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Application of monoliths for downstream processing of clotting factor IX

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Abstract

In this paper, the application of monolithic columns for downstream processing of different clotting factor IX concentrates is shown. Determination of basic chromatographic conditions as well as investigations on the regeneration of disk- and tube-shaped monolithic columns using human serum albumin as a model protein, were performed. Separation of factor IX and vitronectin, a possible impurity in commercial factor IX concentrates was accomplished using disk-shaped monolithic columns. These same applications were also carried out with identical results on up-scaled tube-shaped monolithic columns. Since these media allow very fast separations, this method can be successfully applied not only to an in-process control of the purification of factor IX but also to other biopolymers from human plasma. Besides, the same application on the up-scaled tube-shaped monolithic column was successfully carried out.

Keywords: Monolithic columns; Stationary phases, LC; Preparative chromatography; Pharmaceutical analysis; Factor IX

1. Introduction

The problems that arise in downstream processing of therapeutic proteins from complex mixtures are, above all, losses caused by the purification procedures, resulting in lower yield in terms of mass and biological activity. Apart from reduced yield, manipulations during downstream processing can cause minor modifications in the structure of the target protein. The modifications will not necessarily impair the activity of the product, but they may affect its antigenicity in an adverse fashion. The problem applies not only to proteins from human plasma but also to those from cell cultures [1–3].

In designing a production process, the most important factors are the speed of the process and the ability for fast in-process analysis [4,5]. The use of modern filtration and chromatographic techniques, such as sterile filtration and ultra/diafiltration (UF/DF), ion-exchange chromatography (IEC) and affinity chromatography (AC) allow the production of highly purified concentrates of therapeutic proteins and the isolation of single plasma proteins [1–3,6–8]. However, chromatographic techniques in particular have serious limitations, especially in terms of speed and capacity [9,10]. Although radial columns have reduced considerably the period of time

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required for separation [11], these methods still carry the risk of unwelcome changes or loss of activity in these sensitive molecules, a risk that can be minimized by careful investigation of the production process. Therefore in the last few decades tremendous effort to improve the column packing materials for high-speed, high-capacity, high-resolution separations of biomolecules was exerted. Chromatography with compact, porous units such as monolithic columns is being used increasingly for analytical and preparative separations of biopolymers with apparent molecular masses (M_r) ranging from several thousand to up to several million [12,13]. These monoliths are also called continuous beds and are cast as homogeneous columns or disks. They can be made of synthetic organic [4,14] or inorganic material [15,16]. Recently, even very large molecules such as plasmid-DNA have been separated with monolithic separation media [17]. Fast isocratic separations of small molecules on monoliths have also recently been reported [18,19]. The basis for fast separations with such media is a reduced mass transport resistance owing to the fact that pore diffusion is practically non-existent [20–22]. Film diffusion from the core of the mobile phase to the surface of the matrix in the interior of a through-pore is the only transport resistance [23,24]. Therefore, the period of time required for the separation is reduced by at least one order of magnitude when compared to conventional columns packed with bulk supports. Consequently, mass transport in monoliths is mainly based on convective interaction, the basis for naming one particular type of these supports as CIM, convective interaction media [15].

This report describes the use of a CIM monolithic column for fast in-process analyses and preparative separations (up-scaling) of pharmaceutically relevant biopolymers such as clotting factor IX. Human factor IX is a vitamin K-dependent multidomain glycoprotein synthesized in the liver. The concentration of factor IX in human plasma is very low. In healthy adults it amounts to 5 μg/ml, which is equivalent to 0.1 μ*M* [25]. The absence or a defect of factor IX causes hemophilia B, a genetic disease in which the clotting cascade is disturbed [25,26]. The molecular mass of factor IX is between 55 000 and 65 000 depending on the method used for its determination [27–30]. Human factor IX displays microheterogeneity with an isoelectric point in the range of pH 4.0–4.6 [28–30]. Because of the low concentration in human plasma, isolation of clotting factor IX has been performed by a combination of different chromatographic methods. However, it has not been possible to remove vitronectin, one of the final contaminants from factor IX [31] purified with conventional gel supports used in the manufacturing process of commercial factor IX preparations such as DEAE-Sepharose [10]. Vitronectin is a glycoprotein of molecular mass 78 000 with a plasma concentration of 0.2–0.4 mg/ml [32] and with an isoelectric point in the range of 4.75–5.25 [33]. This report investigates the application of CIM monolithic columns for the separation of vitronectin from factor IX and fast in-process control of factor IX. Besides, it focuses on the optimization and capacity of the process as well as on the stability of the resin in sanitation.

2. Experimental

2.1. Chemicals

PD-10 columns prepacked with Sephadex G-25 were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Rabbit anti-human factor IX (polyclonal), mouse anti-vitronectin (monoclonal, VIT-2 clone), *o*-phenylendiamine dihydrochloride (OPD) tablets, α-monothioglycerol, nitrobluetetrazolium (NBT), 5-bromo-4-chloro-3-indolylphosphate (BCIP), $AgNO_3$, were all purchased from Sigma (St. Louis, MO, USA). Mouse anti-human factor IX (monoclonal, ESN 3) was obtained from American Diagnostica (Greenwich, CT, USA).

Human serum albumin (HSA) was purchased from Behringwerke (Marburg, Germany). Bovine albumin fraction V was purchased from Serva (Heidelberg, Germany). 4–20% Tris–glycine gels, Tris–glycine sodium dodecylsulfate (SDS) running buffer, Tris–glycine SDS sample buffer, wide range protein standard and pre-stained standard were purchased from Novex (San Diego, CA, USA). Nitrocellulose transfer membrane was obtained from Schleicher and Schuell (Dassel, Germany). Fractogel EMD BioSEC (S) and other chemicals of analytical reagent grade were purchased from Merck (Darmstadt, Germany).

