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Isolation of plasma proteins from the clotting cascade by heparin affinity chromatography

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ABSTRACT

The use of heparin affinity chromatography for the isolation of plasma proteins from the clotting cascade is described. The separation is carried out with heparin agarose and, in parallel operations, with different rigid gels on a polymer base. The quality of the separation and the reproducibility of the results were investigated and the stability of the materials at high pH was tested. The affinity supports were used for the isolation of antithrombin III from human plasma and for the separation of factor IX from factor X, after partial purification by anion-exchange chromatography. The isolation of antithrombin III from human plasma served as a model. The non-specific bindings were investigated, together with the resistance of the support when treated with 0.2 and 0.5 M sodium hydroxide. Heparin agarose has low non-specific bindings, but it cannot be exposed to high pH. The supports on a polymer base are resistant to high pH, up to 13.7. However, they may remain slightly hydrophobic, and the hydrophobicity of the matrix leads to an increase in non-specific bindings. When antithrombin III is isolated, the non-specific bindings result in contamination of the final product. The lack of resistance of the matrix at high pH causes a weaker binding of antithrombin III, and the product is eluted at lower and lower sodium chloride concentrations. The results can be indicative of the behaviour of the support in the separation of factor IX from factor X. High non-specific bindings will lead to contamination of the factor IX product and consequently to low specific activity. Insufficient resistance of the support at high pH will result in failure to separate the two clotting factors satisfactorily. The separation can be monitored by heparin high-performance membrane affinity chromatography (HPMAC). Contamination of the sample, which occur in sodium dodecyl sulphate-polyacrylamide gel electrophoresis are detected within minutes by fast heparin HPMAC.

INTRODUCTION

Heparin has been used for some time in the isolation of plasma and membrane proteins, having been immobilized first to soft gels and later also to rigid gels [1,2]. The most frequent use of heparin affinity chromatography (AC) involves the isolation of antithrombin III from human plasma [1,3]. Recently this method has been used as the last step in the isolation process of human blood clotting factor IX [4].

Agarose-based supports with immobilized heparin have also been used successfully for the above-mentioned purification process. The column lasts a long time and non-specific binding is low [5], and

therefore reproducible separations are achieved. An important disadvantage is that the soft gels cannot be treated with 0.2-0.5 A4 sodium hydroxide. This in turn creates a risk of pyrogenic substances accumulating on the column and contaminating the product after several runs.

This paper deals with applications of AC, using heparin on soft, agarose-based gels and heparin immobilized to hard, polymer-based gels and to membranes. The hard gels allow higher flow-rates and consequently shorter purification times. Their chemical stability is much greater than that of the soft gels, and they can be treated with 0.5 M sodium hydroxide.

The isolation of antithrombin III serves as a model investigation for determining the non-specific bindings of the gel and its behaviour after treatment with sodium hydroxide. These model investigations indicate whether or not the gel can also be

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used in the last step of factor IX purification, namely the separation of factor IX from factor X from the concentrate resulting from the previous step, partial purification by preparative anion-exchange HPLC. The option of fast process monitoring by heparin high-performance membrane AC (HPMAC) was also investigated.

EXPERIMENTAL

Chemicals

Human plasma of blood group A + was provided by Octapharma (Hurdal, Norway) as fresh frozen plasma. Virus-inactivated plasma after treatment with 1% (w/v) Triton X-100, 1% (w/v) tri-n-butyl phosphate at 30°C [6] was applied to the column for the isolation of antithrombin III.

Chemicals of analytical-reagent grade were purchased from Merck (Darmstadt, Germany), Serva (Heidelberg, Germany) and Sigma (Munich, Germany).

Clotting assays, solvent, detergent and protein determination

A one-stage coagulation assay for factor IX was performed by mixing the corresponding clotting plasma with the diluted sample and incubating it in an activator (lipid extract and kaolin). After incubation, coagulation was stopped by adding calcium chloride solution. The time required for the clot to form was measured. Each test was calibrated against factor TX concentrate (Octapharma, Vienna, Austria), which in turn was calibrated against the latest WHO standard.

For the factor X assay, the plasma that is deficient of the corresponding clotting factor was mixed with the diluted sample and incubated. After incubation, clotting was terminated by adding calcium thromboplastin. The time required for the clot to form was measured. Each test was calibrated against standard human plasma, which in turn was calibrated against the latest WHO standard.

Factor IX, X-deficient plasma and all other reagents were purchased from Behring (Marburg, Germany). Coagulation tests were carried out with an Amelung (Lemgo, Germany) koagulometer.

