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Application of semi-industrial monolithic columns for downstream processing of clotting factor IX

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Abstract

It has been shown in a previous study that monolithic columns can be used for downstream processing of different concentrates of clotting factor IX [K. Branović et al., J. Chromatogr. A 903 (2000) 21]. This paper demonstrates that such supports are useful tools also at an early stage of the purification process of factor IX from human plasma. Starting with the eluate after solid-phase extraction with DEAE-Sephadex, the use of monolithic columns has allowed much better purification than that achieved with conventional anion-exchange supports. The period of time required for separation is also much reduced. In up-scaling experiments, separations are carried out with 8, 80 and 500 ml columns. A volume of 1830 ml of DEAE-Sephadex eluate, containing a total of 27.6 g of protein and 48 500 IU of factor IX is applied to the 500 ml monolithic column. This corresponds to a separation on a pilot scale. The results of this separation after up-scaling are comparable to those obtained with the 8 ml column on a laboratory scale.

Keywords: Monolithic columns; Preparative chromatography; Factor IX; Vitronectin

1. Introduction

Therapeutic proteins are almost always isolated from very complex starting materials. This applies to proteins from natural sources as well as to proteins synthesized with recombinant technology. Human plasma is one of the natural raw materials, from which a number of therapeutic proteins are isolated. Some of these proteins, among them clotting factors and clotting inhibitors, are found in plasma in very small concentrations only [1]. Besides, many substances have very complex structures and are sensitive towards cleavage and other modifications during isolation [2,3]. Some of the losses during downstream processing may be caused by the purification procedures, resulting in lower yield in terms of mass and biological activity. Manipulations during the production process can cause minor changes in the structure of the target substance. The modifications will not necessarily impair the in vitro activity of the product, but they may affect its antigenicity in an adverse fashion [4,5].

In process optimization, the most important factors are reproducibility, yield and speed of the process, but also the possibility of fast in-process control [6].

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Modern filtration and chromatographic techniques allow the production of highly purified concentrates of therapeutic proteins, either of recombinant or of natural origin [1,2,7]. However, the applied chromatographic techniques may have serious limitations, especially with regard to speed and capacity [8,9].

Therefore, over the last two to three decades, vast efforts have been made to improve the chromatographic techniques and column packing materials in order to achieve high-speed, high-capacity and high-resolution separations of biomolecules [10–13].

Continuous chromatography is one of the options for optimizing chromatographic separations for higher speed and productivity. Iberer et al. [14] have carried out continuous, annular chromatography (CAC) of plasma proteins into size-exclusion mode. The productivity of the continuous process in this case has been even higher than the productivity of the optimal batch process. In a subsequent paper, the same group has shown that the separation of clotting factor IX and vitronectin (VN) from a commercial factor IX concentrate can be performed by CAC in the anion-exchange mode. However, due to technical and constructive shortcomings of the continuous chromatographic device used in the experiments, the productivity of the optimal batch process has exceeded that of the process with preparative CAC [15].

Previously, Branovic et al. [16] have reported the use of monolithic columns for the separation of factor IX and VN from plasma derived factor IX (pdFIX) concentrates in anion-exchange mode. Following appropriate up-scaling experiments, 20 000 IU of factor IX have been separated on an 8 ml tube-shaped monolithic column. The period of time required for the separation was much shorter compared to conventional supports such as DEAE-Sepharose Fast Flow.

A subsequent paper reports further up-scaling of the separation process using monolithic columns up to 500 ml of column volume. Instead of highly purified pdFIX concentrates, which apart from factor IX only contain larger quantities of VN [17], samples with lower specific activities have been used in this case, taken at very early stages of factor IX downstream processing.

2. Experimental

2.1. Clotting factor IX

The factor IX samples were intermediate products (eluate from DEAE-Sephadex A-50) from the production of the pdFIX concentrate Octanine F by Octapharma (Vienna, Austria). The production process is described elsewhere [18].

Sephadex eluate was diafiltrated in binding buffer (20 m*M* Tris–HCl, pH 7.4) before application to the different scaled monolithic columns. For the 8 ml tube PD10 columns from Amersham Biosciences were used according to the manufacturer's instructions. For the large-scale experiments (80 and 500 ml tube) the Sephadex eluate was diafiltrated by a Filtron minisette system (PALL, Dreieich, Germany) using polyether sulfone membranes with an M_r cut-off of 10 kD.

The applied amount of diafiltrate is indicated in Tables 1–4.