All chemicals for factor IX clotting assay were purchased from Instrumentation Laboratory Co. (Lexington, MA, USA). The factor IX concentrates (a commercial factor IX concentrate, factor IX laboratory batch and factor IX eluate after heparin Sepharose CL 6B [10]) were from Octapharma (Vienna, Austria). The factor IX laboratory batch was further purified by size-exclusion chromatography described elsewhere [34]. The water used for preparing eluents was twice distilled (Octapharma). Buffers used for the high-performance liquid chromatography (HPLC) system were filtered over a 0.45-μm filter (Millipore, Vienna, Austria).

2.2. Instrumentation

A BioLogic gradient chromatography system, consisting of two bio-compatible pumps, an injection valve with a 50-μl polyether ether ketone (PEEK) sample loop, a UV detector (280 nm), conductivity detector and a controller, was purchased from Bio-Rad (Hercules, CA, USA).

2.3. Columns

CIM disk monolithic columns bearing strong (quaternary amine – QA) and weak (diethylaminoethyl – DEAE) anion groups were used throughout the experimental work (BIA Separations, Ljubljana, Slovenia). CIM disk monolithic columns consist of a 3×12 mm I.D. disk-shaped poly(glycidylmethacrylate–co-ethylenedimethacrylate) highly porous polymer matrix that is seated in a non-porous self sealing fitting ring. The bed volume of one disk is 0.34 ml, 60% of which resides in the highly interconnected, flow through pores. The disk-shaped matrix (stationary phase) is inserted in commercially available PP or polyacetal housing (BIA Separations) and connected to a HPLC system.

In contrast to disk-shaped monolithic columns, where the flow of the mobile phase is in the axial direction, the scale-up units (the so-called CIM tube-shaped monolithic columns – tubes) are constructed in a different way. The monolithic material is made of the same polymer and has the same structural characteristics as the disk-shaped material, the geometry is, however, different. The tube monolith is actually an annulus with the inner diameter of 1.1 mm and the outer diameter of 15.0 mm. The length of the annulus is 45.0 mm. Thus, the bed volume of the tube is 8.0 ml. The monolithic tube is placed in a specially constructed PP or polyacetal housing. A mobile phase incoming into the monolithic column is directed into a helical groove drilled inside the body of the housing that serves as a distributor. In this way, the mobile phase is distributed over the entire outer surface of the monolith. The construction of the helical groove is such, that the mobile phase is forced to penetrate into the monolithic annulus and to run from the outer surface of the monolithic material in a radial direction into the central hole where it is collected. The mobile phase exits through the bore of the outlet end-fitting which is connected to the UV–Vis detector by means of PEEK or PTFE capillaries (0.5–1.0 mm I.D.).

2.4. Buffers

The binding buffer used for the determination of the dynamic binding capacity of factor IX and for the separation of different factor IX preparations on CIM DEAE and QA monolithic columns was a 20 m*M* Tris–HCl buffer, pH 7.4. As the elution buffer, a 20 m*M* Tris–HCl buffer with 1 *M* sodium chloride, pH 7.4 was used.

2.5. Methods

2.5.1. Electrophoretic techniques

Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out under reducing and non-reducing conditions according to Laemli [35] using a Novex system. Separations were carried out for 1 h at 200 V on 4–20% Tris–glycine polyacrylamide gels. The amount of protein applied was 500 ng per lane. Protein bands were made visible by silver staining [36]. Immunoblot of electrophoretically separated proteins was performed against polyclonal and monoclonal anti-factor IX antibodies and monoclonal anti-vitronectin antibodies. Antibody–antigen complexes were detected as colored bands by BCIP and NBT.

2.5.2. Determination of factor IX activity

A one-stage clotting assay for factor IX was performed by mixing factor IX-deficient plasma with

the diluted sample, in the presence of lipid extract and kaolin as activators. Coagulation was triggered by adding $CaCl_2$ and the time required for a clot to form was measured. The measurements were carried out with an ACL300 coagulometer from Instrument Laboratory (Milan, Italy).

3. Results and discussion

3.1. Testing the reproducibility of the dynamic binding capacity of factor IX and HSA on QA and DEAE disk-shaped monolithic columns after the regeneration procedure

Two different matrices bearing weak (DEAE) and strong (QA) anion-exchange groups were tested for their capability of binding factor IX and HSA. Additionally, the possibility of an efficient regeneration of both types of columns, that is essential for reproducible work, was checked. For the determination of the dynamic binding capacity of HSA on a disk, HSA was dissolved in the binding buffer in a concentration of 1 mg/ml and approx. 15 mg of albumin/run was applied on a disk at a flow-rate of 3 ml/min. Determination of dynamic binding capacity of factor IX on a disk was performed as follows: the commercial factor IX concentrate (with a specific activity of 65 IU/mg) was gel-filtrated on the PD-10 columns using the binding buffer and the final activity was adjusted to 80 IU/ml. Around 1000 IU/run was applied on the column at a flow-rate of 3 ml/min in each run. In both cases, bound proteins were eluted with buffer containing 1 *M* NaCl. The column was then flushed with 0.5 *M* sodium hydroxide (10 column volumes) and thoroughly washed with the binding buffer until the pH from the column effluent reached the value of 7.4. This same procedure was repeated five times. The binding capacity was determined at 10% breakthrough [37]. In five consecutive runs, the capacities of factor IX and HSA on the QA disk were 1983 ± 120 IU/ml and 32 ± 1.2 mg/ml, respectively. The capacities of factor IX and HSA on the DEAE disk were 1690 ± 64 IU/ml and 32 ± 2.9 mg/ml, respectively. It can be seen, that the capacities of HSA were almost the same on both disk matrices. On the other hand, the binding capacity for factor IX on a QA disk was about 20% higher than on a DEAE disk. The capacity of both proteins on the two columns, QA and DEAE monolithic columns, remained practically constant from run to run demonstrating in this way a possibility of an efficient regeneration procedure using 1 *M* sodium chloride and 0.5 *M* sodium hydroxide.

