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Note

Separation of vitamin K-dependent coagulation factors on sulphated cellulose

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We have recently reported that a new sulphated cellulose chromatography medium is capable of separating the vitamin K-dependent clotting factors II, IX and X [1]. The total protein binding capacity and selectivity of the cellulose medium for specific proteins is similar to that reported using other sulphated media [2–4]. Heparin Sepharose and related sulphated Sepharose derivatives such as dextran sulphate Sepharose have been used for large-scale preparation of a number of proteins including antithrombin III (ATIII), lipoproteins and factor IX [4]. There are potential advantages when considering sulphated cellulose as a less expensive alternative medium to heparin Sepharose for larger-scale chromatographic processes [1]. We now report a pilot-scale procedure for selective purification of factor IX using a sulphated derivative of hydroxypropylated cellulose.

EXPERIMENTAL

Materials

Pooled cryosupernatant plasma, which was anticoagulated with citrate phosphate dextrose anticoagulant (CP2D), was obtained from the Auckland Regional Blood Transfusion Centre. Factor IX concentrate was prepared as previously described [5]. When reconstituted with 10 ml of sterile water the concentrate contained 36 U/ml factor II, 30 U/ml factor X and 26 U/ml factor IX. Heparin Sepharose was purchased from Pharmacia (Uppsala, Sweden). The sulphated cellulose (5.2 mequiv. sulphate per ml) was obtained from Phoenix Chemicals (Waitaki Biosciences, Christchurch, New Zealand). Monospecific antiserum to selected human plasma proteins were obtained from Dakopatt (Glostrup, Den-

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mark) and Behring (Marburg, F.R.G.). All other chemicals were analytical reagent grade from Sigma (St. Louis, MO, U.S.A.) or Serva Chemicals (Heidelberg, F.R.G.).

Methods

The chromatographic separations were carried out with a custom-made acrylic column (500 mm×90 mm, gel volume 1.38 l) equipped with an adjustable top adaptor. A constant flow-rate of 34 cm/h (36 ml/min) was maintained using a Masterflex peristaltic pump (Cole-Palmer, Chicago, IL, U.S.A.). The eluate was monitored for protein at 280 nm (UV1 monitor equipped with an industrial flow cell, Pharmacia) and pH and conductivity (Triac, Auckland, New Zealand). A load-to-column volume ratio of 2.5 U factor IX per ml of sulphated gel was maintained for all purifications. Starting material was dialysed for 2 h against starting buffer (20 m*M* sodium acetate, 200 m*M* sodium chloride, 2.5 m*M* calcium chloride, pH 6.0) immediately prior to application to the column. The column was washed with starting buffer and then eluted sequentially with starting buffer containing 0.20, 0.30, 0.45 and finally 0.8 *M* sodium chloride. The recovered peaks

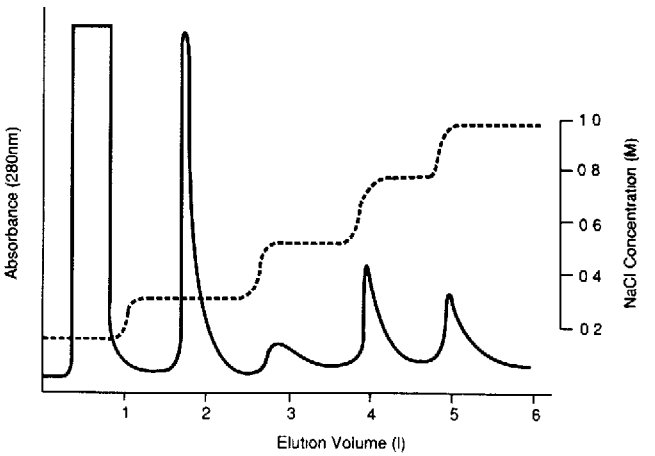

Fig. 1. Separation profile of factor IX concentrate on cellulose sulphate (5.2 mequiv./ml).

