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Improved virus safety and purity of a chromatographically produced Factor IX concentrate by nanofiltration☆

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Abstract

A virus removal system based on tangential flow filtration was introduced into a Factor IX production process. Beside the intended virus reduction potency of filter membranes, an additional purification effect could be achieved. This purification effect was evaluated in detail by means of sodium dodecyl sulphate-polyacrylamide gel electrophoresis and size-exclusion HPLC. High-molecular-mass impurities were retained by the membrane, thus increasing the specific activity of the product.

1. Introduction

Human plasma is a highly complex mixture of more than 100 known proteins of biological importance. Its fractionation is a major tool for the production of blood-derived therapeutics. Since the pioneering work of Cohn et al. [1] in the nineteen forties, two trends in plasma fractionation can be observed:

Simple precipitation steps have been replaced by increasingly sophisticated chromatographical methods, e.g., ion-exchange and hydrophobic interaction chromatography or affinity chromatography, where a large variety of ligands is applied, including monoclonal antibodies. In the past decade these techniques have been perfected to such an extent that today virtually pure products are available, whose specific activities approach the theoretical values [2–4]. The aim of these efforts is to supply patients with therapeutics of the highest possible purity, thus increasing the desired potency and at the same time minimizing possible undesired side effects.

Another trend has been triggered by problems concerning the safety of the product, especially the risk of transfer of infectious agents, e.g., viruses. A large number of measures is taken nowadays to rule out or at least to minimize this inherent risk of blood products. The measures taken include donor selection and screening. This is very important, since plasma pools may consist of thousands of individual blood donations. However, even if screening is carried out with maximum care, infection of the plasma pools cannot be ruled out, as the blood may have been given prior to seroconversion. Therefore all plasma pools have to be regarded as potentially

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pathogenic. It follows that procedures to remove or inactivate viruses have to be part of the actual manufacturing process.

As chromatographic steps have evolved as major separation techniques in plasma fractionation, the removal of viruses through chromatographic means was investigated.

Reduction of contaminations by viruses with a factor between 10 and 10^6 have been described. However, a degree of uncertainty remains concerning the consistency of performance of large-scale chromatographic columns, and it is generally accepted that the removal of viruses should not depend on chromatographic methods alone. Therefore other methods are added to individual production processes, which are especially designed for the removal or inactivation of viruses, using physical or chemical principles, or a combination of both.

The methods that have been used so far include mild terminal heat, severe terminal heat, steam treatment, pasteurization, solvent/detergent (S/D)-treatment and nanofiltration [5–7]. None of these methods can claim to provide the ultimate answer to the problem of virus safety. This is due to several factors, among which the wide variety of virus strains with different behavior, structure and resistance is found, but also the necessity not to reduce the yield or even more important the quality of the product. As far as coagulation factors are concerned this means that the introduction of thrombogenicity or neoantigenicity has to be avoided.

A recent approach to achieve overall reduction values in the order of 10^{10} for lipid-enveloped and 10^6 for non-enveloped viruses is to combine two independent methods so that the log reduction values can be calculated on a cumulative basis. In this study the same approach is chosen for a routinely produced high-purity factor IX preparation. The first virus inactivation method is S/D-treatment, the second is nanofiltration, which is based on tangential flow filtration.

S/D-treatment was originally described by Horowitz *et al.* (5) and was soon applied worldwide. Consequently considerable experience concerning effectiveness and safety has been accumulated. However, this does not apply to virus filtration. Therefore, it was decided here not only to investigate the virus reduction potential of this method by using common model viruses, but also to evaluate possible changes in the product by means of radial immune diffusion (RID), sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and size-exclusion chromatography.

2. Experimental

2.1. Factor IX production

Factor IX is routinely produced from human cryopoor plasma at the Octapharma production facility (Vienna, Austria). Batch size was 1300 kg, production roughly follows the method of Brummelhuis [8]. The initial step is a solid-phase extraction of the cryopoor plasma with DEAE-Sephadex A50, followed by further chromatographic purification on DEAE-Sepharose FF. At this stage the product is subjected to the S/D treatment with 1% Tween 80 and 0.3% tri-*n*-butylphosphate for 6 h at 30°C. The S/D reagents are then removed, and separation of Factor IX, mainly from Factor X, is performed by means of affinity chromatography on Heparin-Sepharose CL 6B. The eluate of the affinity column undergoes virus filtration [9]. After this, the appropriate concentration is adjusted by means of ultra/diafiltration. A sterile filtration follows, and after lyophilisation the final product is obtained.

