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Note

Affinity high-performance liquid chromatography on heparin-Glc-Spheron[a]

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Heparin, which is a sulphuric ester of a complex carbohydrate containing glucosamine glucuronic acid, has a wide range of important biological functions [1]. It is an anticoagulant that acts mainly by selective binding to antithrombin III, a plasma proteinase inhibitor, thereby increasing its inhibitory activity. Heparin also helps in inactivating several other vitamin K-dependent coagulation factors.

Owing to its polyanionic nature, heparin interacts with many cationic biological compounds, and the interactions have been used as a basis for purification by affinity chromatography on soft gels [2–4]. Heparin preparations are polydispersed with a molecular mass ranging from 5000 to 30 000, with an average of *ca.* 12 000–15 000. During the past decade many attempts have been made to separate heparin into defined subfractions. The fractions differ in many of their biological actions, and the individual components exhibit distinct chemical and biological activities [5,6].

Affinity chromatography could serve as an analytical tool to detect and measure the functional binding characteristics of individual heparin fractions. We have attempted to immobilize heparin on a rigid high-performance affinity support, and have compared two different ways of coupling.

EXPERIMENTAL

A macroporous hydrophilic chromatographic material, based on a highly cross-linked copolymer of hydroxyethylmethacrylate with ethylenedimethacrylate with immobilized glucose (Glc-Spheron, Lachema, Brno, Czechoslovakia, *ca.* 100 mg of glucose per 1 g of the gel) was seleced as the support matrix. The

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spherical particles had a size of 25 μm. The porosity was characterized by the molecular mass exclusion limit, and found to be 1000–3000 (relative molecular mass). The human coagulation factor IX concentrate (Bebulin, Immuno, Vienna, Austria), which contains at least 50-fold more factor IX than fresh plasma (0.85 I.U. of factor IX in 1 mg of protein), and unfractionated heparin (heparin sodium, SPOFA, Prague, Czechoslovakia), containing 25 000 I.U./mg (WHO standard), were commercial products.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and fast protein liquid chromatography (FPLC) (Pharmacia, Uppsala, Sweden) [7] were used to check the purity of fractions obtained. SDS-PAGE was performed with a modified discontinuous buffer system [8] on a gradient gel. Single coagulation factor assays were used (Boehringer Mannheim, Mannheim, F.R.G.) to estimate factors II, VII, IX and X.

RESULTS AND DISCUSSION

Two different methods of heparin immobilization on Glc-Spheron (15 μm particle size) were used.

(1) The gel was suspended in 1 *M* NaOH and activated with epichlorhydrin [9] at 60°C for 2 h. After cooling and washing of the activated gel with distilled water, heparin was coupled at pH 9.0 (borate buffer, room temperature, 48 h). The preparation was designated as Heparin-Glc-Sepharon I (HGS I). The amount of immobilized heparin was 4 mg/ml of the gel.

(2) The sugar part of the gel was activated with 1 *M* sodium peroxidate [10] at

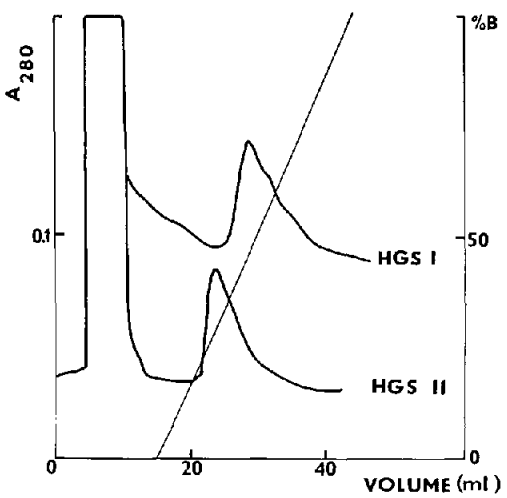

Fig. 1. Compartison of affinity chromatography of crude human coagulation factor IX concentrate on HGS I and HGS II. Column, 5 × 1.4 cm I.D.; sample volume, 500 ml; flow-rate, 1 ml/min; buffer A, 0.01 *M* phosphate (pH 7.3)–0.15 *M* NaCl; buffer B, 1.50 *M* NaCl in buffer A.

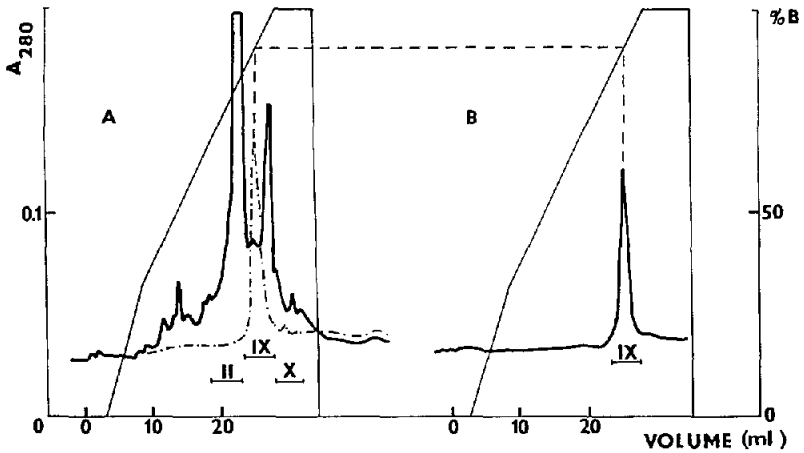

Fig. 2. Elution profiles on Mono Q HR 5/5 anion exchanger. Sample volume, 200 μl; flow-rate, 1 ml/min; buffer A, 0.05 *M* imidazole (pH 6.0); buffer B, 0.7 *M* NaCl in buffer A. Activities of individual coagulation factors are indicated by the bars. (A) Crude human coagulation factor IX concentrate (FIXC); (B) a fraction from the separation of FIXC on HGS I containing factor IX.