TABLE I

ANALYSIS OF VITAMIN K-DEPENDENT PROTEIN FRACTIONATION ON SULPHATED CELLULOSE

Fraction	Total protein		Total factor II		Total factor X		Total factor IX	
	mg	%	U	%	U	%	U	%
Load	3476	100	3600	100	3000	100	2600	100
Peak 1	2335	67	1210	34	0	0	0	0
Peak 2	395	11	800	22	270	9	0	0
Peak 3	195	6	0	0	0	0	0	0
Peak 4	263	8	0	0	660	22	390	15
Peak 5	176	5	0	0	480	16	910	35

were concentrated by tangential flow ultrafiltration (Ultrasart, Sartorius, Heidelberg, F.R.G.). Following the elutions the column was regenerated with one column volume of 0.2 *M* sodium hydroxide and sufficient five-fold concentrated starting buffer to return the pH to starting conditions.

Coagulation assays for factors II, IX and X were performed using Dade (U.S.A.) deficient substrates and standard one-stage techniques [6–8]. Chromogenic substrate assays for factors II and X, using chromozyme TH (Boehringer, Mannheim, F.R.G.) and S-2222 (KabiVitrum, Stockholm, Sweden) were performed on a Shimadzu UV160 recording spectrophotometer as previously reported [1]. Standard in vitro thrombogenicity testing was carried out as described [9–11]. Semiquantitative immunodiffusion was performed by the method of Ouchterlony [12].

RESULTS

The profile of the step gradient elution of factor IX concentrate on sulphated cellulose is shown in Fig. 1. The total protein and coagulation factor analysis and yield of purified factor IX are summarised in Table I. The final 0.8 *M* elution peak contained 35% of the loaded factor IX with small amounts (16%) of factor X. Thrombogenicity tests of the final concentrated factor IX fraction revealed no detectable activated components.

DISCUSSION

We have previously compared heparin Sepharose and cellulose sulphate (5.2 mequiv./ml) using the linear gradient elution profile originally described for the optimal separation of vitamin K-dependent proteins on heparin Sepharose [3]. In this system both media appeared to resolve factors IX and X into discrete peaks. However, when the cellulose sulphate was scaled up and a step gradient applied at higher flow-rates (> 10 cm/h) the factor X usually separated into two peaks one of which copurified with factor IX. Attempts to completely resolve the factor IX from the factor X by manipulation of the salt concentration and buffer pH were unsuccessful. Further modification of the step elution procedure as now described has resulted in pilot-scale purification of a factor IX concentrate which is completely free of factor II and contains only small amounts of contaminating factor X. The product is non-thrombogenic when tested by accepted standard in vitro thrombogenicity assays. The yield and specific activity on a semi-production pilot scale were similar to those reported using the sulphated derivatives of Sepharose [13]. The sulphated cellulose material has a positive cost advantage relative to the more commonly used heparin Sepharose gel and more readily withstands harsh regeneration conditions without alteration in attainable flow-rate and ability to resolve factor IX [1].

Our experience with chromatographic separation on sulphated cellulose has enabled identification of critical determinants for the optimal performance of the sulphated cellulose medium. These include a pre-treatment of the media with pooled normal human plasma (10 mg total protein per ml of gel) before use in a factor IX concentrate purification. During factor IX separation procedures the

eluted peaks should be collected into 25 m*M* sodium citrate to immediately chelate the calcium ions and concentration of the peaks should be rapid using a technique such as tangential flow ultrafiltration which avoids protein denaturation.

A potential disadvantage of the presently available sulphated cellulose media is the relatively low effective molecular mass exclusion limit (88 000) of the gel at high flow-rates [1]. At flow-rates which are acceptable for production scale it is most likely that only the sulphate residues on the external surface of the bead are involved in protein binding. This significantly reduces the potential loading capacity of the media. Development of a cellulose particle with improved porosity could be expected to improve the acceptability of this medium for production-scale chromatography.

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