Tri-n-butyl phosphate and Triton X- 100 were determined by the methods described by Horowitz *et al.* [6]. The protein in the samples was determined

according to the procedure of Lowry *et al.*[7] or according to Smith *et al.*[8], using a protein determination kit manufactured by Pierce (Rodgau, Germany). Bovine serum albumin was used as a standard.

Factor IX purification

The initial steps for the enrichment of factor IX were carried out with the method described by Brummelhuis [9]. The only modification was the replacement of DEAE-Sephadex with DEAE-Sepharose Fast Flow (Pharmacia, Vienna, Austria) in the second run of anion-exchange chromatography. The amount used in our large-scale production process was 1000 kg of plasma per run.

The samples for heparin affinity chromatography were taken from our production process, after partial purification by two anion-exchange steps [9].

Chromatographic equipment

The iron(III) ion-free HPLC system consisted of two pumps, a programmer, a spectrophotometer with a deuterium lamp and a Rheodyne loop injection valve (all from Knauer, Berlin, Germany). A BioPilot system (Pharmacia) was used for semi-preparative and preparative chromatography. The salt gradient in heparin affinity chromatography was controlled by measuring the osmotic pressure (Osmomat 030 cryoscopic osmometer; Gonotec, Berlin, Germany).

Supports and buffers for affinity chromatography

For fast heparin HPMAC, a QuickDisc separation unit with immobilized heparin was used (Säulentechnik Knauer, Berlin, Germany).

The following supports were used for semi-preparative and preparative heparin AC: Heparin Sepharose (Pharmacia), Bio-Gel Heparin (Bio-Rad, Vienna, Austria) Eupergit Heparin (Röhm Pharma, Weiterstadt, Germany) and Toyopearl Heparin 650 M (TosoHaas, Stuttgart, Germany). The supports were packed into glass columns of different dimensions (Saulentechnik Knauer or Pharmacia).

A 10–20 mM sodium citrate buffer (pH 7.4) was used for sample application and equilibration. Sodium chloride was added to both the washing buffer (about 0.25 mol/l) and the elution buffer (about 0.45 mol/l) until the desired osmolarity was reached at 500 and 900 mOsmol.

Sodium dodecyl sulphate-polyacrylamide gel *electrophoresis (SDS-PAGE)*

The dialysed and freeze-dried samples were dissolved in 62.5 mM Tris–HCl buffer (pH 6.8) containing 3% (w/v) of SDS, 5% (v/v) of mercaptoethanol, 10% (v/v) of glycerol and 0.001% (w/v) of bromophenol blue. In other experiments, 10-30 μl of sample were taken from the collected fractions after chromatographic separation and mixed with buffer containing five times higher concentrations of the above-mentioned substances. The amount of the buffer taken for the experiments was measured in such a way as to yield the original concentration after dilution with the sample. SDS-PAGE was carried out by the Laemmli method [10] using a mini system (Protean, Bio-Rad) or in the case of larger gels, 15 cm long, a Hoefer system (Hoefer Scientific Instruments, Vienna, Austria); 10% minigels or 5–15% large gradient gels were used. The staining of the gels was performed with 0.1% Coomassie Brilliant Blue. The amount of applied protein was between 2 and 20 μg per lane when a mini system was used and between 5 and 50 μg for large gels.

RESULTS

Isolation of antithrombin III

Antithrombin 1111 can be isolated directly from the plasma by heparin HPAC. A step gradient with buffers of 500 or 900 mOsmol is used to remove the non-specifically bound proteins and the weakly bound proteins, respectively. The antithrombin III is subsequently eluted by a step gradient with 2 M sodium chloride.

Fig. 1 shows the isolation of antithrombin III from human plasma by means of heparin HPAC. The plasma is first virus inactivated with solvent–detergent (see Experimental) and then applied to a column with immobilized heparin. The weakly bound proteins are washed out by two step gradients at 500 and 900 mOsmol. The antithrombin III is subsequently eluted with 2 M sodium chloride and tri-n-butyl phosphate. The solvent tri-n-butyl phosphate and the non-ionic detergent Triton X-100 do not bind to the column. Any remnants of solvent or detergent are removed by the first washing. The amount of Triton X-100 and tri-n-butyl phosphate in the final product was always less than 1 ppm. The multiple treatment with sodium hy-

