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Note

Chromatofocusing of pyridoxalated and polymerized human haemoglobin

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Chromatofocusing offers a new approach to analytical and preparative fractionation of heterogeneous protein samples according to the different p*I* values of their individual components[1,2]. It combines classical isoelectric focusing with column chromatography. In the present paper we describe results obtained for pyridoxalated and polymerized stroma-free haemoglobin (SHF-P and SFH-PG, respectively), which are being investigated as one of the potential blood substitutes for infusion therapy[3]. The results were compared to those obtained by means of thin-layer analytical isoelectric focusing, where it was possible to detect up to about 23 protein fractions[4].

MATERIALS AND METHODS

Pyridoxalated human haemoglobin SFH-P and its polymerized derivative SFH-PG were prepared according to refs. [3–5]. Before application to the chromatofocusing column, the samples were equilibrated with the eluent [Polybuffer™ 96 (Pharmacia, Uppsala, Sweden) diluted 1:9 with degassed distilled water and adjusted with acetic acid to pH 6.0] by using gel filtration on Sephadex G-25. Chromatofocusing was done according to the manufacturer's instruction manual[6]. The Polybuffer exchanger PBE™ 94 gel (Pharmacia) was settled and equilibrated with the starting buffer, 0.025 mol/l Tris–acetic acid, pH 8.3, at a relatively low linear flow-rate in two different columns. The bed dimensions were 15 × 0.9 cm and 27 × 1.6 cm, respectively. All buffers were degassed before use. The samples were applied by first running 5 ml of eluent, followed by 5 ml of SFH-P or SFH-PG. The applied sample was then eluted with Polybuffer, pH 6.0. The absorbances of the eluted proteins were measured at 280 nm using a Unicam spectrophotometer. The pH gradient was established by measuring the pH in each collected fraction using a Radiometer PHM 64 pH meter (Radiometer, Copenhagen, Denmark).

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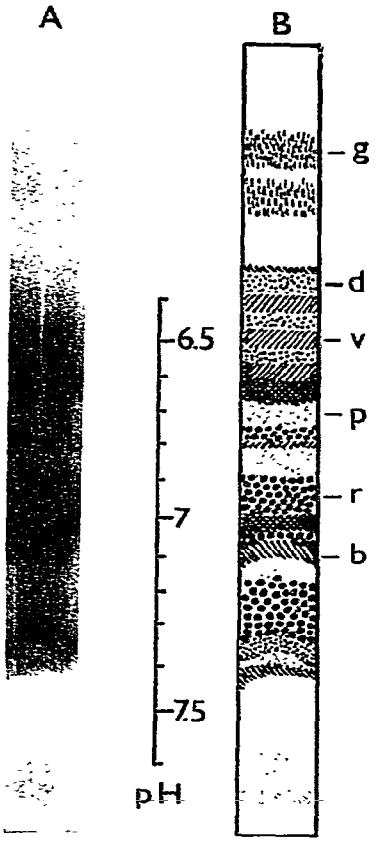

Fig. 1. Chromatofocusing of pyridoxalated human haemoglobin (SFH-P). A, Photograph of the column (panchromatic negative) 5.5 h after the start; B, scheme of A with zone colours (g = grey-green; d = deep purple; v = violet-purple; p = pink; r = red; b = brown). Bed dimensions: 15 × 0.9 cm. Flow-rate: 2.5 ml cm^{-2} h^{-1}.

RESULTS AND DISCUSSION

Fig. 1 shows the development of a SFH-P pattern 5.5 h after the start, just before the first haemoglobin fraction began to leave the column (15 × 0.9 cm). About 21–25 haemoglobin bands were usually observed, having different tints of red, deep purple, pink, brown and violet. (The black and white photograph is a poor guide to the degree of separation achieved.) The comparison of the positions and sequence of the main bands with their p*I* values (estimated from Fig. 2, curve 1) enabled the construction of an approximate pH scale valid for the column at this time. The assumed pH gradient was relatively smooth and linear. The two grey-green zones near to the start remained at that position even after all other fractions had been eluted. Their elution was difficult and usually incomplete, and they cannot unambiguously be assigned.

The large number of differently coloured zones was due chiefly to the simultaneous presence of the oxygenated and deoxygenated forms of various pyridoxa-

lated and native haemoglobin subfractions, as well as of their ferro and ferri forms which mutually differ in their p*I* values. After elution from the column into open test-tubes, all fractions (except the brown-red ferrihaemoglobins) rapidly gained the typical bright red coloration of oxyhaemoglobin. In our previous flat bed isoelectric focusing (IEF) study of SFH-P and SFH-PG in the presence of atmospheric oxygen[4] all pyridoxalated fractions with p*I* values less than 6.8 also had a bright red colour. Evidently, the deep purple and violet bands in the column correspond to the deoxy forms of haemoglobin subfractions and derivatives.