2.2. Chemicals

o-Phenylenediamine dihydrochloride (OPD) tablets, α-monothioglycerol, nitrobluetetrazolium (NBT), 5-bromo-4-chloro-3-indolylphosphate (BCIP), $AgNO_3$, were all purchased from Sigma (St. Louis, MO).

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). The water used for preparing eluents was twice distilled (Octapharma). Buffers used were filtered over a 0.45-μm filter (Millipore, Vienna, Austria).

2.3. 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 BioRad (Hercules, CA). This system was used to run the 8 ml tubes.

An AKTA system was used for preparative isola-

tion of factor IX (Amersham Biotech, Uppsala, Sweden) on 80 ml tubes.

Preparative HPLC system consisting of two Wellchrom K-1800 pumps and a K-2500 UV detector was from Knauer (Berlin, Germany) and used for the separation on 500 ml tubes.

2.4. Columns

The tube shaped monolithic columns (CIM® ''convective interaction media'') made of highly porous poly (glycidyl methacrylate–co-ethylene dimethacrylate) were from BIA Separations, Ljubljana, Slovenia. Tube-shaped monoliths at volumes of 8, 80 and 500, respectively were used.

Column constructions and details about up-scaling are described elsewhere [16,19]

2.5. Buffers

The binding buffer used for the separation of Sephadex eluate on CIM DEAE monolithic columns was a 20-m*M* Tris–HCl buffer, pH 7.4 (Buffer A). As the elution buffer, a 20-m*M* Tris–HCl buffer with 2 *M* sodium chloride, pH 7.4 was used (Buffer B). The step gradients were performed online by mixing the binding buffer with the elution buffer to 17.5, 27.5 and 100% B with the gradient formers of the chromatographic system used.

2.6. Methods

2.6.1. Determination of factor IX coagulation 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).

2.6.2. Determination of other coagulation factors

Clotting factors II, VII and X were determined in duplicate using commercial one-stage clotting assays from Instrumentation Laboratories (Milan, Italy).

Factor VIIa was quantified by a prothrombin time-based clotting assay using a test-kit from Diagnostica Stago (Asnieres, France).

2.6.3. Determination of proteolytic activity

Proteases with arginine specificity cleave the substrate S-2288 *p*-nitroaniline. The proteolytic activity is determined spectrophotometrically at 450 nm by measuring the rate of the released *p*-nitroaniline. The substrate was purchased from Chromogenix/Instrumental Labs. (Milan, Italy).

2.6.4. Enzyme-linked immunosorbent assay (ELISA) for determination of vitronectin

ELISA was carried out with a commercial available vitronectin enzym immuno assay in accordance with the manufacturer's instructions (Tokara Biomedicals Shiga, Japan). The first antibody is immobilized and binds the corresponding antigen VN.

2.6.5. Sodium dodecyl (lauryl) sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblot

SDS–PAGE was performed according to the method of Laemmli [22]. Sample preparation included the addition of reducing sample buffer and boiling for 10 min. Then 2 μg protein for SDS–PAGE and 500 ng protein per lane for blotting were applied. The electrophoresis chamber, blot module and the 4–20% precast polyacrylamide gradient gels were from Novex (San Diego, CA). The precision protein standard was from Bio-Rad. Gels were silver stained for 60 min or electroblotted for 60 min onto a 0.2-μm nitrocellulose membrane from Schleicher and Schuell (Dassel, Germany) using blotting buffer. Monoclonal antibody (mAb) against factor IX ESN1 was purchased from American Diagnostica (Greenwich, CT). The mouse anti-VN mAb was obtained from Sigma. The respective secondary antibodies were alkaline phosphatase conjugated and also obtained from Sigma. Bands were visualized using nitrobluetetrazolium and 5-bromo-4-chloro-3-indolylphosphate as substrates.