droxide, to which the support Toyopearl Heparin is exposed in the experiments, does not impair the chromatographic performance of the column. A similar behaviour towards the exposure to sodium hydroxide is observed with the Eupergit Heparin support (Fig. 1, right-hand side, only SDS-PAGE is shown). When Toyopearl Heparin is used, only antithrombin III is eluted. However, with Eupergit Heparin additional bands appear in the eluate with 2 M sodium chloride. They represent mainly a polypeptide with an apparent relative molecular mass (M_r) of 220 000, and another polypeptide slightly larger than antithrombin III and a smaller polypeptide with an apparent M_r of about 35 000 (see SDS-PAGE in Fig. 1). The contaminants can be removed by chromatography with Toyopearl Heparin, when washed with diluted sodium chloride solution. The column with heparin agarose has lost to a great extent its binding capacity to antithrombin III after being treated only twice with 0.2 M sodium hydroxide for 2 h. This leads to a shift in the elution of this protein. Antithrombin III is eluted already in the second peak, that is, by the buffer with 900 mOsmol (see SDS-PAGE in Fig. 1).

Separation of clotting factor IX from factor X

The separation of the clotting factor IX from factor X is the last step in the isolation process of factor IX from human plasma (see Experimental and ref. 4). By anion-exchange chromatography an enriched factor IX fraction is obtained. Virus inactivation using the solvent-detergent method is carried out after elution from the anion-exchange column. Factor X is the main contaminant still present in the sample after anion-exchange HPLC. As can be seen in Table I, the sample contains almost identical activities of both factor IX and factor X after partial purification by anion-exchange HPLC.

Factor X is separated from factor IX by heparin AC. Either factor X does not bind at all to the column, or it is washed out in the washing step, using the buffer of 500 mOsmol (see Fig. 2 and Table I). Neither solvent nor detergent binds to the heparin column, and any remnants of these substances are removed in the subsequent washing step with the application buffer. Factor IX is subsequently eluted in a second step, using a step gradient at 900 mOsmol (see Fig. 2). All the investigated supports perform in a way similar to that shown in Fig. 2. Dif-

ferences between the supports exist with regard to yield and specific activity or enrichment (see Table I).

Of the four supports listed in Table I, three were investigated further. Bio-Gel Heparin was excluded from subsequent studies, as the first experiments showed that neither the yield of factor IX nor its enrichment was satisfactory (see Table I).

Table 11 shows the performance of the heparin affinity supports, after undergoing five treatments
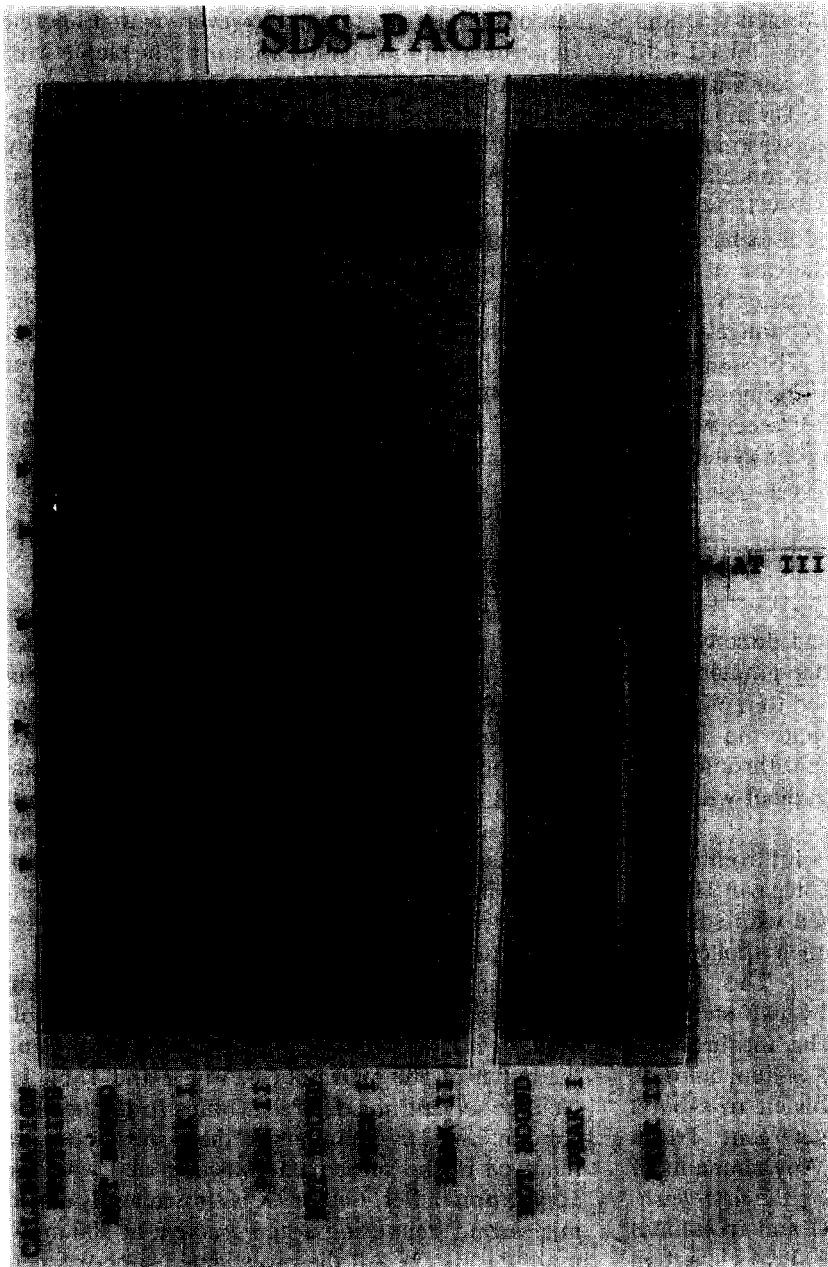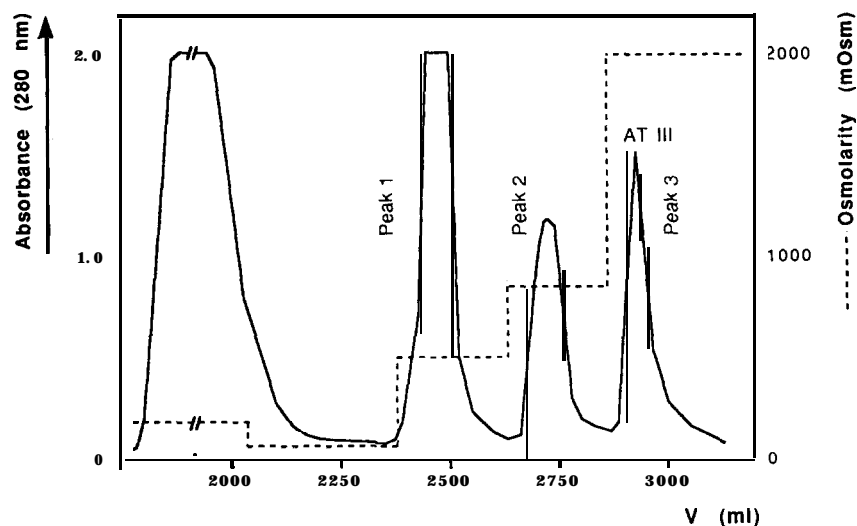

