particularly effective system uses a Waters μBondepak C_{18} packing with a slight gradient of perchlorate-phosphate-methanol-acetonitrile mixtures. A chromatogram with human β, α, $^{A}\gamma^{T}$, $^{G}\gamma$, and $^{A}\gamma^{I}$ chains is finished in 80 min, and quantitation of the various chains is possible.

High performance liquid chromatography (HPLC) may be applied to various tasks in the study of hemoglobin to provide analytical data, separations, and preparations with much facility. Tryptic peptides of hemoglobin may be separated easily by HPLC on several types of column packing and with a variety of solvent systems (reviewed in (1)). These parameters in various combi-

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nations provide many dimensions to accomplish difficult separations, and the methods should be equally applicable to other types of cleavage products. The application of HPLC to the study of hemoglobin is not limited to the separation of peptides. Thus, Congote et al. (2) and Shelton et al. (3) describe the separation of the α, β, $^{G}\gamma$, and $^{A}\gamma^{I}$ globin chains by HPLC with the same type of column packing but with different solvent systems. Huisman and collaborators have modified the system of Shelton et al. (3) so that the $^{A}\gamma^{T}$ chain may be separated from the $^{G}\gamma$ and $^{A}\gamma^{I}$ chains and have applied their procedure to various problems (4-7). Here we report an investigation of the separation of globin chains which has considered the effects of varied combinations of column packing and solvents. Particular emphasis was placed on devising methods for the separation of the three γ chains.

MATERIALS AND METHODS

The HPLC equipment consisted of two Altex Model 110A Solvent Metering Pumps, an Altex Mixer 400-02, an Altex 420 Microprocessor, a Waters U6K Universal Injector, an Altex-Hitachi Model 155-10 UV-Vis Variable Wavelength Detector, and a Linear Instruments Corpn. (Irvine, CA) single channel recorder.

The various column packings were: Altex Ultrasphere ODS and CN, DuPont Zorbax C_8, CN, and TMS, and Water μBondepak C_{18}. Dimensions were 4.6 x 250 mm for all except the Waters column (3.9 x 300 mm).

The solvents were various mixtures of a phosphate buffer [49 mM KH_2PO_4 (6.66 g per liter) and 5.4 mM H_3PO_4 (0.37 ml 85% H_3PO_4 per liter)], a perchlorate solution [0.15 M $NaClO_4$

(18.3 g per liter)], 85% H_3PO_4, methanol (MCB OmniSolv), and acetonitrile (Baker's HPLC). Water was doubly deionized and distilled in glass. Solvents were not filtered or degassed. Neither pre-columns nor guard columns were used.

The most effective separations were made with a gradient between Solvent A which was 80:5:15:0.1 (*v/v/v/v*) of perchlorate solution - methanol-acetonitrile-H_3PO_4 and Solution B which was a 20:5:75:0.1 mixture. These solvents with the exception of the added methanol are patterned after Meek (8). The gradient was begun with 66% B and was increased to only 70% B in 80 min at a flow rate of 1.5 ml per min at room temperature. At the completion of the gradient, the column was purged with 100% B and then reequilibrated with 66% B. These were the conditions for all figures below.

Hemoglobin solutions were prepared from saline-washed cells by hemolysis for 30 min at room temperature with water and carbon tetrachloride equal to 1 and 0.4 times, respectively, the packed cell volume. After double centrifugation at 4° and 30,000 g for 30 min, the solution was diluted with at least 10 times its volume of water and filtered through an 0.5 μm cellulose type filter (Rainin Instrument Co., Woburn, MA). A volume (20 to 100 μl) which contained 0.2-0.3 mg of hemoglobin was injected on to the chromatographic column.

Percentages were calculated by planimetry.

Identification of peaks was made by comparing the appearance, disappearance, and proportions of peaks in mixtures of known hemoglobin composition.