3. Results and discussion

3.1. Separation of DEAE-Sephadex eluate from the production process of factor IX on an 8 ml monolithic tube

In experiments carried out so far it has been shown that 20 000 IU of factor IX in a commercial factor IX concentrate can be separated from VN on an 8 ml monolithic tube [16]. VN is practically the only contaminant found in this factor IX concentrate [17]. In the experiments shown here, a DEAE-Sephadex eluate has been used. This intermediate product is obtained at an earlier stage of the production process and consequently contains a number of other plasma proteins [18,20]. Apart from factor IX and VN, this material also contains the vitamin K-dependent clotting factors II, VII and X and the clotting inhibitors protein C and S [20]. Fig. 1a–c and Table 1 show the results of a separation on an 8 ml monolithic tube at different flow-rates. As is seen in Fig. 1, a practically identical separation was achieved, when the flow-rate was increased from 8 to 24 ml/min. However, the period of time required for separation was reduced from 25 min to 9 min. The specific activity of factor IX (contained in fraction 2, see Table 1) and its yield was even slightly better at high flow-rates. Proteolytic activity in factor IX containing fraction was highly reduced. However, this reduction was not dependent on flow-rate (not shown here). It is remarkable that the specific activity of factor IX was about 28 IU/mg of protein (see Table 1), when DEAE monoliths were used. This figure is higher by almost one order of magnitude than the one obtained with DEAE-Sepharose FF, when used at the same stage of the production process. A combination of pseudo-affinity chromatography using hydroxyaminopropyl tentakel gel (HAP) and hydrophobic-interaction chromatography (HIC) with octyl-Sepharose results in a intermediate product with similar specific activity (about 15 IU/mg protein). However, the content of other vitamin K-dependent proteins such as factors II, VII and X is also significantly higher if such a purification strategy is used. Very high content of measured factor VII indicates a possible activation of this clotting factor in the HIC step [18].

As shown in Table 3 proteolytic activity and activities of clotting factors II, VII and X are significantly reduced after chromatographic separation with DEAE monoliths. However, they are still detectable. It means that a subsequent purification step such as an additional anion-exchange chromatography or heparin affinity chromatography is needed to get a high-purity product with a specific activity higher than 200 IU factor IX/mg protein. Such a high specific activity in the last chromatographic step could be reached because the bulk of the contaminating proteins was removed in a previous step, anion-exchange chromatography with monolithic support [9,16].

By SDS–PAGE and westernblot against factor IX it was confirmed that peak 2 (within Fig. 2a,b, lane 3) mainly contains factor IX. Since no bands at approximately M_r 30 000 corresponding to factor IX heavy chain can be seen, no detectable activation of this clotting protein took place. Vitronectin was successfully separated from factor IX and is found in peak 1 and 3 which was confirmed in the westernblot against VN (within Fig. 2c, lanes 2 and 4).

3.2. Up-scaling experiments

In subsequent experiments, up-scaling options were tested on an 80 ml DEAE monolithic tube. The resulting separation is practically identical with that achieved on an 8-ml tube (not shown here). A total of 4239 mg of protein is applied, with 4860 IU of factor IX. It follows that specific activity at the beginning is 1.15 IU of factor IX/mg of protein. The yield of factor IX in this case was 78.8%, the specific activity of the eluate was 20.8 IU of factor IX/mg of protein (see Table 2). In the experiment, the removal of contaminating proteases, other vitamin K-dependent proteins and of VN was also measured. The results are shown in Table 3. The proteases, the activated factor VII (factor VIIa) and the factor X were almost fully separated, whereas the amount of VN was reduced by a factor of more than 20.

Such results are not achieved with a DEAE-Sepharose column [9]. When this procedure is chosen, DEAE-Sepharose removes proteases, factor VIIa and partly factor II. Factor X and VN remain in the DEAE eluate and cannot be separated from factor IX in this step. Only in a subsequent step with Heparin-Sepharose affinity chromatography is factor X sepa-