The column had been equilibrated and eluted by deaerated (*i.e.*, deoxygenated) Polybuffer, while the SFH-P samples were not deoxygenated before and during application to the column. Thus, the respective oxy–deoxy equilibria were established on the column during chromatofocusing. Under such "partly anaerobic" conditions there is a rough but important analogy between the oxy- and deoxyhaemoglobins in the column and those present in the periphery of the blood stream. In contrast to IEF, chromatofocusing may give more reliable information on the expected heterogeneity of SFH-P and SFH-PG "*in vivo*". This heterogeneity involves not only the proteins "as such" but also the state of their haem groups. On the other hand, the different p*I* values of oxy- and deoxyderivatives make it difficult to compare the patterns achieved by chromatofocusing under anaerobic and by IEF under aerobic conditions.

Fig. 2 shows that the general shapes of the elution patterns of two batches of SFH-P were similar, although differences in details were found. A smooth pH gradient was formed during chromatofocusing on both columns. A better separation of the SFH-P subfractions was achieved on the larger column, where the flow-rate per cm^2 was 3.2 times slower. A slow flow-rate was important also during sample application in order to prevent irregularities and distortions of the horizontal positions of bands. Direct observation of the fractionation of SFH-P on the column gave markedly more qualitative information than the elution curves alone (when measured at 280 nm). There was a discrepancy between chromatofocusing and thin-layer analyti-

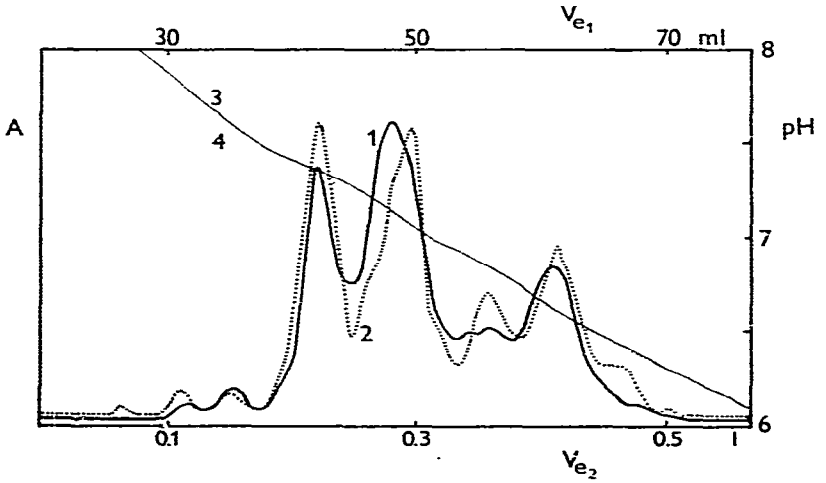

Fig. 2. Elution curves of pyridoxalated haemoglobin (SFH-P). 1 = SFH-P60 (the same batch and run as in Fig. 1), elution volumes V_{e_1} (upper scale); 2 = SFH-P61, run on a 27 × 1.6 cm bed at a flow-rate of 8 ml cm^{-2} h^{-1}, V_{e_2} values; 3 and 4 = pH gradients corresponding to curves 1 and 2, respectively.

cal IEF in the content of native and pyridoxalated haemoglobin subfractions in SFH-P. Thus, with IEF[4] more than 60% of haemoglobin was modified into acid derivatives of $pI < 6.8$; chromatofocusing suggested only about 40%. This difference might be due to the loss of pyridoxalated protein bound in the dark g zones in the column (Fig. 1). Generally similar patterns were achieved also during chromatofocusing of polymerized SFH-PG which had been treated with 10 mg glutaraldehyde per gram of haemoglobin[4], lyophilized with sucrose[7] and stored for 4 weeks at 20°C (batch No. SFH 56 PG/10).

It can be concluded that column chromatofocusing gives very good results when investigating the heterogeneity of chemically modified (pyridoxalated and polymerized) haemoglobins of new variants of blood substitutes. Even though there is a complication with SFH-P and SFH-PG in that the column is contaminated by an unidentified coloured material, the informational output of chromatofocusing is similar and in certain respects even higher than that of isoelectric focusing. However, thin-layer analytical IEF has the advantage of being suitable for the simultaneous analysis of large numbers of very small samples. The two methods could therefore be used in parallel.

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