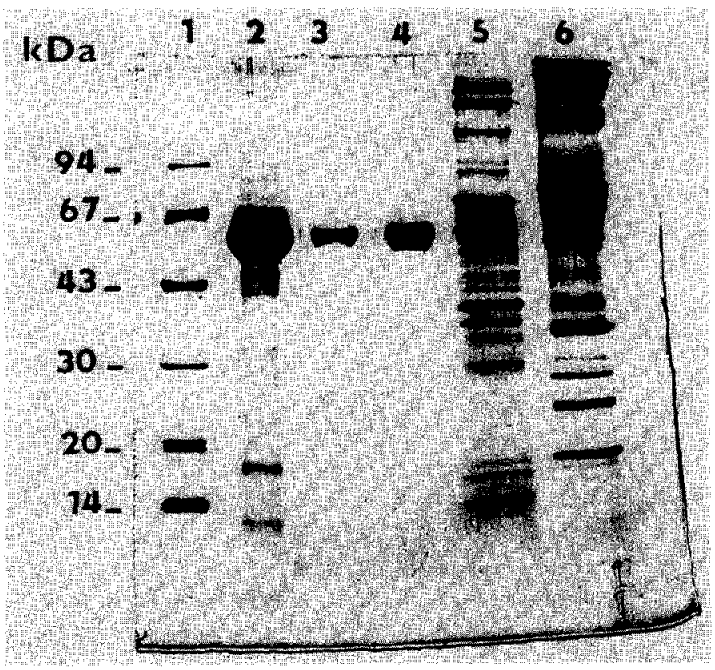

Fig. 3. SDS-PAGE (7–15% gel, Coomassic blue staining) of (1) LMW calibration kit, (2) antithrombin III purified from human plasma on heparin-Sepharose, (3) antithrombin III purified from human plasma on HGS I, (4) antithrombin III purified from human plasma on HGS II, (5) slightly bound plasma proteins on HGS II and (6) slightly bound plasma proteins on HGS I.

room temperature for 2 h, and heparin was immobilized at pH 6.0 for 12 h. The bonds were stabilized by reduction with sodium borohydride (pH 9.0) to increase the chemical stability of the bond. This preparation was designated as HGS II, and contained 1 mg/ml of immobilized heparin in the gel.

A sample of plasma was chromatographed on a column (5 × 1.4 cm I.D.) packed with the tested materials to check the ability of the immobilized heparins to interact with plasma proteins. Antithrombin III had the highest affinity from plasma proteins and factor IX had the highest affinity for immobilized heparin on both tested materials of vitamin K-dependent coagulation factors. We separated a commercial preparation of factor IX on both gels to investigate more closely the affinity of the two immobilized heparins for several plasma coagulation factors. The amount of factor IX in the concentrate was *ca.* 2% of the total protein content, and considerable amounts of factors II and X were present. Higher ionic strength was needed to elute the bound proteins from HGS I than from HGS II (Fig. 1), probably resulting from the different modifications of the support matrix and suggesting the contribution of the matrix–protein interactions. By increasing the ionic strength of the starting buffer, however, conditions were found using HGS I under which factors II, VII, and X activities eluted with the starting buffer. Factor IX activity retained on the matrix and was eluted with a sodium chloride gradient (20 m*M* Tris, pH 7.8, 0.2 *M* NaCl). The preparation was homogeneous according to anion-exchange chromatography (Fig. 2) and immunoblotting [11] after SDS-PAGE. The purification factor calculated as compared to factor IX concentrate was *ca.* 60, and the recovery 80%.

The capacity of HGS I for antithrombin III was *ca.* 2 mg/ml of the gel and for HGS II *ca.* 1 mg/ml. The change in the flow-rate from 1 ml/min (with a back-pressure of less than 0.2 MPa) to 0.5 ml/min did not essentially influence the resolution. Antithrombin III was purified by one-step chromatography in reasonable purity (Fig. 3).

In conclusion, immobilized heparin on both matrices retained the ability of selective reaction with antithrombin III. The preparations render a high degree of recyclization possible (more than 100 runs so far) without any loss of adsorptive capacity or changes in the mechanical properties of the carrier. High stability and consistent results are obtained. They seem to be good candidates for high-performance affinity chromatography materials to measure molecular interactions of various heparin fractions with the coagulation proteins. They allow characterization of bimolecular interactions on a microscale with a good resolution.

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