2.2. Virus filtration

Virus filtration is performed according to the recommendations of the manufacturer (Millipore). The filtration technology is based on a PVDF membrane, rendered especially hydrophilic by a patented method (Viresole). The filter device is available with 300 cm^2 for lab scale experiments and with 9000 cm^2 for production scale experiments. It is operated by

means of two peristaltic pumps, one controlling a tangential cross flow (retentate) of the protein solution to be filtered, the other controlling the filtration rate (permeate). Samples were taken before filtration (initial product, IP), after filtration (final product, FP) and finally from the remaining portion of the solution that did not pass through the filter (final retentate, FR).

2.3. *Analytical techniques*

Protein measurements were performed according to Bradford [10] with a Hitachi U2000 spectrophotometer. Chemicals were from BioRad. Radial immune diffusion (Behring) was used to determine C4 and inter-α-trypsin inhibitor.

Factor IX estimation was performed in a one-stage coagulation assay with an Amelung KC4A coagulometer or automatically with an ACL 300 apparatus from Instrumentation Laboratory. Coagulation factor deficient plasmas, calibration plasmas and other substances were either from Behring or Instrumentation Laboratory.

Virus reduction measurements: The data concerning virus removal were obtained from the manufacturer of the filter (Millipore) and from our own experiments. These were performed according to established EC-guidelines [11]. The following widely used model viruses were included in the studies: SV 40, Reovirus 3, Polio, Sindbis, Pseudorabies, VSV, Theiler's virus, Canine Parvo and Yellow fever.

(SDS-PAGE) was performed in order to monitor the additional purification that occurs during the filtration process. In general the method of Laemmli [12] was followed. Gradient gels contained 4.5 to 15% polyacrylamide (BioRad). A BioRad 3000xi power supply and a Hoefer Scientific Instruments electrophoretic chamber were used. Gels were run for approximately 3 h at 200 to 240 V at 12°C. Staining was carried out with Brilliant Blue G (Sigma). Dried electropherograms were scanned with a BioRad GS670 imaging densitometer.

Chromatographic equipment: the microprocessor controlled, iron(III) ion free, HPLC system consisted of two pumps, a spectrophotometer with a deuterium lamp and a loop injection valve (all from Knauer, Berlin, Germany). A BioPilot system (Pharmacia, Vienna, Austria) was used for semi-preparative and preparative chromatography when necessary. The salt gradient in the chromatographic steps was controlled by measuring the osmotic pressure (Osmomat 030 cryoscopic osmometer, Gonotec, Berlin, Germany).

Supports and buffers for chromatographic separations: DEAE-Sepharose Fast Flow (Pharmacia) was used for anion-exchange chromatography, both on an analytical and on a preparative scale. Affinity chromatography was carried out with an analytical or a preparative Heparin-Sepharose support (Pharmacia). The chromatographic method has been described in detail elsewhere [13]. Analytical size-exclusion HPLC was performed on a tandem consisting of a Zorbax 6F-250 and a Zorbax 6F-450 BioSeries column with a pre-column (Säulentechnik, Knauer). The dimensions of the columns were 250 × 9.4 mm I.D. each. The buffer in size-exclusion HPLC was 50 m*M* Tris-HCl, pH 7.2, with 200 m*M* NaCl. The other chromatographic conditions are listed in the legends to the figures.

3. Results

Factor IX can be isolated from human plasma as described. Nanofiltration is included in the manufacturing process in order to increase viral safety of the product. Apart from virus elimination a certain degree of protein retention was observed during filtration. In Table 1 the data of a representative Factor IX production batch are shown. The Factor IX content, protein concentration and specific activity are listed for the starting material, the main intermediate stages and the final product. The data confirm previous results from lab-scale experiments. The specific activity of the product is increased by a factor of ca. 1.5 after nanofiltration.