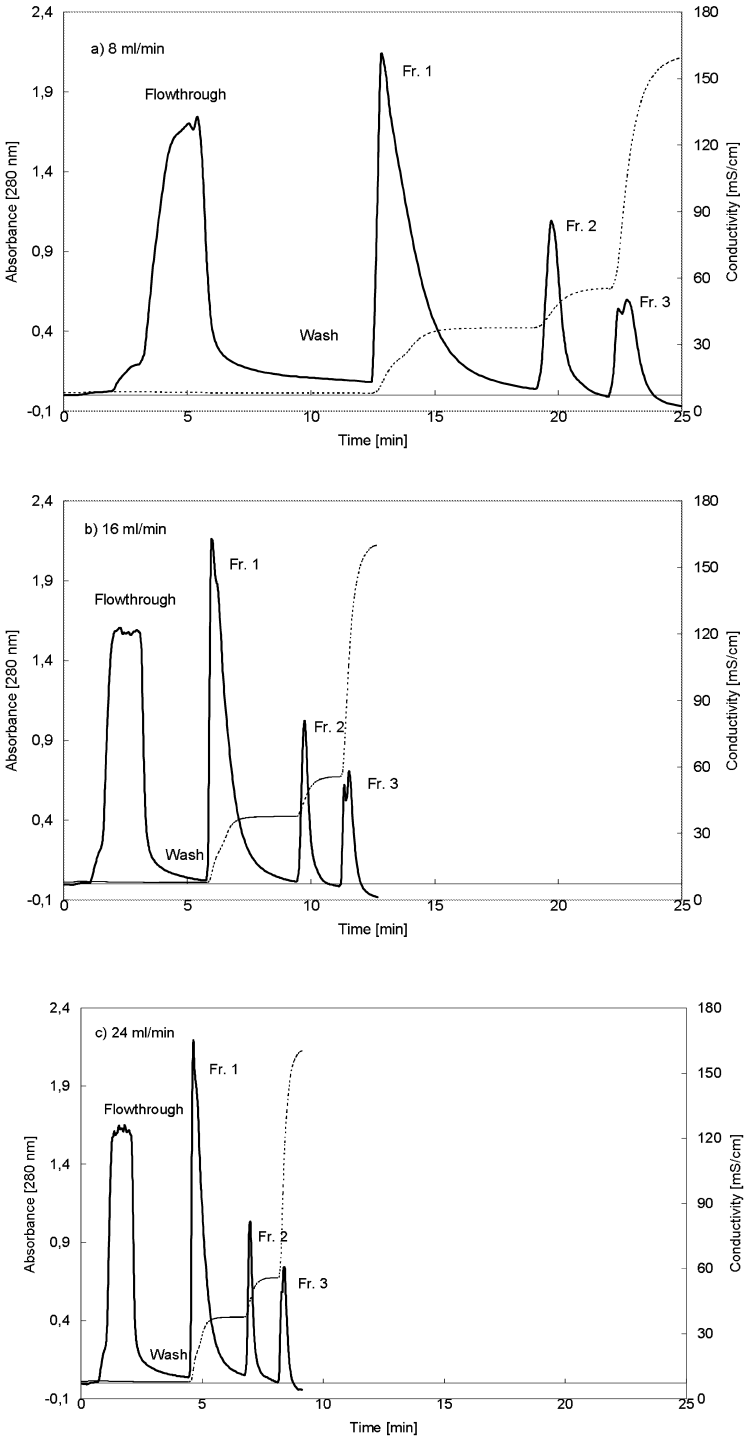

Fig. 1. Separation of the in-process sample from factor IX production (eluate from DEAE-Sephadex) on an 8 ml CIM-DEAE tube-shaped monolithic column. Influence of different flow-rates on the separation. Chromatographic data: flow-rate is indicated in the figure, back pressure in all cases less than 0.2 MPa, room temperature. Absorbance ———, conductivity - - -. For further information cf. also Table 1.

Table 1
Separation of the eluate from DEAE-Sephadex on an 8 ml CIM-DEAE tube-shaped monolithic column. The flow-rate used was 24 ml/min

	Volume (ml)	Factor IX activity (IU/ml)	Total factor IX (IU)	Protein concentration (mg/ml)	Factor IX specific activity (IU/mg protein)
Starting material*	45.0	12.7	571.5	9.1	1.4
Not bound (FT)	43.6	0.02	0.9	2.9	0
Wash	61.7	0.02	1.2	1.7	0
Fraction 1	57.4	0.35	20.1	2.2	0.2
Fraction 2	34.1	15.2	518.3	0.55	27.6
Fraction 3	19.9	0.05	1.0	0.8	0.1

Yield=94.6% of factor IX activity and 95.8% of total protein were recovered. *DEAE eluate after solid-phase extraction from cryo-poor human plasma (cf. Ref. [18]).

rated. The application of this isolation schema with bulk supports means that VN is practically the only contaminant that remains in the factor IX concentrate. It can be removed only by nanofiltration with the Viresolve filter [17,21].