3.2. Optimization of chromatographic conditions for an analytical in-process control of factor IX using monolithic columns

To investigate the possibility of using QA and DEAE disk for an in-process control as well as the separation of vitronectin from factor IX, two different factor IX preparations were analyzed: a commercial factor IX preparation (''FIX CP'' with the specific activity of 65 IU/mg) and a factor IX laboratory batch (''FIX LB'' with the specific activity of 35 IU/mg). The first factor IX preparation was produced in Octapharma production facilities and the second was a laboratory batch from the R&D Department with a high content of accompanying plasma proteins and therefore low specific activity. Both samples were applied to a QA disk and analyzed under the conditions of a linear gradient mode of sodium chloride (see Fig. 1A). One sharp peak (eluted with 46% of buffer B) and one broad peak (eluted with 57% of buffer B) were obtained. Analysis of fractions by performing the factor IX clotting assay indicated that all factor IX activity was present in the first peak.

In Fig. 1B the analysis of both factor IX preparations on a QA disk using a step gradient mode with 40, 70 and 100% of buffer B is shown. Both factor IX samples were separated in two fractions – one peak was eluted with 40% buffer B and the second one with 70% of buffer B. For both samples, factor IX activity was found in the first peak. Although the clotting activities of both samples were the same, the absorbance of FIX LB (with a high content of other plasma proteins) in the peak eluted with 40% buffer B was greater than that of FIX CP. This meant that in the case of FIX LB, accompanying proteins were co-eluted in the first peak indicating that under the conditions used it was not possible to separate them from factor IX on a QA disk.

In Fig. 2A the analysis of FIX LB and FIX CP on

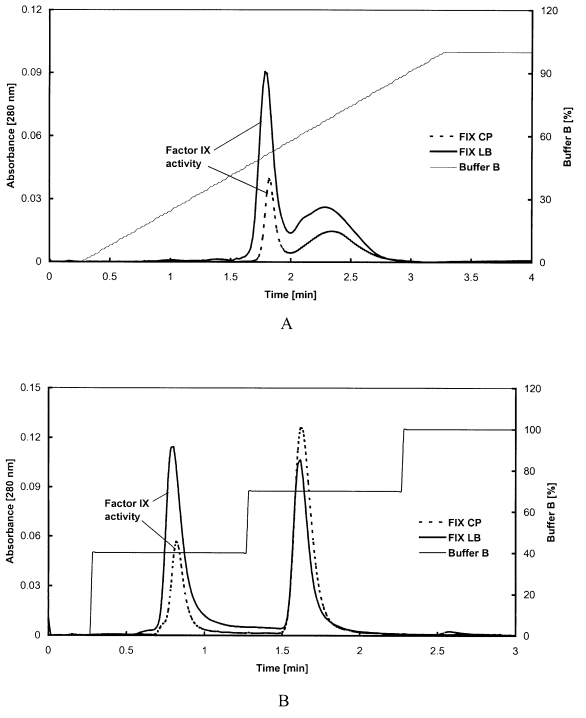

Fig. 1. Optimization of chromatographic conditions for the separation of factor IX from accompanying plasma proteins in FIX CP and FIX LB on a QA disk. 5.5 factor IX IU of each concentrate (FIX CP and FIX LB) was applied to a QA disk. Flow-rate was 4 ml/min. The elution curves of both runs are superimposed. (A) Linear gradient (0–100% of buffer B/3 min), (B) step gradient (40, 70, 100% of buffer B).

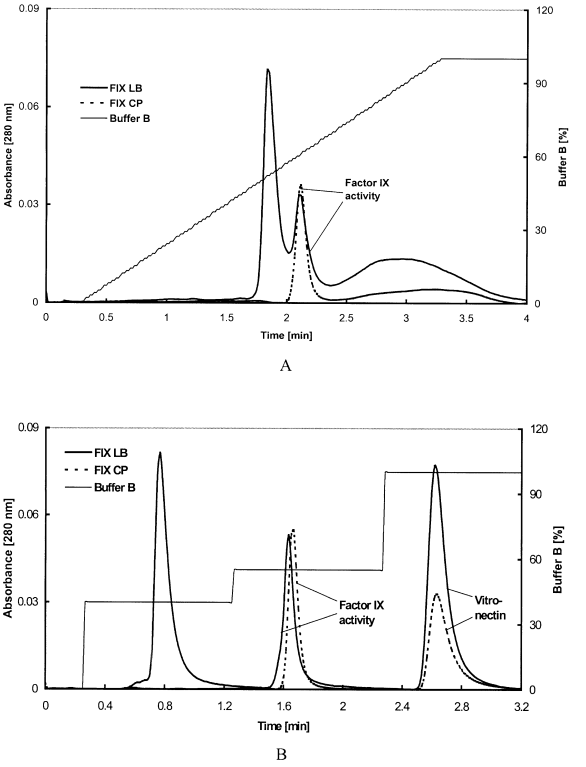

Fig. 2. Optimization of chromatographic conditions for the separation of factor IX from accompanying plasma proteins in FIX CP and FIX LB on a DEAE disk. 5.5 factor IX IU of each concentrate (FIX CP and FIX LB) was applied to a DEAE disk. Flow-rate was 4 ml/min. The elution curves of both runs are superimposed. (A) Linear gradient (0–100% of buffer B/3 min), (B) step gradient (40, 55, 100% of buffer B).