Fig. 1

Fig. 1. Isolation of antithrombin III from human plasma by heparin HPAC. Virus-inactivated human plasma (1500 ml, *ca.* 90 g of protein) was applied to a column with immobilized heparin. The bound proteins were eluted with the step gradient shown. The resulting peaks were pooled and electrophoretically represented. Chromatographic conditions: column dimensions, 200 × 20 mm I.D.; support, Toyopearl Heparin, Eupergit Heparin or Heparin Sepharose CL6B; flow-rate, 20 ml/min (10 ml/min with Heparin Sepharose); pressure, 2-3 bar; room temperature. Previous page: SDS-PAGE of the fractions that were separated on Toyopearl Heparin, Eupergit Heparin or Heparin Sepharose. Calibration proteins (from top to bottom): M_r 211 000, 94 000, 67 000, 49 000, 30 000, 20 000, 14 400. A gradient gel between 5 and 15% was used. The amount of sample loaded was *ca.* 40 μg (peaks 1 and 2) and *ca. 5 μg* (peak 3, purified antithrombin III).

with 0.2 and 0.5 A4 sodium hydroxide for 2 h each time. Although the shape of the chromatogram remains the same (not shown here), the composition of single fractions and the yield of factor IX are significantly different. The most remarkable change in this context is the mixing of factor IX and factor X in the washing step with the buffer of 500 mOsmol. A conspicuous development takes place in the column with Heparin Sepharose. After only two treatments for 2 h each with 0.5 *M* sodium hydrox-

TABLE I

SEPARATION OF FACTOR IX AND FACTOR X BY HEPARIN AFFINITY CHROMATOGRAPHY: COMPARISON OF THE SUPPORTS USED IN THE EXPERIMENTS

After preparative anion-exchange HPLC the sample contains almost identical activities of factors IX and X: the concentration of factor IX is 29-31 U/ml and that of factor X is about 27-30 U/ml.