RESULTS AND DISCUSSION

Figures 1 and 2 depict separations of chains in the hemoglobins of representative adult and cord blood samples. In this

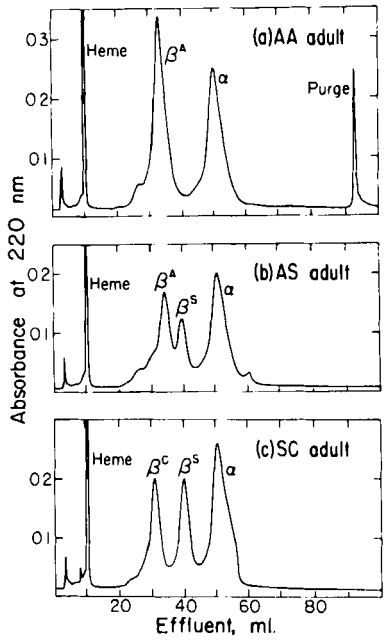

FIGURE 1

Separation of chains of hemoglobins from adults with AA, AS, and AC hemoglobins on a Waters μBondepak C_{18} column. The gradient is described in the text.

perchlorate-phosphate-methanol-acetonitrile system (PPMA), the β chain precedes the α chain as it does in the trifluoroacetic acid-acetonitrile system of Congote et al. (2). Conversely, in the phosphate-methanol-acetonitrile (PMA) system which we initially described, the α chain emerges ahead of the β chain (3). Huisman et al. (4) simplified these separations by using hemoglobin instead of globin as the sample. With PMA developers, conditions may be altered so that the heme emerges either before or after the chains (4, 9); the retention of heme in this system is apparently relatively unaltered by conditions, but the retention of

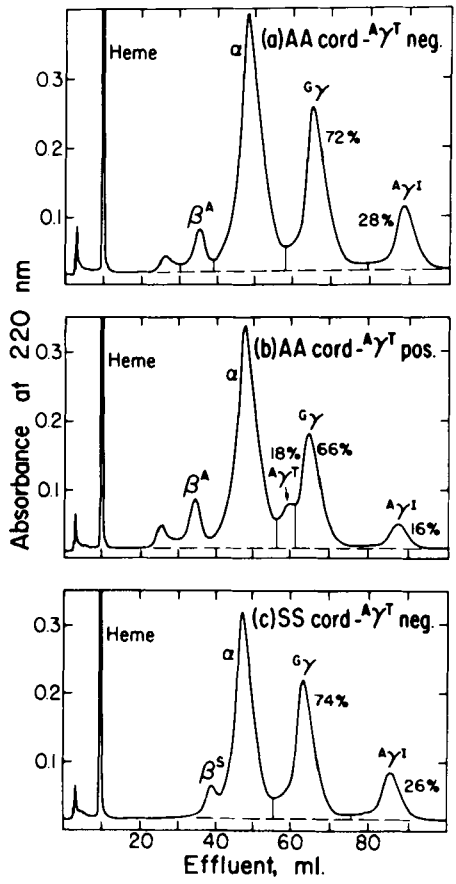

FIGURE 2

Separation of chains of hemoglobins in the cord bloods of infants with AA hemoglobin with and without $^{A}\gamma^{T}$ chains and of an SS infant. Conditions as in Fig. 1.

the chains is changed. The present PPMA system has the advantage of a rapid and effective elution of heme. Although Meek's solvents have 0.1M perchlorate, there seems to be a slight advantage in the use of 0.15M perchlorate for the chain separations.

The α and β^{A} chains separate well. A separation of β^{A}, β^{S}, and β^{C} chains can be achieved, although for no pair is the

separation complete. Although numerous variations in elution pattern and solvents have been tried, the depicted separation of β^A, β^S, and β^C chains is the best that has been achieved. Perhaps, a reduction in the amount of sample might improve the separation.

If γ chains are absent as in the samples in Fig. 1, the chromatograms are complete in about 45 min (70 ml). In Fig. 1(a), the gradient was terminated early and the developer shifted to Solvent B to purge the column; the final peak is material that was removed by the purge. In the other panels of Figs. 1 and 2, the purge is not shown, but was done at 120 ml when the gradient was complete.