a DEAE disk is demonstrated. Both samples were analyzed on a DEAE disk by applying the same linear gradient mode as in the case of a QA disk. By using this linear gradient, FIX LB was separated into three fractions. The first, second and third fractions appeared as peaks eluted, respectively, in 48, 58 and 68% of buffer B. Factor IX activity was found in the second peak eluted with 58% of buffer B. FIX CP was separated into only two fractions (the same as on a QA disk), first (with factor IX activity) eluting at 58% of buffer B. In contrast to QA disk, factor IX bound stronger to a DEAE disk making it possible to separate the impurities in FIX LB from factor IX.

Fig. 2B shows the application of a step gradient on a DEAE disk with 40, 55 and 100% of buffer B. FIX LB was separated in three well resolved peaks, whereas FIX CP could be separated into only two peaks. Factor IX activity was found only in the protein fraction eluted with the step gradient at 55% of buffer B. The separated fractions were collected and subjected to SDS–PAGE under reducing conditions and immunoblot analysis with polyclonal antibodies against factor IX and monoclonal antibodies against vitronectin (Fig. 3A–C). At least three protein bands between molecular masses of 66 300 and 55 400 in the FIX CP were resolved by SDS–PAGE (Fig. 3A, lane 1). In addition to protein bands in the same size, FIX LB contained proteins of molecular mass in the range of approx. 200 000. (Fig. 3A, lane 4). Separated peaks from FIX CP showed one or two protein bands with a molecular mass close to that of factor IX (Fig. 3A, lanes 2 and 3). Distribution of protein bands in three separated peaks from FIX LB is illustrated in the same figure,

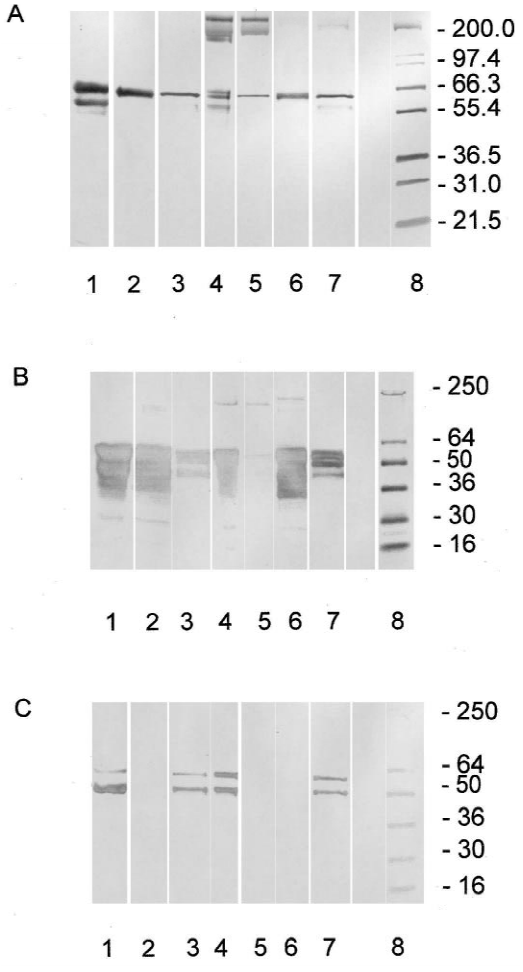

Fig. 3. SDS–PAGE and immunoblot during optimization of chromatographic conditions for factor IX purification on a DEAE disk (see Fig. 2B). A 500-ng amount of protein was applied to each lane. (A) 4–20% SDS–PAGE (under reducing conditions), (B) immunoblot with polyclonal antibodies against factor IX, (C) immunoblot with monoclonal antibodies against vitronectin. The following samples were applied to the gels: 1=FIX CP, 2=peak I of FIX CP, eluted with 55% of buffer B (see Fig. 2B), 3=peak II of FIX CP, eluted with 100% of buffer B (see Fig. 2B), 4=FIX LB, 5=peak I of FIX LB, eluted with 40% of buffer B (see Fig. 2B), 6=peak II of FIX LB, eluted with 55% of buffer B (see Fig. 2B), 7=peak III of FIX LB, eluted with 100% of buffer B (see Fig. 2B), 8=molecular size marker.

on lanes 5–7. In immunoblots using polyclonal antibodies against factor IX, reactive factor IX bands are visible in both the starting material as well as in the separated peaks (Fig. 3B, lanes 1–7). However, immunoblots with monoclonal antibodies against vitronectin confirmed vitronectin in both the starting material (FIX CP and FIX LB, Fig. 3C, lanes 1 and 4) and in the peak eluted with 100% of buffer B (Fig. 3C, lane 3 in the separation of FIX CP and lane 7 in FIX LB separation). Vitronectin was not found in the peak where factor IX activity was measured (Fig. 3C, lane 2 in FIX CP and lane 6 in FIX LB separation). These results confirmed that factor IX and vitronectin were eluted, respectively, in 55 and 100% of buffer B. As seen in Figs. 1 and 2, all analyses were completed within 3–4 min. Depending on the technical possibilities of the HPLC system (time constant of detector, short connection from injection to detector), this time scale could be further shortened by 2–3-fold. Because of this very fast analysis time, the method can be successfully applied to an in-process control of a purification process consisting of several steps, especially on the preparative scale.