In order to demonstrate the additional purification achieved by filtration, SDS-PAGE was performed with samples taken before and after

Table 1
Factor IX and protein concentration and specific activity during production process

Sample	Factor IX (IU/ml)	Protein (mg/ml)	Specific activity
Cryopoor plasma	1.2	59.2	0.02
Post solid-phase extraction	39.5	32.9	1.2
Post anion-exchange chromatography	54.0	19.4	2.8
Post affinity chromatography	33.3	0.4	83
Post nanofiltration	107	0.9	119

filtration. The eluate of the affinity column shows a fairly pure product with only minor impurities. After filtration traces of proteins with high molecular masses far above 100 kDa are substantially reduced. Surprisingly some trace impurities with low molecular masses in the range of ca. 10 to 15 kDa are reduced too. The findings are confirmed by the SDS-PAGE image obtained from a sample of the final retentate. After filtration and subsequent diafiltration of the product a minor amount of Factor IX solution remains in the retentate outlet of the filter. In this retentate both kinds of impurities are found enriched compared to the initial product, see Fig. 1a.

The reduction of impurities is seen in the densitogram of the SDS-PAGE gel, Fig. 1b. A trace protein with a high molecular mass (on the left hand side of the diagram) is reduced in the filtered product and enriched in the final retentate compared to the initial product. The same is observed for some impurities with low molecular masses in the range around 15 kDa.

Complement component C4 and inter-α-trypsin inhibitor were measured by means of RID. Both proteins could be detected in the unfiltered product at a level of ca. 0.04 mg/ml, which is very close to the lower detection limit of the method. After filtration neither C4 nor inter-α-trypsin inhibitor were detected by RID.

Viresolve nanofilter membranes are available with two pore sizes. These have nominal exclusion limits of 70 and 180 kDa, respectively. As virus filtration depends mainly on a sieving mechanism, virus retention is more pronounced with the 70 kDa membrane. Commonly used model viruses were included in the study. The results of virus removal potency of the 70 kDa membrane are given in Table 2.

Both types of membranes are capable of removing traces of proteins with high molecular masses from the Factor IX solution, thus increasing the specific activity of the product. The ability to retain proteins also causes some loss of product, which again is more pronounced in the case of the 70 kDa membrane. In Table 3 data are given on the concentrations of Factor IX and protein as well as on specific activity and yield after filtration, including information about means and standard deviations (S.D.). The data concern each of three consecutive batches of Factor IX production applying 70 and 180 kDa membranes.

In order to check the stability of the nanofiltered Factor IX product, the same test program was performed as for the unfiltered product. Samples are held at 37°C for 3 months, at room temperature for 24 months and at 4°C for 36 months. The following parameters are checked regularly: solubility, visual control, pH value, Factor IX potency, stability in solution, L-lysine-HCl, L-arginine-HCl, NAPTT, heparin, loss on drying and sterility. During the three-month period at 37°C, no statistically significant difference could be observed between the filtered and unfiltered product. The investigation at room temperature and at 4°C is still ongoing. So far, there was also no difference detectable indicating that the filtration procedure does not affect the stability of the Factor IX preparation.

The following in vitro and in vivo tests were performed to compare the thrombotic potential of the filtered product versus the unfiltered one. Fibrinogen clotting time, thrombin generation