The next stage in up-scaling, separation on a 500-ml monolithic unit, is shown in Fig. 3. Here, 1830 ml of DEAE-Sephadex eluate is applied, with a total of 27.6 g of protein and 48 500 IU of factor IX. This amount corresponds to between 5 and 10% of that of a factor IX production batch. The results of the experiments are shown in Table 4. The yield of factor IX in this case was about 54%, the specific activity of factor IX was 23.8 IU/mg of protein. The slightly lower yield of factor IX is probably due to the less than optimal flow-rate of 50 ml/min (see also above). Experiments with higher flow-rates could not be performed for the time being for technical reasons. The specific activity of 23.8 IU of factor IX/mg of protein is roughly identical with the

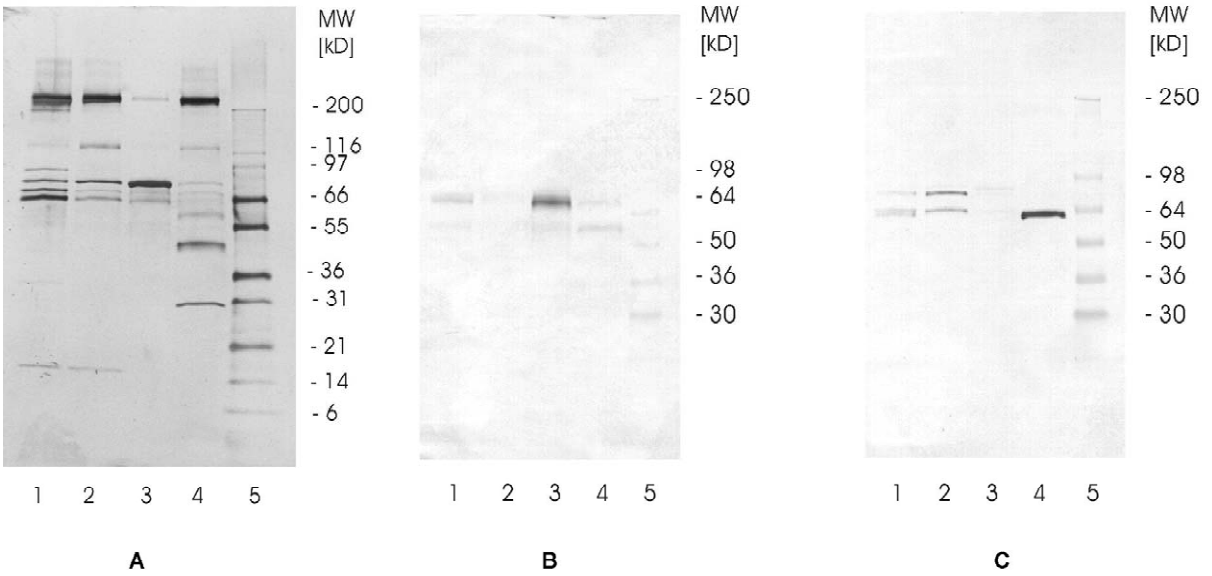

Fig. 2. SDS–PAGE and immunoblot of fractions collected during separation of Sephadex eluate on a 8 ml CIM DEAE tube (see Fig. 1). (A) SDS–PAGE, (B) immunoblot against factor IX, (C) immunoblot against VN. 4–20% gradient gels were used. For SDS–PAGE 2 μg protein and for immunoblots 500 ng protein were applied to each lane. Lane 1 shows the starting material, lane 2 peak 1, lane 3 peak 2 (factor IX enriched peak), lane 4 peak 3 (VN enriched peak) and lane 5 the molecular mass standard proteins.

Table 2
Separation of the eluate from DEAE-Sephadex on an 80 ml CIM-DEAE tube-shaped monolithic column. Analytical data regarding factor IX yield and activity. Results shown here are representative for four chromatographic runs

	Volume (ml)	Factor IX activity (IU/ml)	Factor IX Ag (IU/ml)	Total factor IX (IU)	Protein concentration (mg/ml)	Factor IX specific activity (IU/mg protein)	Factor IX Ag factor IX activity
Starting material*	270	18.0	22.3	4860	15.7	1.15	1.24
Not bound (FT)	272.6	0.0**	–	5.2	5.84	–	–
Wash	324.6	0.05	–	10.1	3.7	–	–
Fraction 1	171.1	2.8	–	479.1	5.0	0.56	–
Fraction 2	98.2	32.6	43.3	3201.3	1.6	20.8	1.33
Fraction 3	131.0	0.2	–	23.6	1.6	0.13	–

Yield=78.8% of factor IX activity and 94.7% of total protein were recovered. *DEAE eluate after solid-phase extraction from cryo-poor human plasma (cf. Ref. [18]). **Traces.