3.3. Scale-up to a DEAE 8-ml tube

3.3.1. Testing the reproducibility of the dynamic binding capacity of HSA on a DEAE tube after the regeneration procedure

From the previous results it was observed that dynamic binding capacity decreased if the regeneration procedure was not performed between two consecutive determinations (data not shown). Therefore, the dynamic binding capacity of HSA on a DEAE tube was again determined as a function of the regeneration procedure. To determine if an efficient scale-up to an 8-ml DEAE tube was possible, the dynamic binding capacity was determined in five consecutive runs. HSA was dissolved in the working buffer and the concentration was adjusted to 3 mg/ml. The flow-rate was 8 ml/min and approx. 300 mg of HSA/run was applied to the tube. The binding capacity was determined at 10% breakthrough. Regeneration with 1 *M* sodium chloride and 0.5 *M* sodium hydroxide was performed between different runs in the same way as on the disk. After each regeneration step, the tube was thoroughly washed with the binding buffer to obtain a correct pH. Results show that capacity was very constant and varied between 28.2 and 30.0 mg/ml (29.1 ± 0.9 mg/ml).

3.3.2. Determination of the dynamic binding capacity of factor IX on a DEAE tube

The capacity of factor IX on the 8-ml DEAE tube was determined in the same way as on the disk except the flow-rate was 8 ml/min. Applied factor IX preparation had a specific activity of 150 IU/mg (in contrast to commercial factor IX preparation which had 65 IU/mg) due to an additional size-exclusion chromatographic step on Fractogel EMD BioSEC (S) gel [31].

During the run different pools were collected and analyzed for factor IX activity and protein content. Bound proteins were eluted by using 1 *M* sodium chloride. Almost 44 000 IU of factor IX was applied on a DEAE tube and 77% of this amount was found in the eluted peak. The remaining activity of 20% was found in the other pools giving a total recovery of 97%. The capacity of factor IX on a DEAE tube was calculated in two different ways: from the breakthrough curve at 10% and by determination of factor IX activity in the eluted peak. The capacity from breakthrough curve was 3197 IU/ml. However, from the amount of the bound factor IX, a higher binding capacity of 4230 IU/ml was calculated. For comparison, the same material was used for determination of dynamic capacity on a DEAE disk. Dynamic binding capacity measured from breakthrough curve was 3360 IU/ml and from the eluted peak of factor IX preparation was 5350 IU/ml. By opposing the capacities on a DEAE disk and a DEAE tube, it can be recognised that the capacity of the tube was about 20% lower than that of the disk. These lower capacities may be due to insufficient saturation of the tube with factor IX since the application of factor IX was stopped before a plateau of the breakthrough curve was reached.

These capacity studies showed higher dynamic binding values than that described in Section 3.1 where 1690 IU per ml disk was determined. Different specific activities of the factor IX concentrates used for the determination were made responsible for that effect. A specific activity of 150 IU/mg from the studies described here face a specific activity of 65 IU/mg in the previous experiments. Therefore, the use of factor IX concentrates with higher specific activities led to increased dynamic binding capacities. This effect was expected since factor IX concentrates with lower specific activity contain other plasma proteins which might bind. In our case it was shown that the main protein impurity was vitronectin that has also an isoelectric point in the acidic range. In capacity studies vitronectin competes with factor IX for binding to the anionic ligands.

3.3.3. Preparative separation of vitronectin from factor IX on a DEAE tube

It was already shown that it was possible to separate factor IX and vitronectin from commercial concentrate of factor IX on DEAE disk using 55% of buffer B for factor IX elution (Fig. 2B). The same application was scaled-up on a DEAE tube. For this purpose, factor IX containing eluate from heparin-Sepharose (specific activity 153 IU/mg) was used.

Fig. 4 illustrates the elution profile after application of 20 000 IU of factor IX (60% of total capacity) on a DEAE tube. When bound proteins were eluted with 55 and 100% of buffer B, two well separated peaks were obtained. Results show that factor IX activity was mainly found in the first peak (93%) and that the specific activity of factor IX had increased from 153 to 190 IU/mg of protein.

Additional analysis by SDS–PAGE under reducing and non-reducing conditions (Fig. 5A), and by immunoblot using monoclonal antibodies against factor IX (Fig. 5B) confirmed that factor IX was eluted with 55% of buffer B. However, results on immunoblot with monoclonal antibodies against vitronectin (Fig. 5C) revealed that a part of the vitronectin was eluted with a lower salt concentration (55% B) than that needed for DEAE disk. However,

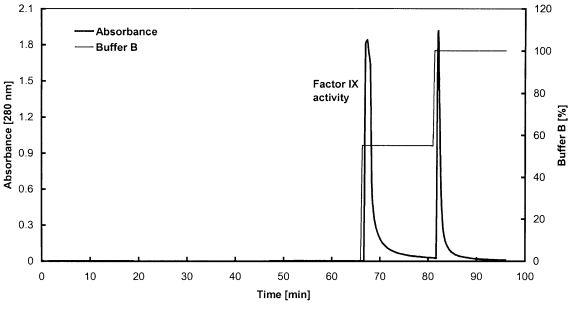

Fig. 4. Separation of factor IX from vitronectin on a DEAE tube. A 20 000-IU amount of factor IX with specific activity of 153 IU/mg was applied to a DEAE tube. Flow-rate was 8 ml/min and bound proteins were eluted with 55 and 100% of buffer B.