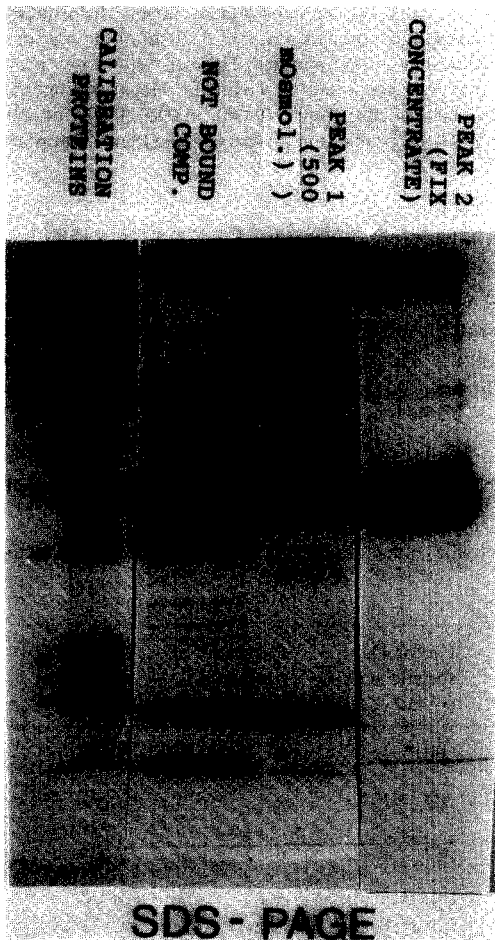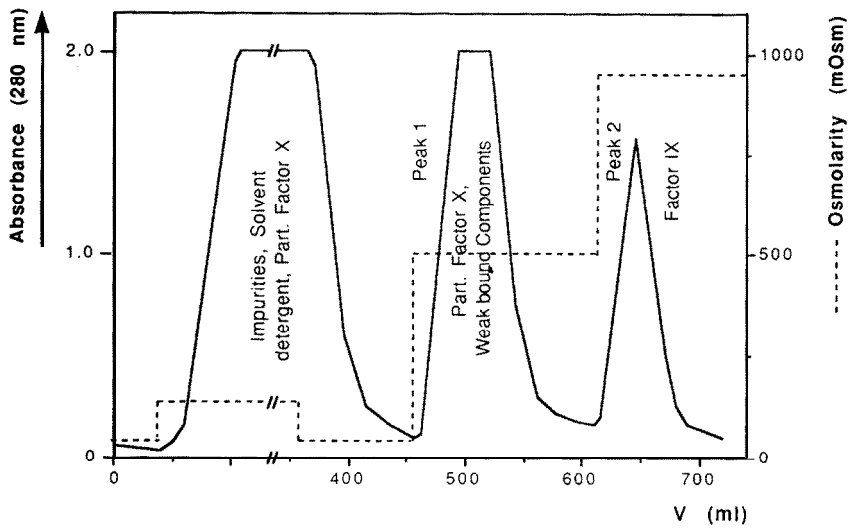| Support	Factor X in flow-through (%)	Factor X in peak I (%)	Factor X in peak II (%)	Factor IX yield (%)	Factor IX, specific activity (U/ml)	Factor IX in peak I (%) (loss)	Factor IX enrichment
Heparin Sepharose	5	93	2	50–65	80	5	21.3 ×
BioGel Heparin	3	90	7	30–40	62	8	16.5 ×
Eupergit Heparin	8	90	2	60–85	82	6	21.9 ×
Toyopearl Heparin	80	19	0	80–92	122	2	32.5 ×

Fig. 2. Separation **of** clotting factor IX from clotting factor X by heparin HPAC. Eluate from anion-exchange HPLC (150 ml, with about 3000 units each of factor IX and factor X) was dialysed, virus inactivated by the solvent-detergent method and applied to a heparin column. The column was washed with 20 mM citrate buffer (pH 7.4) (at ca. 50 mOsmol). Factor X and factor IX were subsequently eluted with a step gradient of sodium chloride. The respective activities of factor IX and factor X in each peak were determined in clotting assays. The separated peaks were represented electrophoretically, using a mini gel system. Factor X does not bind at all or only weakly to the heparin column, and appears either in the flow-through fraction or in the first peak, eluted by a buffer of 500 mOsmol. The highly enriched factor IX is eluted in the second peak by a buffer of 900 mOsmol. Chromatographic conditions: column dimensions, 100 × 20 mm I.D.; support, Toyopearl Heparin; flow-rate, 10 ml/min; pressure, 1-2 bar; room temperature. SDS-PAGE; calibration proteins (from top to bottom): M_r 21 1 000, 119 000, 98 000, 80 600, 64 400, 44 600, 38 900. A mini gel (10%) was used. The amount of sample loaded was 15 μg (peak I) or 10 μg (peak 2, factor IX concentrate).

