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Note

Rapid high-performance liquid chromatographic method for the separation of the three types of γ-chain of human fetal haemoglobin

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In 1968 Schroeder *et al.*[1] described a heterogeneity of human fetal haemoglobin resulting from the presence of either a glycyl (Gγ) or an alanyl residue (Aγ) in position 136 of the γ-chain. In 1976, Ricco *et al.*[2] discovered another type of γ-chain heterogeneity in which the isoleucyl residue in position γ 75 was replaced by a threonyl. Aγ and Gγ chains are products of two non-allelic structural genes, while AγI and AγT chains are produced by alleles of the Aγ chain gene.

The determination of the γ-chain heterogeneity of haemoglobin F is of interest for genetic and population studies[3]. It may also yield some information about the biological and clinical polymorphism of haemoglobinopathies[4].

The first determinations of the Gγ and Aγ chain ratio were conducted by various chromatogrpahic procedures followed by amino acid analyses. Improvement in the methodology, brought by high-performance liquid chromatographic (HPLC) and by electrophoretic techniques have allowed extensive studies. Congote *et al.*[5] and Shelton *et al.*[6] described the separation of the α, β, Gγ and AγI globin chains by HPLC on a C_{18} 100-Å porosity column packing but with different solvent systems. Huisman *et al.*[7] made some modifications to the system of Shelton *et al.*[6] so that the three types of γ-chains may be separated in 3 h. More recently a perchlorate–phosphate–methanol–acetonitrile system was proposed by Shelton *et al.*[8] which, in 80 min, allowed a qualitative detection of the AγT chain.

In the present communication we describe a modification of the HPLC procedure using a 300-Å porosity column packing and trifluoroacetic acid (TFA)–acetonitrile–methanol as eluent. This procedure allows, in 80 min, with a reduced flow-rate as compared to the previously described methods, the quantitation of the three types of γ-chains. In addition, the completely volatile elution system described may be of interest for preparing salt-free material for further investigations.

Blood was drawn in heparinized tubes and washed red blood cells were lyzed with water. Haemoglobin solutions were purified with toluene and stroma removed by centrifugation (13,000 *g*; 5 min). Globin was prepared by the acid–acetone method. For each sample, 5–10 mg of globin were prepared. Before analysis the freeze-dried globin was dissolved in distilled water at a concentration of *ca.* 5 mg/100 μl.

An Aquapore RP 300 column (25 × 0.46 cm) (Brownlee Labs., Sante Clara, CA, U.S.A.) protected by a guard column was used. With a flow-rate of 1 ml/min,

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a non-linear gradient (Fig. 1) was developed in 80 min. Solvents: A, TFA (0.3% in water)–acetonitrile–methanol (54:36:10), B, water–acetonitrile–methanol (45:50:5). All the solvents were HPLC grade; TFA was spectrophotometric grade.

After reequilibration of the column in solvent A for 10 min, a 20-μl aliquot of the globin solution was applied and the elution program started.

A Chromatem 800 HPLC machine (Touzart et Matignon, Vitry, France) equipped with two Altex pumps was used. Detection was performed at 280 nm (Shimadzu UV detector) and the areas of the peaks were determined using an ICAP 5 integrator (Delsi, France). In calculating the ratio of γ-chains it was assumed that their molecular absorption coefficients are identical.

RESULTS AND DISCUSSION

The chromatographic analysis of globin subunits by reversed-phase HPLC has been, up to now, carried only on C_{18} hydrocarbon ligands, particle size 10 μm, nominal pore size less than 100 Å. The 300-Å porosity packing is known to be more hydrophilic and therefore results in a quicker elution and sharper polypeptide peaks[9].

In this system we found that the haem emerges rapidly before the chains and that the β-chains precede the α-chains as in the perchlorate–phosphate–methanol–acetonitrile system of Shelton *et al.*[8] or in the TFA–acetonitrile system of Congote *et al.*[5].

In a first set of experiments we used a linear gradient of from 36% acetonitrile in 0.3% TFA to 50% acetonitrile in water. In 45 min the separation of β, α, Gγ and Aγ was clear but there was not between α and Gγ so as to distinguish AγT. Therefore we modified both the slope of the gradient and the solvent system. Methanol was added in order to increase the retention of the chains. Since our main interest was in isolating the γ-chains, we chose to elute rapidly the α- and β-chains using a sharp increase of the gradient slope at the beginning of the chromatography and then to proceed with a mild gradient.