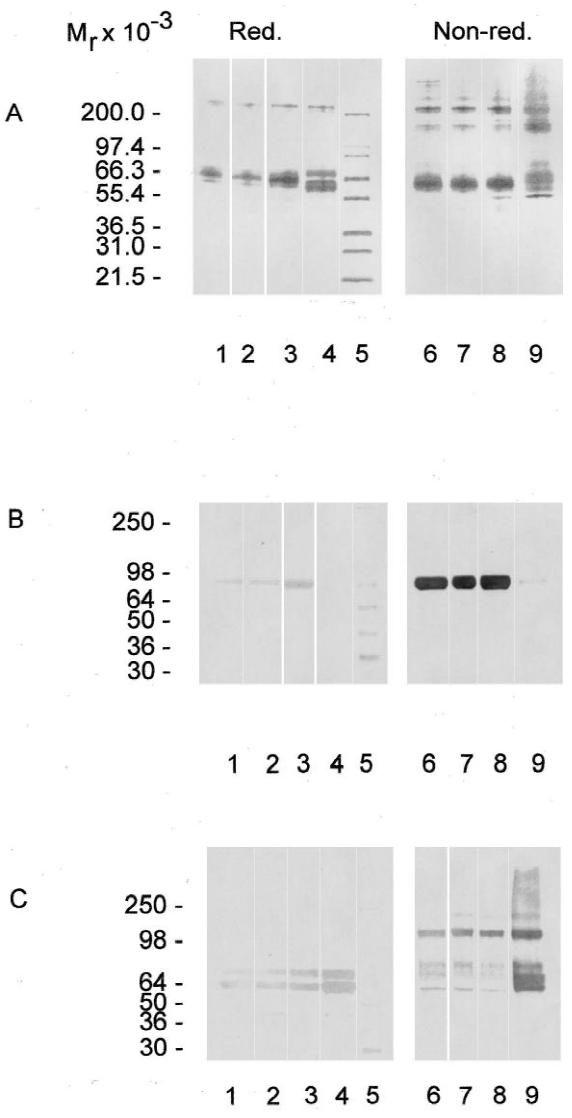

Fig. 5. SDS–PAGE and immunoblot of eluted peaks during separation of factor IX from vitronectin on a DEAE tube (see Fig. 4). A 500-ng amount of protein was applied to each lane. (A) 4–20% SDS–PAGE under reducing and non-reducing conditions, (B) immunoblot with monoclonal antibodies against factor IX, (C) immunoblot with monoclonal antibodies against vitronectin. The following samples were applied to the gels under reducing conditions: 1=FIX LB before gel-filtration, 2=FIX LB after gel-filtration, 3=peak eluted with 55% of buffer B (see Fig. 4), 4=peak eluted with 100% of buffer B (see Fig. 4), 5=molecular size marker. The same samples were applied to the gels under non-reducing conditions (lanes 6–9).

most of the amount of vitronectin was found in the peak eluted with 100% of buffer B.

Rapid separation on the DEAE disk confirmed the results from SDS–PAGE and immunoblot analyses. In this experiment, both peaks (the first one eluted with 55% and the second one eluted with 100% of buffer B) were desalted and applied to a DEAE disk. Results are presented in Fig. 6. In the first peak from DEAE tube, factor IX and vitronectin were identified according to their retention times (Fig. 6A). In the second peak from DEAE tube, only vitronectin was detected (Fig. 6B). Here the efficacy of DEAE disk as a very fast and efficient analytical tool is demonstrated.

Because there was only partial separation of factor IX from vitronectin in the first step of ion-exchange

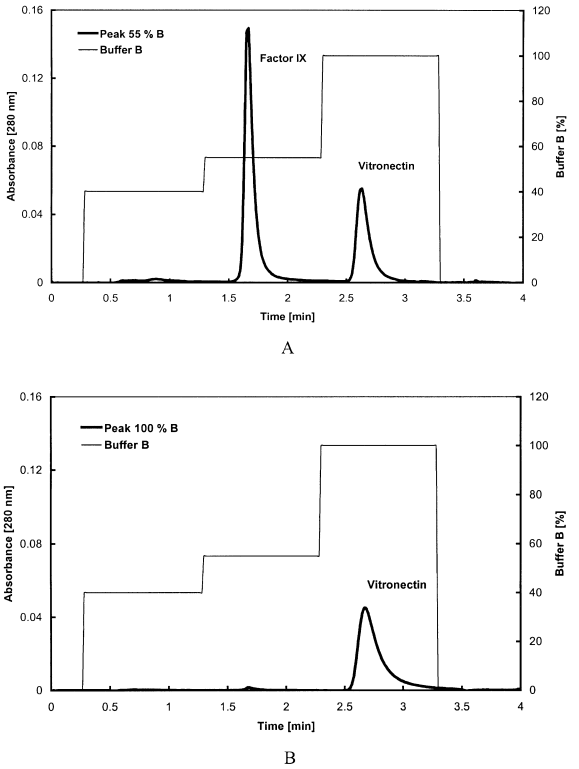

Fig. 6. In-process control of eluted peaks during separation of factor IX from vitronectin on a DEAE tube (see Fig. 4) on a DEAE disk. Eluted peaks from tube were desalted on PD-10 columns and 100 μl was applied to a DEAE disk. Flow-rate was 4 ml/min and bound proteins were eluted with 55 and 100% of buffer B. (A) Peak eluted with 55% of buffer B, (B) peak eluted with 100% of buffer B.