The $^G\gamma$ and the $^A\gamma^I$ chains separate well (Fig. 2(a) and (c)). The $^A\gamma^T$ chain is qualitatively detectable, but variation in conditions has not produced a separation that is more satisfactory for quantitative determination. Despite the inadequate separation of the $^A\gamma^T$ chain, the calculated quantity on the basis of the dashed baseline is exactly the average of 39 $^A\gamma^T$ positive samples of cord hemoglobin (10). The present PPMA method has the advantage over the extended PMA modification of Huisman et al. (5) in that the $^A\gamma^T$ chain can be detected in an 80-min instead of a 160-min chromatogram. If the $^A\gamma^T$ chain is detected by the PPMA method, its quantitation could then be substantiated in a duplicate by the modified PMA method, if necessary (5). If the $^A\gamma^T$ chain is absent, the $^G\gamma$ to $^A\gamma$ ratio may be calculated readily. The percentages of $^G\gamma$ and $^A\gamma$ chains as given in Figs. 2(a) and (c) are in excellent agreement with the frequently quoted 3:1 ratio in the newborn. The identity of the small peak that precedes the β^A peak in Figs. 2(a) and (b) is unknown. It may be identical with the incompletely separated material at the leading edge of the β peak in Figs. 1(a) and (b).

In calculating the ratio of γ chains, it is reasonably assumed that their molecular extinction coefficients are identical. However, the quantitation of various hemoglobins in a mixture should be possible by this method, if the relative molecular extinction coefficients of the non-α chains are used. The relative coefficients which are known for 280 nm and commonly applied in chain separations by the Clegg-Naughton-Weatherall procedure, cannot be used here because absorbance at 220 nm is determined. Nevertheless the relative coefficients should be calculable from present data. Because α and non-α chains are present in molar equivalents, an equation such as $\alpha = A\beta + B\gamma$ should apply where α, β, and γ represent the integrated absorbance of a peak and A and B are constants. Likewise, it follows that the percentage of any one hemoglobin (for example, Hb-A) would be $\text{Hb-A} = \frac{A\beta \times 100}{\alpha}$. In Fig. 1(a), the $B\gamma$ term is zero, and the ratio of the areas of the α and β peaks gives a value of 0.87 for A. When this value of A is used with the data from Fig. 2(a), B equals 1.06. The exact value and accuracy of these constants needs to be established by a series of replicate determinations with chromatograms like that in Fig. 1(a) and with others like that in Fig. 2(a) but with a varied ratio of β and γ chains.

When a sample of isolated Hb-A_2 was chromatographed by the present procedure, the δ chain emerged at the position of the β^S chain. The quantity of Hb-A_2 in the normal adult is too small for the δ chain to produce a definite peak between the β and α peaks (Fig. 1(a)), but the presence of the δ chain may be responsible for the fact that the valley between the β and α peaks does not return to baseline.

Although the present procedure should prove useful in some aspects of the study of hemoglobin, there are certain undesirable

phases: it is relatively time-consuming (although less so than the modified PMA method and monumentally less than the Clegg-Naughton-Weatherall separation), the peaks are relatively broad and generally somewhat asymmetrical (hence, valleys may not return to baseline), and the separation of the $^{A}\gamma^{T}$ chain is minimal. Yet, at present, it is the best of the many modifications that were tried and that would permit, at least, the qualitative detection of the three types of γ chain. On the other hand, other conditions might be useful in specific instances. For example, a better separation of α and β^{S} chains might be achieved if the separation of γ chains were inconsequential. Only brief mention can be made of the many variables that were tried and their corresponding effects.

The gradient is so slight that development is almost isocratic, although no isocratic procedure was satisfactory: the percentage of perchlorate solution in the 80-min period of development decreases from 40.4 to 38 while that of acetonitrile increases from 54.6 to 57, and methanol and H_3PO_4 are constant.