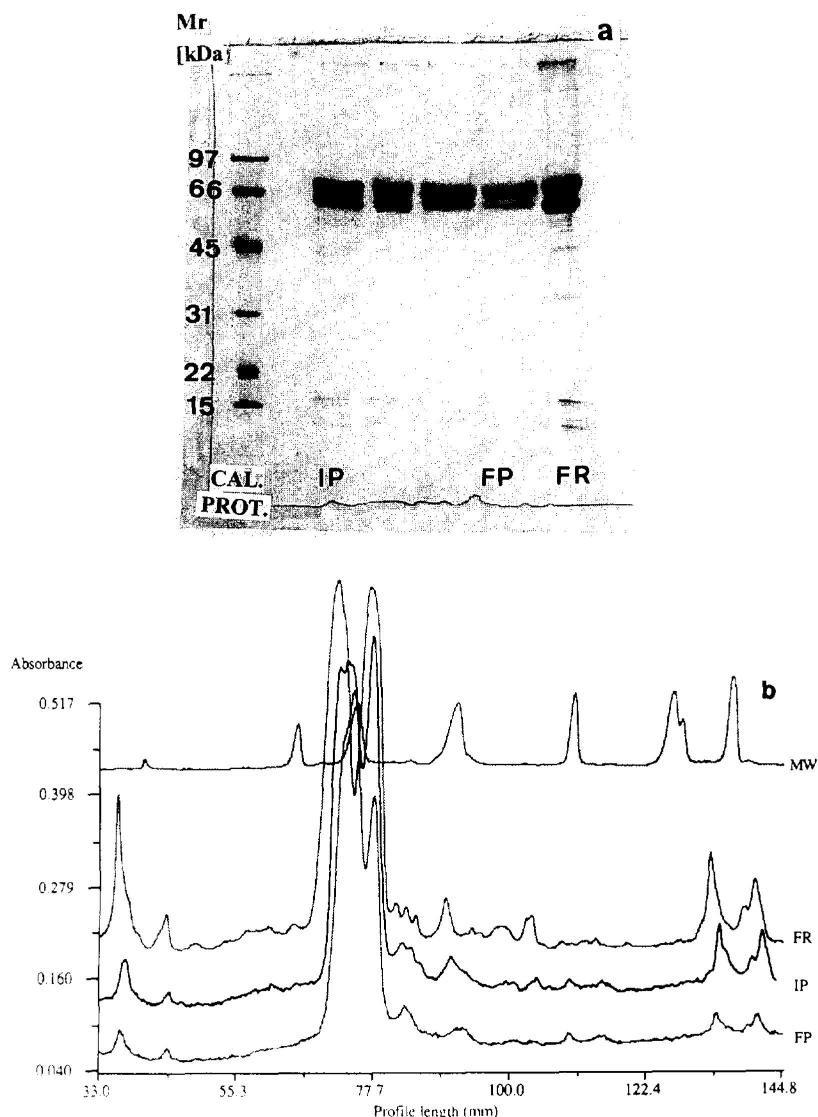

Fig. 1. (a) Electrophoretical analysis of Factor IX before (initial product, IP) and after (final product, FP) virus filtration. Molecular mass standards were 97, 66, 45, 31, 22 and 15 kDa, respectively. High-molecular-mass impurities are concentrated in the final retentate (FR). (b) Densitogram of SDS-PAGE shown in (a). High-molecular-mass impurities are reduced in the final product (FP) and enriched in the final retentate (FR) compared to the initial product (IP).

time and non-activated partial thromboplastin time were measured in vitro. The in vivo thrombogenicity has been evaluated in the venous stasis model according to Wessler in rats. Details of all methods are described elsewhere [14]. The filtered Factor IX preparation did not show any enhanced thrombogenic potential compared to the unfiltered product.

4. Discussion

To guarantee virus safety of blood derived therapeutics, various measures are taken. Recently virus reduction values of 10 log for lipid enveloped and 6 log for non-enveloped viruses were defined [15].

In order to contribute to virus safety, a step

Table 2
Validation of virus removal by nanofiltration

Virus	Diameter (nm)	LRV
Polio	33	3.5
SV-40	48	5.7
Sindbis	54	7.4
Reovirus-3	78	7.2
Pseudorabies	150–200	>5.5
Vesicular Stomatitis	70–85	>7.5
Theiler's virus	28–30	3.7
Canine parvo	18–26	5.2
Yellow fever	40–50	5.6

Lipid-coated as well as non lipid-coated viruses were investigated using a 70 kDa membrane. Log reduction values (LRV) between 3.7 and 7.2 were obtained.

for the removal of viruses based on tangential flow nanofiltration was used in the production process of a Factor IX concentrate with high purity. Apart from the effectiveness of the method for removing viruses from protein solutions, which has been investigated elsewhere [7,11], the object of the experiments was to find out whether or not the filtration procedure causes further changes in the product. Apart from the danger of infectiousness two more concerns apply to coagulation products in particular. As is the case with other drugs, which may have to be administered to a patient as long as he lives, the