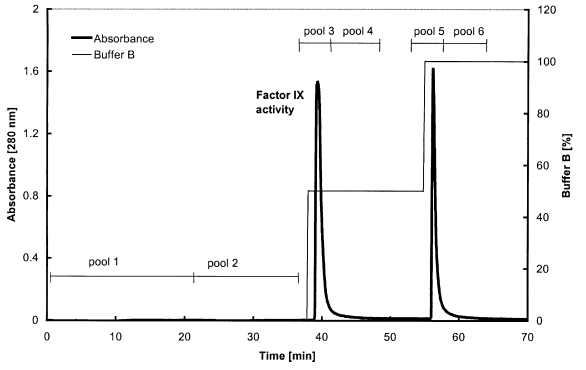

Fig. 7. Separation of factor IX from vitronectin on a DEAE tube (rechromatography). An 8000-IU amount of factor IX from the first chromatography and with a specific activity of 190 IU/mg was applied to a DEAE tube. Flow-rate was 8 ml/min and bound proteins were eluted with 50 and 100% of buffer B.

chromatography, the first eluted peak from the previous experiment containing both factor IX and vitronectin was desalted and applied to a DEAE tube (rechromatography). During the optimization process it was confirmed that factor IX could be separated from vitronectin on a DEAE tube using 50% of buffer B (data not shown). This condition was used for the elution of bound factor IX in rechromatography on a DEAE tube (see Fig. 7). As shown in Table 1, when 8000 IU of factor IX was applied to a DEAE tube, 96% of factor IX was found in the first peak eluted with 50% B (Table 1, pool 3) versus $<4\%$ in the second peak eluted with 100% B. Analysis of the starting material and the eluted peaks by SDS–PAGE under reducing conditions (Fig. 8A) identified factor IX as protein band with molecular mass between 66 300 and 55 400 in both starting materials (for the first chromatography and for rechromatography, Fig. 8A, lanes 2 and 3) and in the first peak eluted with 50% B (Fig. 8A, lane 4). In immunoblots labeled with monoclonal antibodies against vitronectin, reactive vitronectin bands are visible in the starting material (Fig. 8B, lanes 2 and 3) as well as in the fraction eluted with 100% B (Fig. 8B, lane 5). However, vitronectin was not found in the first peak where factor IX was eluted (Fig. 8B, lane 4). In-process control on a DEAE disk using already described optimized chromatographic conditions (see above) was also performed. When the first peak from rechromatography was applied on a DEAE disk, only one peak eluted with 55% of buffer B was obtained (Fig. 9A). The second peak from rechromatography was applied on a DEAE disk and one peak eluted with 100% of buffer B was observed (Fig. 9B). Taking into consideration the results during optimization procedure of monolithic columns for factor IX separation, it was concluded that the first eluted peak on a DEAE disk contains factor IX and the second peak vitronectin. This means that a complete separation of factor IX from vitronectin was achieved by performing rechromatography on a DEAE tube. However, it can be speculated that a complete separation of factor IX and vitronectin could be obtained in only one ion-exchange chromatography step by applying the optimized gradient conditions or a lower amount of factor IX.

In Table 2, the increase of specific activity during purification of factor IX on DEAE tube is shown. After the first step, the specific activity was increased from 153 to 190 IU/mg. After the second step, the

Table 1
Analysis of collected pools during rechromatography on DEAE tube (separation of factor IX from vitronectin, see Fig. 7)

Sample	Volume (ml)	Factor IX activity (IU/ml)	Total activity (IU)	Protein concentration (mg/ml)	Total protein (mg)	Specific activity (IU/mg)	Recovery of total activity (%)
Applied material	163	49.9	8133.7	0.27	44.0	185.5	100
Pool 1	168.5	0.02	3.37	0.02	3.4	1	0.04
Pool 2	107.2	0.02	2.14	0.02	2.1	1	0.03
Pool 3	24.7	316	7805.2	1.0	24.7	316	96
Pool 4	45.3	2.3	104.19	0.02	0.9	115	1.3
Pool 5	22.0	0.73	16.1	0.49	10.8	1.5	0.2
Pool 6	56.6	0.05	2.83	0.02	1.1	2.5	0.03
Σ Pools			7939.34		43.0		97.6