TABLE II

SEPARATION OF FACTOR IX AND FACTOR X BY HEPARIN AFFINITY CHROMATOGRAPHY: COMPARISON OF THE SUPPORTS AFTER REPEATED TREATMENT WITH SODIUM HYDROXIDE

support	Factor X in flow-through (%)	Factor X in peak I (%)	Factor X in peak II (%)	Factor IX yield (%)	Factor IX specific activity (U/ml)	Factor IX in peak I (%) (loss)	Factor IX enrichment (peak II)
Eupergit Heparin"	3	93	3	50–70	65	10	17.3 ×
Toyopearl Heparin"	16	82	1	65-80	92	5–7	24.5 ×
Heparin Sepharose[b]	20	75	2	20–30	50	60-70	— [b]

[a] Treated five times, each time 2 h, with 0.5 M sodium hydroxide.
[b] Treated twice, each time for 2 h, with 0.2 M sodium hydroxide; factor IX was chiefly eluted in the first peak, mixed with factor X.

ide, the material binds factor IX only weakly and loses almost all of its selectivity between the factors IX and X (see Table II).

Heparin high-performance membrane affinity chromatography

Fig. 3 illustrates an option for fast process monitoring by means of heparin HPMAC. The eluate from the last peak of the HPAC column, containing purified antithrombin III (*cf*, Fig. 1), is diluted, applied to the Quick Disk Heparin separation unit and analysed with the same step gradient as used in the experiment shown in Fig. 1 (antithrombin III purification). The contamination in the antithrombin III preparation after using the Eupergit Heparin column, as detected by SDS-PAGE (see Fig. 1), ap-
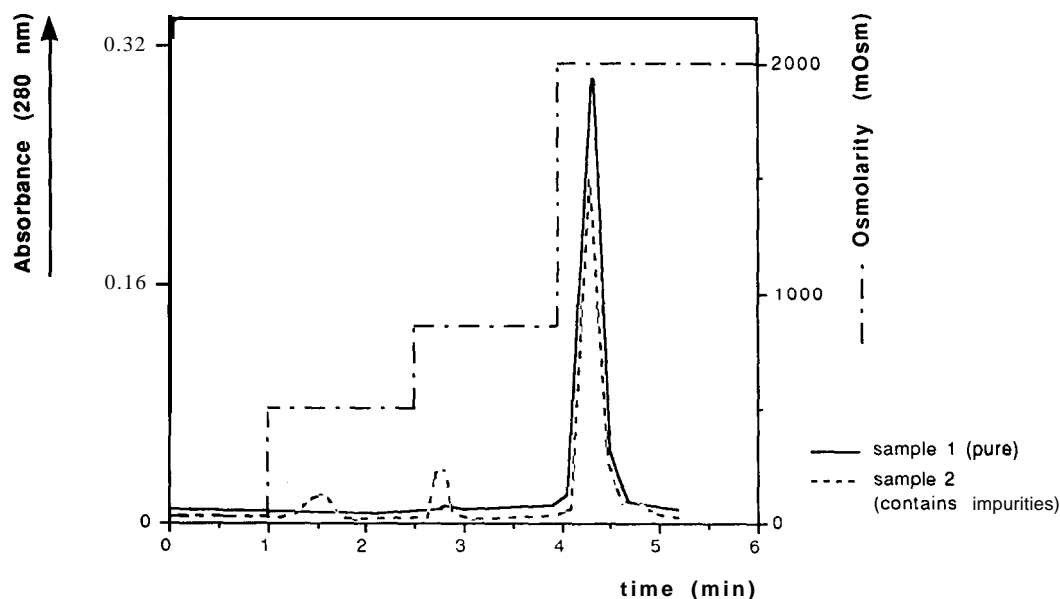