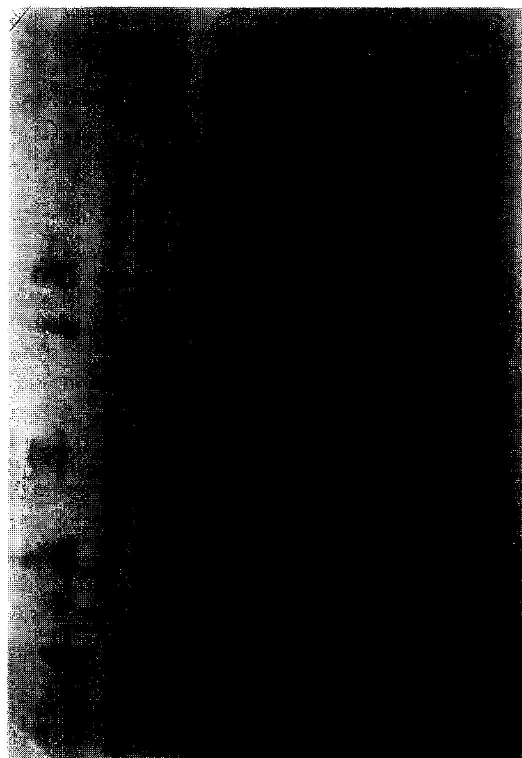

Fig. 2. Immunoblot of Factor IX samples. Lane A: Factor IX before nanofiltration; lane B, C: samples after nanofiltration. The reaction of the anti-Factor IX monoclonal antibody remains the same before and after filtration indicating that no detectable degradation occurs.

Table 3
Factor IX, protein and specific activity of each of three batches produced with Viresolve 70 and 180 respectively and after nanofiltration

Membrane Type	Batch No.	Before nanofiltration			After nanofiltration			Recovery after nano-filtration (%)
		Factor IX (IU/ml)	Protein (mg/ml)	Spec. act.	Factor IX (IU/ml)	Protein (mg/ml)	Spec. act.	
180 kDa	1	27	0.28	96	52	0.24	217	97
180 kDa	2	35	0.3	117	49	0.31	158	89
180 kDa	3	34	0.3	113	56	0.34	130	96
Mean±S.D.				109±11			168±44	94±4
70 kDa	1	33	0.4	83	116	0.6	193	80
70 kDa	2	30	0.2	150	116	0.7	166	76
70 kDa	3	26	0.2	130	57	0.3	190	93
Mean±S.D.				121±34			183±15	83±9