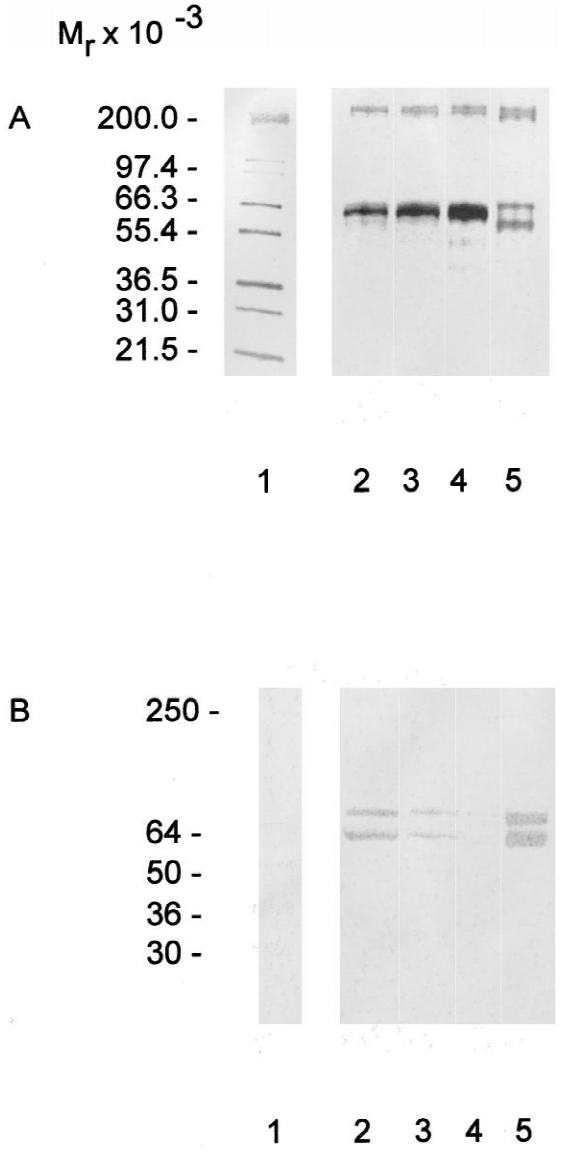

Fig. 8. SDS–PAGE and immunoblot of eluted peaks during separation of factor IX from vitronectin on a DEAE tube (rechromatography, see Fig. 7). A 500-ng amount of protein was applied to each lane. (A) 4–20% SDS–PAGE under reducing conditions, (B) immunoblot with monoclonal antibodies against vitronectin. Following samples were applied to the gels: 1= molecular size marker, 2=factor IX starting material for the first chromatography, 3=factor IX starting material for the rechromatography, 4=peak eluted with 50% of buffer B (see Fig. 7), 5=peak eluted with 100% of buffer B (see Fig. 7).

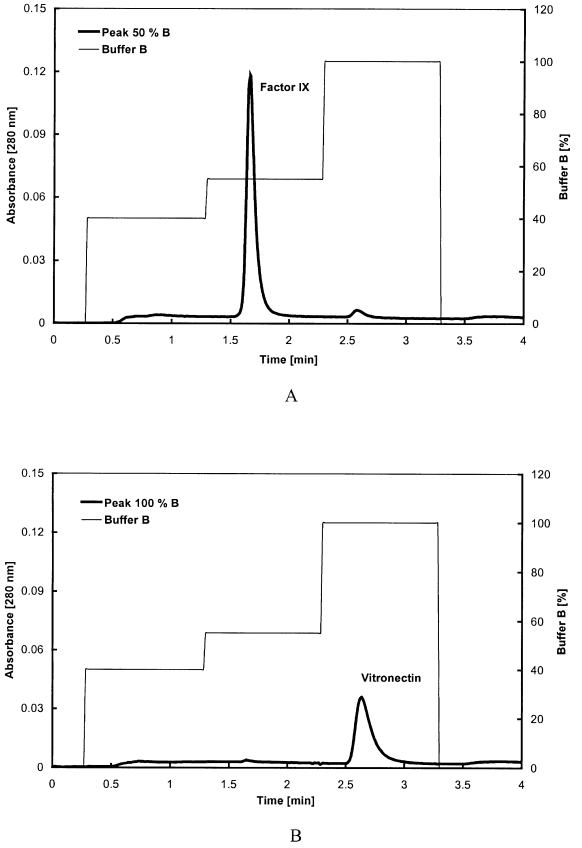

Fig. 9. In-process control of eluted peaks during separation of factor IX from vitronectin on a DEAE tube (rechromatography, see Fig. 7) on a DEAE disk. Eluted peaks from tube were desalted on PD-10 columns and 100 μl was applied to a DEAE disk. Flow-rate was 4 ml/min and bound proteins were eluted with 55 and 100% of buffer B. (A) Peak eluted with 50% of buffer B (see Fig. 7), (B) peak eluted with 100% of buffer B (see Fig. 7).

theoretical specific activity of 316 IU factor IX/mg protein was reached and vitronectin was successfully removed from factor IX during this step.

4. Conclusion

(1) The dynamic binding capacity of HSA on both disk and tube as well as on both types of anionic exchangers (strong and weak) was shown to be reproducible, proving that the regeneration procedure used (1 *M* NaCl and 0.5 *M* NaOH) was successful

Table 2
Summary of factor IX separation from vitronectin by two subsequent chromatographic steps on a DEAE tube

Sample	Factor IX activity (IU/ml)	Protein concentration (mg/ml)	Specific activity (IU/mg)
Starting material	46	0.30	153
The first chromatography on a DEAE tube (peak with factor IX activity)	358	1.88	190
The rechromatography on a DEAE tube (peak with factor IX activity)	316	1.00	316

and efficient. The dynamic binding capacity of HSA on both types of monolithic columns (disk and tube) were constant and comparable (about 30 mg/ml monolith).

(2) The dynamic binding capacity of factor IX on disk was 20% higher on the QA disk compared to the DEAE disk. One reason may be the different properties of weak (DEAE) and strong (QA) ionic exchangers. A comparison of the dynamic binding capacities of factor IX on the DEAE disk and DEAE tube shows a higher capacity on the disk. Chromatographic conditions responsible for a 20% lower capacity of factor IX on the DEAE tube when compared to the DEAE disk have to be investigated in further experiments.

(3) The DEAE disk showed better chromatographic properties for the separation of impurities from factor IX than the QA disk. The advantageous chromatographic behavior and the very short analysis time thus makes these monolithic columns a very useful analytical tool for fast factor IX in-process control.

(4) The optimal chromatographic conditions described in this paper made it possible to separate vitronectin from factor IX using monolithic columns in a commercial plasma derived factor IX concentrate. This finding is important because these two proteins were not separated in some commercially available factor IX concentrates that are purified by performing chromatography on conventional ion exchangers. Because of this, vitronectin is one of the contaminant of factor IX in commercial concentrates. A complete separation of factor IX and vitronectin was achieved on a disk in one-step and on a tube by two subsequent chromatographic steps. These results were confirmed by conventional analytical methods such as SDS–PAGE and immunoblot as well as by fast in-process control using the DEAE disk.

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