Table 3
Separation of the eluate from DEAE-Sephadex on an 80 ml CIM-DEAE tube-shaped monolithic column. Analytical data regarding removal of contaminating proteins

	Volume (ml)	Proteolytic activity (IU/ml)	VN (μg/ml)	Factor VII (IU/ml)	Factor VIIa (mU/ml)	Factor X (IU/ml)	Factor II (IU/ml)
Starting material*	270	1100	638.0	5.9	3255	16	15.7
Not bound (FT)	272.6	9.2	27.9	0.07	23.4	0.28	n.d.**
Wash	324.6	11.0	98.6	0.5	223.9	0.34	0.05
Fraction 1	171.1	165.0	277.0	17.1	9123***	29.0***	22.7
Fraction 2	98.2	212.2	77	0.1	7.4	0.4	0.3
Fraction 3	131.0	3700	637	0.0	95	0.0	0.0

Recovery=VN 107.6%, factor II 92.6%, factor X 120%***. *DEAE eluate after solid-phase extraction from cryo-poor human plasma (cf. Ref. [18]). **Not detected. ***Partially activated.

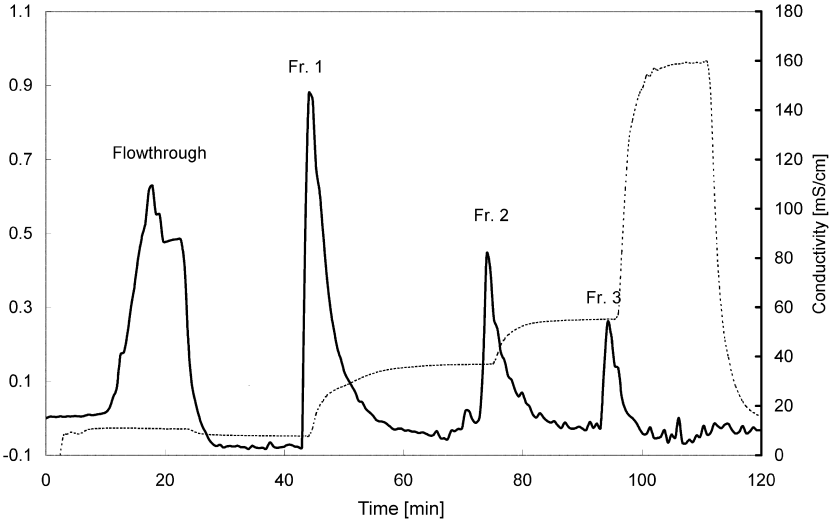

Fig. 3. Separation of the eluate from DEAE-Sephadex in a pilot scale. A 500 ml CIM-DEAE tube-shaped monolithic column was applied. Chromatographic data: flow-rate 50 ml/min, back pressure less than 0.2 Mpa, room temperature. Absorbance ———, conductivity - - -. For further information cf. also Table 4. Mw=Molecular mass; kD=kilodaltons.

Table 4
Separation of the eluate from DEAE-Sephadex on a 500 ml CIM-DEAE tube-shaped monolithic column. Analytical data regarding removal of contaminating proteins

	V (ml)	Factor IX (IU/ml)	Factor IX total (IU)	Protein concentration (mg/ml)	Factor IX specific activity (IU/mg protein)
Starting material*	1829.4	26.5	48480	15.1	1.8
Not bound (FT)	1818.4	0.03	54.6	7.1	–
Wash	3490.5	0.02	69.8	1.4	0.02
Fraction 1	954.7	1.67	1586	5.7	0.3
Fraction 2	390	61.8	24135	2.6	23.8
Fraction 3	605	0.31	187.6	1.9	0.2

Yield=53.7% of factor IX activity and 92.7% of total protein were recovered. *DEAE eluate after solid-phase extraction from cryo-poor human plasma (cf. Ref. [18]).

specific activity obtained with separations on 8 and 80 ml monolithic DEAE columns.

4. Conclusions

Monolithic columns in the anion-exchange mode are useful tools not only for fast analytical separations [6,10,12], but also for preparative separations in the isolation process of factor IX. The best results are achieved if a DEAE-monolithic column is used.

The use of monolithic columns considerably reduces the period of time required for separation. The specific activity of factor IX is increased for almost one order of magnitude.

Up-scaling to the level of pilot-scale has been achieved using a 500-ml monolithic column. The experiments with an industrial scale monolithic column with a volume between 5 and 10 l are already ongoing.

The technical problems to be solved in further up-scaling do not concern the structure of the monolithic column. For the time being they exist in the capacity of the pumps and the construction of the gradient former at high flow-rates.

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