Fig. 3. Process monitoring of the isolation of antithrombin III by fast heparin HPMAC. Sample 1, antithrombin III, obtained by separation with Toyopearl Heparin (solid line); sample 2, obtained by separation with Eupergit Heparin (broken line). A 50-μl volume of sample (ca. 20 μg of protein) was diluted to 500 μl with 10 mM citrate buffer (pH 7.4) and applied to the separation unit. Chromatographic conditions: separation unit, QuickDisk Heparin, 25 mm diameter, 3 mm thickness; flow-rate, 3 ml/min; pressure, 2 bar; room temperature.
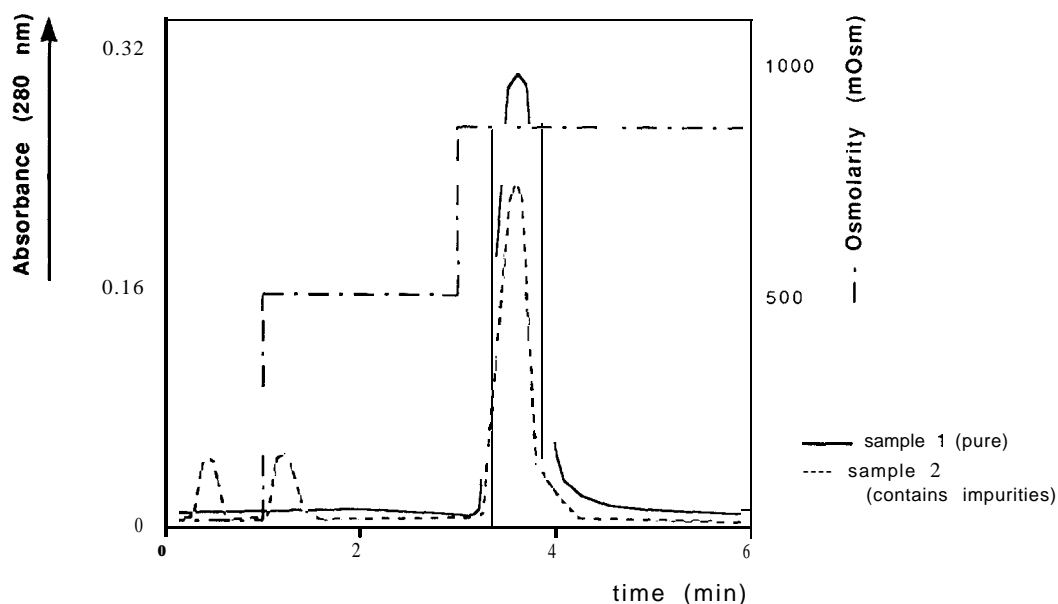
Fig. 4. Process monitoring of the isolation of factor IX by fast heparin HPMAC. Sample I. factor IX without contamination by factor X (full line); sample 2, containing minimal factor X activity apart from factor IX (broken line). A 50-μl volume of sample (cu. 75 μg of protein) was diluted with IO mM citrate buffer (pH 7.4) and applied to a QuickDisk Heparin separation unit. Chromatographic conditions as in Fig. 3

pears in the chromatogram resulting from the heparin HPMAC unit as an additional peak eluted in the second step with a 900 mOsmol buffer.

Fig. 4 shows fast affinity HPMAC using a disc with immobilized heparin, similar to Fig. 3. A sample without contamination by factor X (full line) is compared with a sample with about 5% of contamination by factor X (broken line). The contamination is the result of an impaired performance of the column, resulting from repeated treatment with 0.5 M sodium hydroxide. It can be detected at once through the analytical QuickDisk unit.

DISCUSSION

Heparin AC has considerable advantages over other affinity chromatographic methods when used for the separation of biopolymers. The binding between ligand and ligate is easily dissociated by increasing the salt concentration in the elution buffer. This ensures the stability of the separation medium, and the sample usually retains its biological activity.

The isolation of antithrombin 111 from human plasma by heparin AC has been carried out for some time even on an industrial scale, in order to produce this therapeutically important protein [1,3]. Antithrombin III binds strongly to heparin and is eluted from the column only at very high salt concentrations. In the experiments carried out here, the method was used chiefly to evaluate heparin affinity supports. The model investigations with the isolation of antithrombin III should help to develop further guidelines for choosing appropriate supports for the separation of the clotting factor IX from factor X. Heparin AC is the last and probably the most difficult step in the isolation process of factor IX. Apart from its selectivity requirements, the support has to be resistant at high pH. Treatment with 0.2-0.5 M sodium hydroxide is still the most frequently used and easiest way for column sanitizing, that is, chiefly the removal of bacterial endotoxins. Consequently, the buffers used in the separation of factor IX from factor X, namely sodium chloride of 500 and 900 mOsmol, were also chosen for the removal of the weakly bound proteins in the isolation of antithrombin III.

As in other affinity chromatographic methods,

the matrix used for immobilization plays an important part in heparin AC. The silica gel-based supports were excluded straightaway from these investigations, as they have a low stability even in weakly basic media. Consequently, supports based on natural polymers (agarose) or synthetic polymers were investigated further. The remaining hydrophobicity of the synthetic support is again an important factor. The non-specific bindings in the case of Eupergit Heparin and the poor recovery after separation on Bio-Gel Heparin probably originate in the relatively hydrophobic matrix. Although all the plasma proteins with the exception of antithrombin III are supposed to be washed out by the two previous step gradients, several additional lines appear in the elution peak when this support is used. These polypeptides are seen also in the second washing step, but they cannot be removed completely (see Fig. 1, SDS-PAGE).