Fig. 1A shows the elution pattern obtained with the globin of a thalassemic patient heterozygous for the AγI and AγT gene. Since the peaks are much sharper than with the 100-Å porosity packing a good return to the baseline is observed between the adult chains and Gγ, allowing the distinction of AγT which may them easily be quantitated. The procedure described can be used even when the amount of fetal haemoglobin in the sample is low. It requires only an adjustment of the sensitivity of the detector. For instance, a full scale deflection of 0.64 a.u. was used for a 1-mg sample of cord-blood globin, whereas 0.04 a.u. were required for studying the fetal haemoglobin heterogeneity in patients with sickle-cell disease. Without requiring a preliminary enrichment in fetal haemoglobin, an accurate quantitation was obtained with samples containing amounts as low as 2% of haemoglobin F. Fig. 1B shows an example of a sample containing 3% haemoglobin F.

We have used the same chromatographic technique with lysate, but since the elution pattern is progressively modified by storage of the sample, even when kept frozen, more reproducible results were obtained with globin dissolved just prior to analysis.

The use of a guard column is a must. A regular increase of the pressure was observed, probably due to some plugging of the guard column. Aquapore RP 300

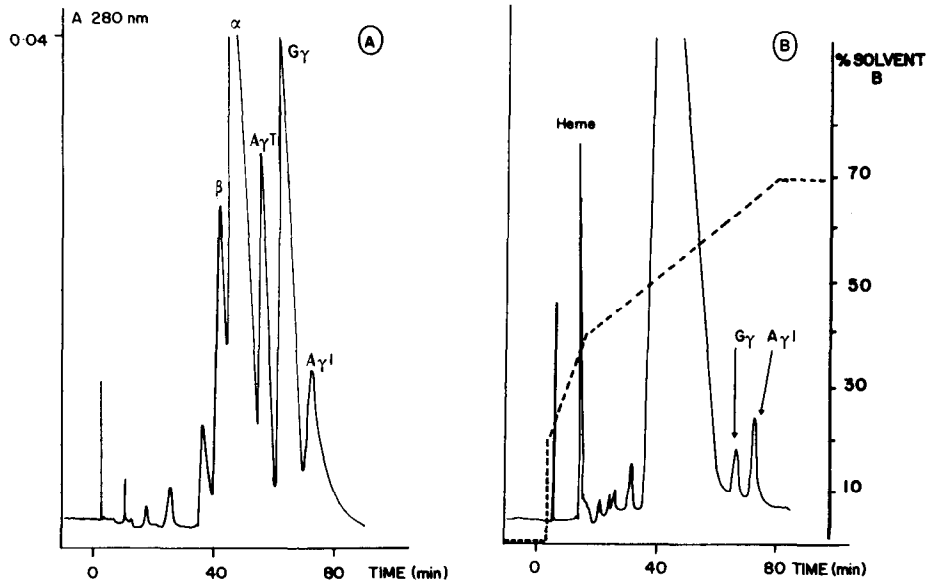

Fig. 1. Elution patterns of globin subunits: A, β-thalassemic patient heterozygous for the AγT and Aγ I gene; B, patient with sickle-cell anaemia carrying 3% haemoglobin F. Experimental conditions: Aquapore RP 300 column; column equilibrated in 100% solvent A; flow-rate 1 ml/min; gradient, 0 to 25% B from 2 to 2.5 min, 25 to 40% B from 2.5 to 14 min, 40 to 70% B from 14 to 75 min, isocratic at 70% B from 75 to 80 min.

columns are used intensively in our laboratory for tryptic peptides and for polypeptide chain separations, and in more than 6 months there was no loss of resolution.

A final point which may be of interest is that all the procedure is performed in a solvent system which may easily be evaporated leaving a salt-free protein. Therefore the desalting step by either chromatography or dialysis may be omitted before further structural studies.

In comparison with the previously described methods, this modified HPLC procedure reduces the cost of each analysis by about 50%.

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