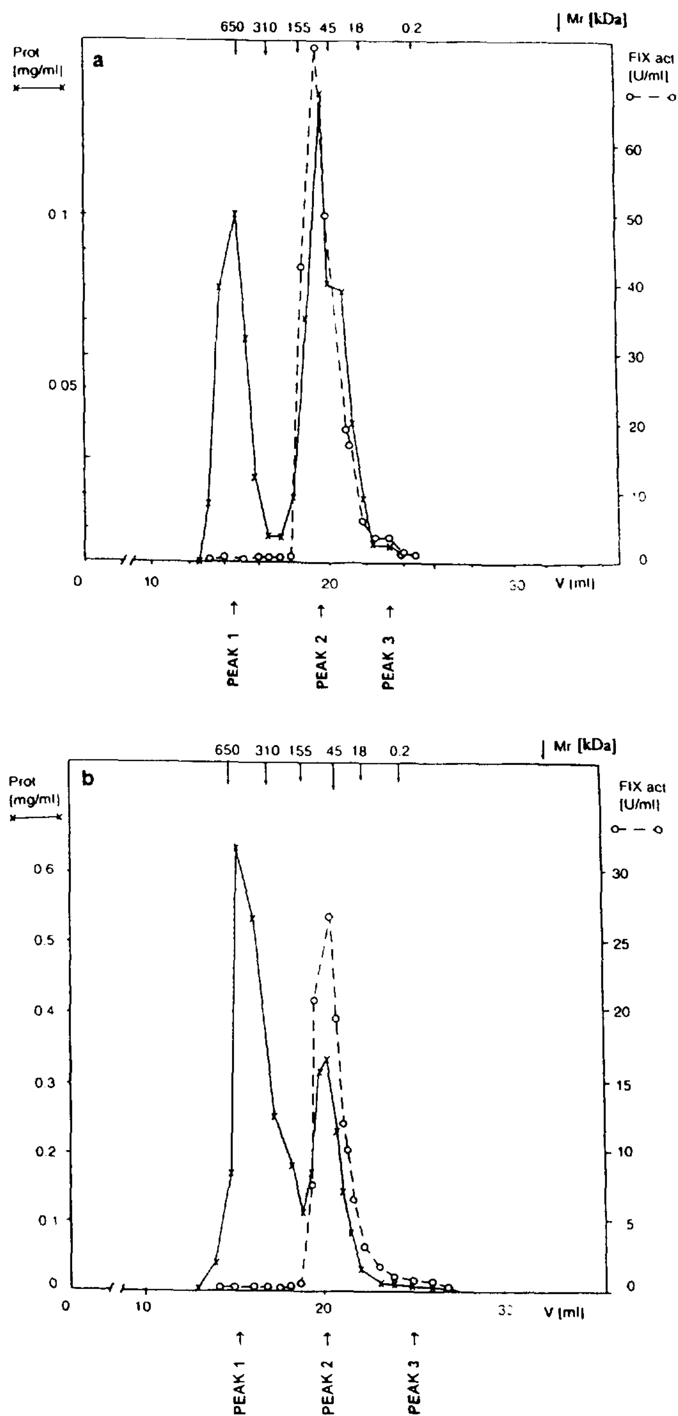

Fig. 3. Size-exclusion HPLC of two Factor IX (F IX) preparations with different degree of purity. (a) Preparation with specific activity of 70 IU Factor IX/mg protein (containing ca. 40% impurities). An aliquot of 200 μl of sample containing 150 IU of Factor IX was applied. (b) Preparation with specific activity of 25 IU Factor IX/mg protein (medium purity preparation). An aliquot of 200 μl of sample containing 150 IU of Factor IX was applied. Chromatographic conditions for both runs: see Fig. 1a.