When the Toyopearl Heparin support is used (see Fig. 1), non-specific bindings are much reduced. Apart from the antithrombin III, no other bands are seen in SDS-PAGE. A disadvantage of the Heparin Sepharose support is its low stability at high pH. This results in increasingly weak binding of antithrombin III after only the second treatment with 0.2 M sodium hydroxide (cf., SDS-PAGE in Fig. 1). New, untreated Heparin Sepharose binds antithrombin III very strongly, similarly to Toyopearl and Eupergit Heparin. Non-specific bindings are low, so that the protein is eluted in a virtually pure state in the third step with 2 M sodium chloride. After treatment with sodium hydroxide, however, part of the antithrombin III is eluted earlier, along with several other proteins, by the buffer of 500 mOsmol (cf., SDS-PAGE in Fig. 1).

On the one hand, the other agarose-based supports survive treatment with 0.5 M sodium hydroxide without difficulty, e.g., DEAE-Sepharose Fast Flow, which was used for the prepurification of factor IX (see Experimental). On the other hand, heparin for its part, if bound to other supports, can also be treated with sodium hydroxide without adverse effects (see above). This indicates strongly that the binding itself that exists between heparin and agarose with this support is unstable at high pH.

Heparin HPAC, when used as the final step in the purification of clotting factor IX from human plasma, cannot be regarded as affinity chromatography in the original sense. The precise nature of the interaction between the heparin and the sample components is not clearly defined. It has to be assumed that ionic interactions are the most important element. Also in this step the solvent–detergent mixture from the virus inactivation is removed from the sample (see Fig. 2 and Results). With such a sophisticated separation, the reproducibility of the results is of utmost importance. Even very small changes in the binding characteristics of the support can have an adverse effect on the separation. Besides, sanitizing of the gel with 0.5 M sodium hydroxide is necessary after each separation in order to remove any pyrogenic substances that may have formed. Heparin agarose and heparin bound to silica gel matrices are less suited for these applications, as they lack stability in basic media (cf., Fig. 1 and Tables I and II). In contrast, other media such as Eupergit Heparin, Toyopearl Heparin and QuickDisk membranes with immobilized heparin withstand repeated treatment with sodium hydroxide. Heparin, immobilized to compact, porous discs, can be used succesfully for preparative purification of factor IX (not shown here). However, this separation technique has so far been used only on an experimental scale and is described elsewhere [11]. The treatment with sodium hydroxide leads to a deterioration in the separation of factor IX from factor X. This applies to all supports, but the phenomenon is least noticeable in the case of Toyopearl Heparin (see Tables I and II). However, the separation performance of all three synthetic supports that were investigated stabilizes after the first contact with 0.5 M sodium hydroxide, and subsequent separations and yields are reproducible.

The performance of the supports in the isolation of antithrombin III from plasma is also indicative of their effectiveness in the separation of factor IX from factor X. If the support concerned shows higher non-specific binding in the isolation process of antithrombin III, as was the case here with Eupergit Heparin (see Fig. 1), the amount of other proteins in the factor IX product will also be higher, and its specific activity will be lower (cf., Table I). The inreasingly weak binding of antithrombin III to the support after treatment with sodium hydroxide indicates that under these conditions the separation between factor IX and factor X will be insufficient, e.g., in the case of Heparin Sepharose (cf., Fig. 1

and Tables I and 11). Preliminary model investigations help in choosing the likely supports. Subsequent experiments can then be carried out specifically, with a reduced number of supports, cutting the cost of the expensive factor IX samples.

The use of heparin HPMAC for fast analyses of the samples can serve as process monitoring during isolation of both antithrombin III and factor IX. Any contamination that may occur is quickly detected as additional peaks (see Figs. 3 and 4). The results obtained in this way agree with those from other analytical methods. When the antithrombin III is contaminated (broken line in Fig. 3), the substances appear as bands in SDS-PAGE.

Contaminants in the factor IX product caused by factor X (broken line in Fig. 4) are also detected in the clotting test. However, both analyses, SDS-PAGE and the clotting test, take much longer to carry out, and the necessary information is available only much later. Using QuickDisk Heparin HPMAC the results are obtained within minutes. This allows an almost immediate response, e.g., modifications of the production process or possibly corrections in the fractioning. Similar proposals for process monitoring exist in connection with other materials that allow fast chromatographic analyses, e.g., ion-exchange chromatography or immunoaffinity chromatography, using perfusion chromatographic supports [12].

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