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Note

Fast protein liquid chromatofocusing of lyophilized native haemoglobin and its chemically modified derivatives

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Chromatofocusing[1–3] is a relatively new technique for protein fractionation. It separates proteins on the basis of charge differences close to their isoelectric points. The combination of this principle with the Pharmacia fast protein liquid chromatographic system (FPLCF) offers better resolution of fractions and a substantial shortening of analysis times.

In the present note we describe our results obtained with FPLCF for the analytical fractionation of native and modified human haemoglobin, an oxygen-carrying plasma expander.

MATERIALS AND METHODS

Lyophilized native human haemoglobin and its derivatives modified with pyridoxal-5-phosphate, borohydride, glutaraldehyde and serum albumin[4] were prepared as before. Before application to the chromatofocusing column, the samples were transferred into the start buffer (0.025 *M* triethanolamine, pH 8.3) by gel filtration with Sephadex G-25 and filtered through a 0.22-μm sterile membrane filter. The sample was then applied on a Mono P HR 5/20 column in the start buffer via a Pharmacia V-7 valve and eluted with 0.21 ml Pharmalyte 8–10.5 + 9.0 ml Polybuffer 96 (adjusted to pH 6.0 using 2 *M* acetic acid). The pH gradient is self-generated by the interaction of the eluent with the charged groups on the gel. The light absorbance of the eluted proteins was measured at 280 nm. The pH gradient was determined by immediate measurement of the pH of the collected fractions using a Radiometer PHM 64 pH meter (Radiometer, Copenhagen, Denmark).

RESULTS AND DISCUSSION

Fig. 1 shows a comparison of typical FPLCF analyses of native human haemoglobin and of its derivatives obtained upon treatment with pyridoxal-5-phosphate, glutaraldehyde, borohydride and serum albumin. The high resolution of this complex protein mixture into at least 9–12 peaks was achieved reproducibly within about 60

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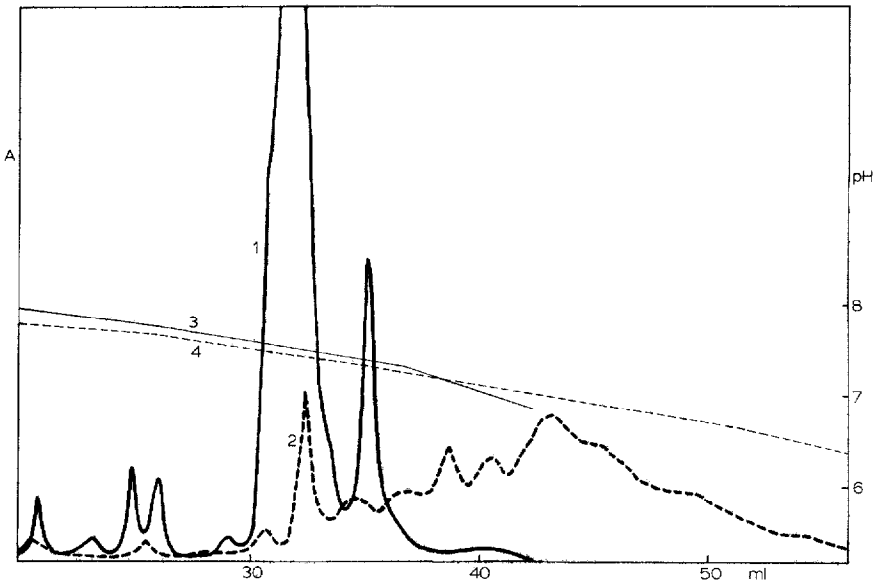

Fig. 1. Fast protein liquid chromatofocusing of native and modified human haemoglobin. Curves: 1, native haemoglobin; 2, haemoglobin treated with pyridoxal-5-phosphate, glutaraldehyde, borohydride and serumalbumin; 3, pH gradient for curve 1; 4, pH gradient for curve 2.

min by using about 25 ml of the Polybuffer. This is a striking advantage over classical "slow" chromatofocusing[5] which lasted about 24 h, needed at least 90 ml of Polybuffer and resulted in poorer resolution. This is evidently due to the fact that the rapid FPLCF analysis prevents diffusion and partial overlapping of the zones. The separation of modified haemoglobin by FPLCF was markedly better than in FPLC with a single salt gradient[6]. However, there is an apparent discrepancy between the p*I* values of characteristic individual peaks of haemoglobin derived from FPLCF as compared to those found in analytical isoelectric focusing (IEF)[4]. The p*I* values in FPLCF (and similarly also in classical chromatofocusing) are higher than in IEF by about 0.4 units. The fractions which were adsorbed on the column at alkaline pH were eluted sooner during stepwise acidification (at higher pH values) than had been expected from the results of IEF. This can be explained by the combined action of two factors: (1) increasing repulsive forces between the positively charged groups of the ion exchanger and of the protein molecules during acidification; (2) decreasing electrostatic adhesion caused by the presence of ions of the Polybuffer. Thus, the p*I* values estimated by FPLCF are shifted to higher pH values. IEF gives more reliable results in this respect.

Nevertheless, FPLCF is undoubtedly another step forward in the field of rapid, reproducible fractionation of very complex mixtures of proteins.

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