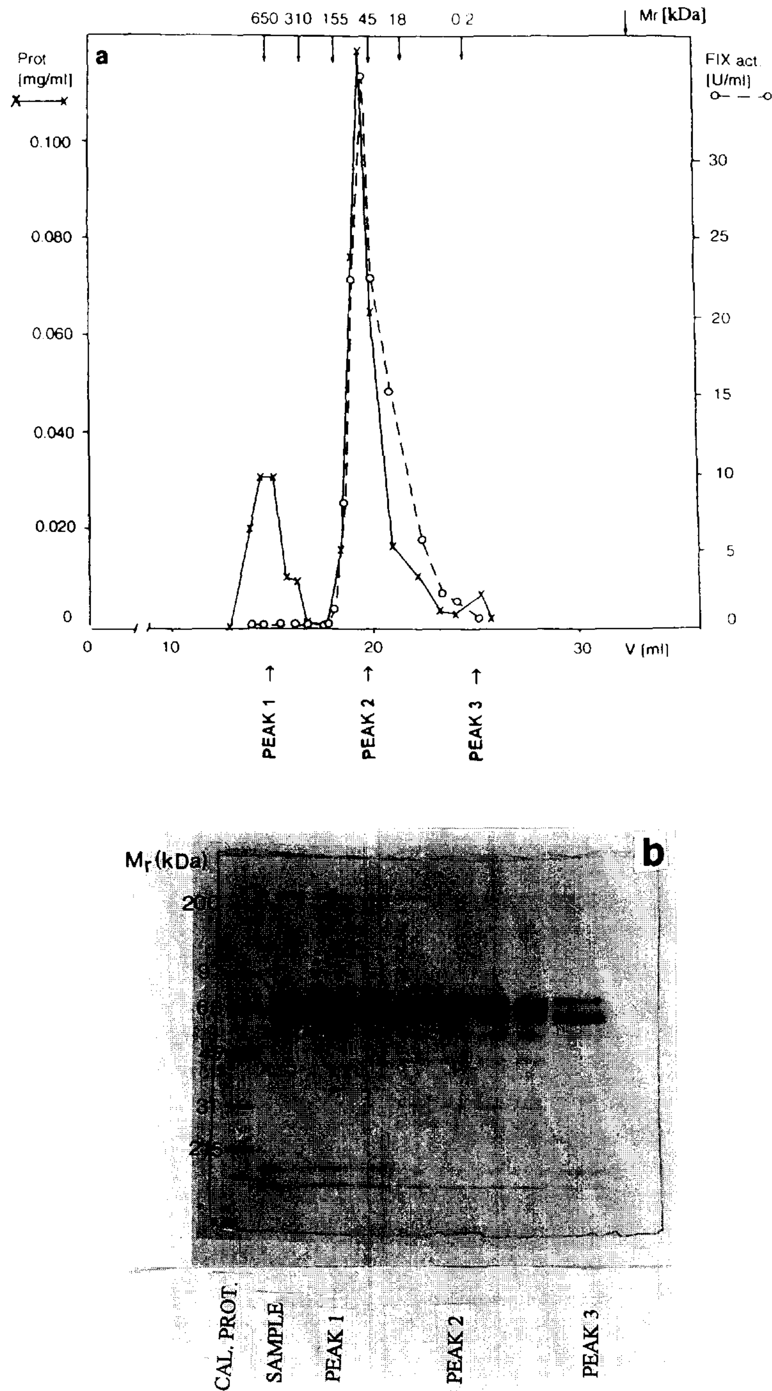

Fig. 4. (a) Size-exclusion HPLC of Factor IX preparation before nanofiltration. An aliquot of 500 μl of sample containing 100 IU of Factor IX was applied to a size-exclusion HPLC column tandem containing a Zorbax GF-250 and a Zorbax GF-450 column. Chromatographic conditions: flow-rate 0.5 ml/min, pressure 3.0–3.2 mPa, room temperature. After fraction collection (0.5 ml fractions), protein concentration and Factor IX clotting activity were determined (see Section 2). (b) SDS-PAGE of corresponding samples, separated by size-exclusion HPLC.