Of the six column packings that were tested, the Waters μBondepak C_{18} is the most satisfactory and is followed by Altex Ultrasphere ODS, and DuPont Zorbax CN and C_8 in that order. The Altex Ultrasphere CN and DuPont Zorbax TMS packings could not be made to provide worthwhile separations with PPMA under varied conditions; PMA developers were not investigated.

Although PMA developers clearly produce desired separations as witnessed by the modifications of Huisman et al. (5), unfortunately, in this method, one is forced into 40 min of isocratic development to remove the heme before the gradient is introduced. If conditions are altered (as they can be) to separate the chains before the emergence of heme, the peaks are relatively

sharp, but the chromatogram must be completed in about 45 min and there is little room for maneuvering to improve separations or to provide space for the detection of the $^{A}\gamma^{T}$ chain. The PPMA system with its rapid removal of heme permits the gradient to be altered and extended as necessary.

As already mentioned, the β chain precedes the α chain in the PPMA system as well as in the trifluoroacetic acid-acetonitrile system of Congote et al. (2) whereas the reverse occurs with the PMA developers. However, there is an exception in the PPMA system with one of the acceptable packings. When the PPMA developers are used with a DuPont Zorbax CN column, the α chain emerges first. This system may have advantages although it was not studied extensively. Thus, if the gradient was from 25 to 50% B in A over 40 min at 1.5 ml per min, the peaks were sharper than in Figs. 1 and 2, and separation of β^{A} and β^{S} chains was about as good. When Solution A had the composition 60:5:35:0.1 PPMA, B was 40:15:45:0.1, and the perchlorate concentration was 0.1 M, a gradient from 42 to 55% B in A over 60 min did separate α, β, $^{G}\gamma$, and $^{A}\gamma$ chains in that order. However, the valleys did not return to baseline. Therefore, further study was not made because an effective separation of the $^{A}\gamma^{T}$ chain seemed unlikely.

Methanol was initially included in the developing system in order to increase the retention of the chains. In some experiments, n-propanol or isopropanol was substituted for methanol, the quantity was varied from 0-10%, and/or gradients were employed. None of these variations was more advantageous than the use of methanol itself.

The hemoglobin chains of other species are amenable to separation by this method. The separation of the chains in the

hemoglobin of a 33-day-old baboon (Papio cynocephalus) is seen in Fig. 3. The γ chains of the baboon do not differ at position 136 as do the human γ chains nor at position 135 as do those of some other primates (11). However, they do have either valine ($^{V}\gamma$) or isoleucine ($^{I}\gamma$) at position 75 (12, 13) as does the orangutan (14). Although the baboon $^{V}\gamma$ and $^{I}\gamma$ chains do not separate as completely as the human $^{G}\gamma$ and $^{A}\gamma$ chains, quantitation is possible. The $^{V}\gamma$ chain approximates 40% at birth, but the percentage increases postnatally (15). The identities of the small peak that precedes the β chain and of the two that follow the α chain are unknown.

Numerous systems, therefore, are available for the HPLC separation of hemoglobin chains. The conditions that are described here were designed to achieve the maximum separation of the common human hemoglobin chains with relative speed. On the other hand, with less complex mixtures or for special

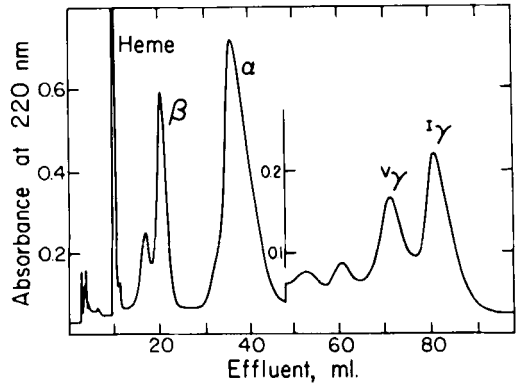

FIGURE 3

Separation of the chains of hemoglobins from a 33-day-old baboon. Conditions as in Fig. 1. At approximately 48 ml, the absorbance setting was changed.

separations, modification of column packings, solvents, or gradients should permit almost any desired separation.

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