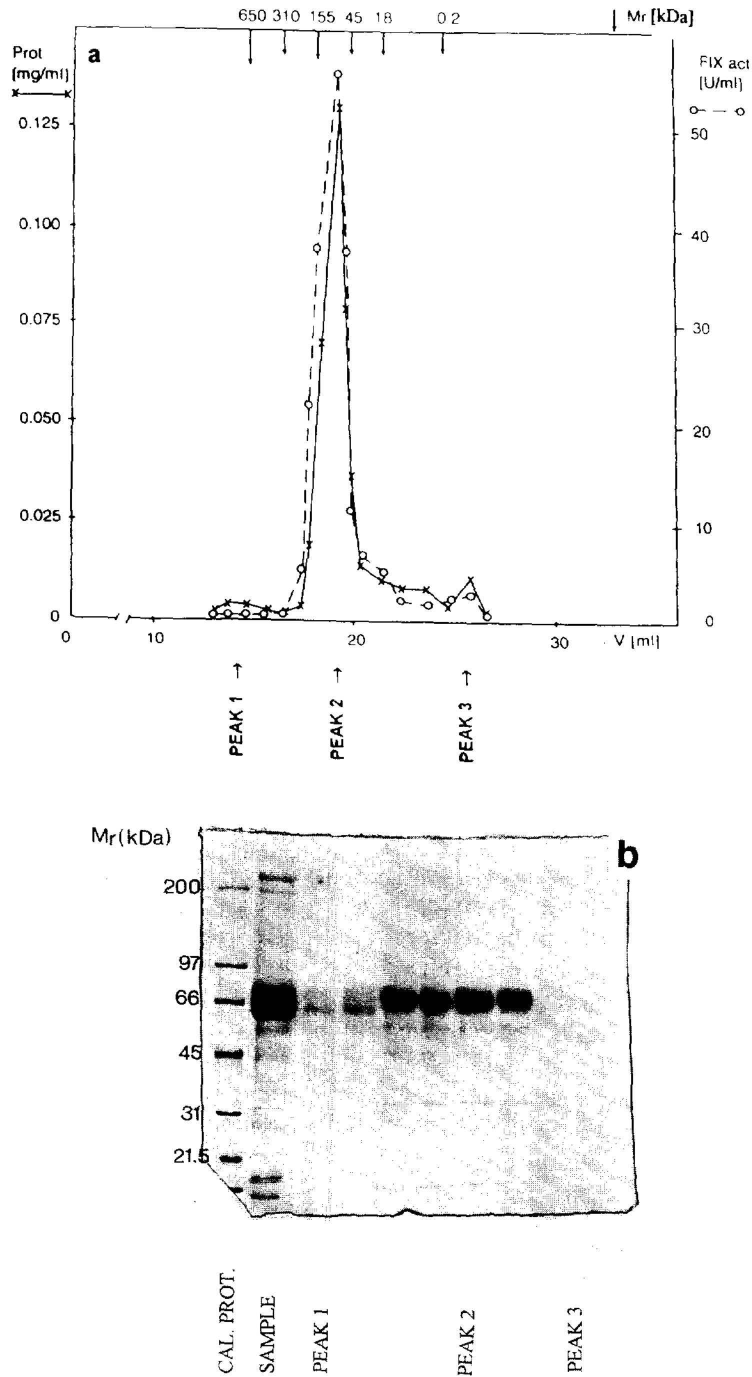

Fig. 5. Size-exclusion HPLC of Factor IX preparation after nanofiltration. An aliquot of 500 μl of sample containing 120 IU of Factor IX was applied to a size-exclusion HPLC column tandem containing a Zorbax GF-250 and a Zorbax GF-450 column. Chromatographic conditions: see Fig. 1a. (b) SDS-PAGE of corresponding samples, separated by size-exclusion HPLC.

antigenicity of clotting factor products has to be considered carefully. Even products with high purity contain tiny amounts of other proteins. They may be irrelevant in short-term treatment. However, long-term exposure to these unnecessary proteins can put a severe strain on the immune system. This is especially true if the trace proteins are not of human origin, as can be the case in products which are derived from transgenic cells or purified by monoclonal antibodies.

Obviously non-human protein contaminants cannot appear in products which are obtained from human plasma. But even in this case some kind of "artificial" immunogenicity may be introduced. This can occur for instance, if products are subjected to thermal treatment during pasteurization. Even if suitable stabilizers are applied, denaturation of the active molecule may lead to effects generally referred to as neoantigenicity. With regard to the filtration process the risk is negligible, because thermal stress is minimal during filtration. An Immunblot of Factor IX before and after filtration shows no influence of the filtration procedure (Fig. 2).

A second major safety concern of clotting products is the possible relation of protein impurities to unwanted side effects such as, most importantly, potential thrombogenicity. A reduction of trace impurities from clotting factor products is therefore of general interest. Here such an effect was observe in the filtration of a Factor IX solution. Its SDS-PAGE performed before and after filtration shows that some trace impurities with high molecular masses are retained by the membrane. The effect is not wholly unexpected, considering the exclusion limits (70 and 180 kDa, respectively) of the membrane. The observation was confirmed by size-exclusion-HPLC, which showed a substantial reduction of the impurities.

Inter-α-trypsin inhibitor (M_r 160 000) and the complement component C4 (M_r 200 000) were earlier found out to contribute to impurities in that range of molecular masses [16]. By means of RID a reduction of these components was detected. Rather more surprisingly some impurities with low molecular masses were also removed from the solution. This effect cannot be explained by the cutoff limits, and is more likely due to some unspecific protein binding of the membrane.

Size-exclusion HPLC has proven to be a fast and reliable method to evaluate the purity of Factor IX preparations, as shown in Fig. 3. How this method can be used is shown in Figs. 4 and 5 where two Factor IX preparations differing in purity are compared. The sample with high specific activity (Fig. 5) contains only negligible amounts of impurities (peaks 1 and 3). The sample with low specific activity (Fig. 3b) accordingly displayed a much larger peak in the high-molecular-mass range (peak 1) compared to the Factor IX peak (peak 2). Currently, this method is being implemented as routine method in our laboratory for the fast in process control of Factor IX production.

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