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Production of highly purified clotting factor IX by a combination of different chromatographic methods ☆

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Abstract

A highly enriched preparation of human clotting factor IX was produced by a combination of adsorption chromatography, hydrophobic interaction chromatography and heparin affinity chromatography. The introduction of adsorption chromatography with a hydroxyaminopropyl support allows the capture step to be carried out directly from the cryoprecipitate-depleted plasma with a chromatographic column in flow-through mode. This replaces the batch procedure used until now. The other two chromatographic steps are designed in such a way that the eluate from the preceding step can be directly applied, without any intermediate treatment of the sample. This cuts the period of time required for the process by almost 50%, and increases the yield considerably. The isolated factor IX contains practically no contaminants and has a specific activity over 200 IU/mg of protein.

Keywords: Factor IX; Glycoproteins; Proteins

1. Introduction

Human clotting factor IX (FIX) is a glycoprotein, which is essential for normal hemostasis. The absence or a defect of FIX causes hemophilia B, a genetic disease in which the clotting cascade is disturbed [1,2]. Structure and amino acid sequence of FIX are similar to those found in other vitamin K-dependent glycoproteins, which play a part in the clotting cascade. Apart from FIX these are clotting factor II (FII), VII (FVII) and X (FX) as well as protein C [2]. FIX is a glycoprotein with an apparent molecular mass between 55 000 and 65 000, the exact figure depending on the method used for its determination [3–7]. Predicted molecular mass value, calculated for the amino acid sequence alone is 47 054, carbohydrate content is about 17% [2]. Because of a rather high portion of acidic oligosaccharides (10 sialic acid residues per molecule), human FIX has a very low isoelectric point. Upon isoelectric focussing in both slab gel and in capillary, human FIX displays microheterogeneity with an isoelectric point in the range of pH 4.0–4.6 [4–6].

The concentration of FIX in human plasma is very low. In healthy adults it amounts to 5 μg/ml, which is equivalent to 0.1 μmol [2]. Administering whole blood or human plasma for the sake of correcting a FIX deficiency will therefore result in volume overloading and an overdose of other proteins, e.g.

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fibrinogen, which occur in the plasma in much higher concentrations.

The first enriched FIX concentrates were produced in the late 1950s. The method was adsorption to barium sulfate of the protein from the plasma. The barium sulfate was later replaced by tricalcium phosphate, a non-toxic salt [8]. Other vitamin K-dependent clotting factors were enriched along with FIX. Hence the name 'prothrombin complex concentrate' (PCC) for such preparations. They are rich in FII, FVII, FIX and FX. Such concentrates were successfully used from the 1960s onwards for treatment of hemophilia B. Infusion with PCC (also called PPSB) was for more than 20 years the method of choice for this kind of treatment [8,9].

A disadvantage of the precipitation methods was that they could not be easily integrated into the plasma fractionation method of Cohn [10]. Only when chromatographic methods were introduced was it possible to fully harmonize the production of PCC with the scheme of the Cohn-fractionation of human plasma. A maximum yield of albumin, IgG and clotting factors, above all FVIII and the vitamin K-dependent factors, was achieved through the introduction of cryoprecipitation by Pool et al. [11]. While the cryoprecipitate representing the FVIII-rich fraction was separated, the supernatant, the so-called cryopoor plasma, was used for the production of PCC. The batch adsorption step using anion-exchange resin causes only a small delay in the main fractionation scheme designed for albumin and IgG [10].

Clinical PCC are good examples for the specific use of different materials with anion-exchange ligands. If a weak anion-exchange resin is used in the first step of the isolation process, the capture step, a so-called 'three-factor' concentrate of clotting factors II, IX and X results. However, if a resin with a strong anion-exchange ligand is used, the product will be a 'four-factor' concentrate, containing FVII together with the three aforementioned clotting factors [12,13]. Until recently, three-factor PCC used to be the drug of choice to prevent and treat bleeding in hemophilia B [9]. Now they are increasingly being replaced by highly purified FIX products [13–15].

Three-factor PCC, obtained in the catching step with weak anion exchange material, has until now been the starting material for further purification of FIX, which was achieved by subsequent steps, usually anion exchange, hydrophobic interaction or affinity chromatography [7,15–17]. The last phase of the production process is always affinity chromatography, carried out either on a heparin support or another polysulphated matrix, or else with anti FIX monoclonal antibodies [7,13,16].

The use of DEAE–Sephadex for batch-adsorption from plasma and subsequent purification with DEAE–Sepharose fast-flow and heparin–Sepharose allows the production of a highly purified FIX concentrate. Such a protocol allows a complete integration into the Cohn scheme for plasma fractionation. The supernatant from DEAE–Sephadex after solid-phase extraction of vitamin K-dependent proteins can be used for the production of albumin and IgG [13,16]. Chromatography of the adsorbed fraction on DEAE–Sepharose fast-flow separated the residual FVII, which was eluted by increasing the ionic strength. FII and FX were removed by affinity chromatography on heparin–Sepharose. The process is structured in such a way that the reagents for virus inactivation, the detergent Tween 80® and the solvent tri-*n*-butylphosphate (TnBP), are removed in the last chromatographic step. The resulting FIX concentrate has a high specific activity (about 100 IU/mg of protein), corresponding to a purification factor from plasma of about 2000–4000. The content of other vitamin K-dependent clotting factors is very low. The contaminants detected were chiefly C4 and inter-α-trypsin-inhibitor [15]. Such highly purified FIX concentrates were successfully used for treatment of hemophilia B patients, even in very high doses [14,18].

Filtration as an additional method for the removal of viruses was introduced at the beginning of the 1990s and has been developed further ever since [19,20]. As FIX is a rather small molecule, it can be separated from larger virus particles [16,21]. We have recently been able to show that nanofiltration, carried out for the sake of removing viruses, will also affect a large portion of the impurities with high molecular masses [16]. Despite this purification, a significant amount of contaminating plasma proteins will still be found in preparations of FIX produced in the aforementioned manner. This also means a somewhat lower specific activity, compared to those preparations that have been produced by using monoclonal anti FIX antibodies [5,22].

In this report, the production of a highly pure FIX

preparation from human plasma is discussed. In order to avoid delays in plasma fractionation, the batch adsorption in the first production step (capture step) is replaced by column chromatography.

2. Experimental

2.1. Production of Factor IX

The production from cryopoor plasma of the FIX preparation Octanine F was carried out at the Octapharma production facility (Vienna, Austria). The product was virus inactivated by the solvent/detergent (S/D) method. The second step for virus removal was nanofiltration with a Viresolve 70 filter (Millipore, Vienna, Austria). Batch size was 1300 kg. The production roughly follows the method of Brummelhuis [10], with modifications that have been described previously [16]. Briefly, the initial step is a solid-phase extraction of the cryopoor plasma with DEAE–Sephadex A-50 (Amersham–Pharmacia, Vienna, Austria), followed by further chromatographic purification on DEAE–Sepharose fast-flow (Amersham–Pharmacia). At this stage, the product is subjected to the S/D treatment with 1% (w/v) Tween 80® and 0.3% (w/v) TnBP for 6 h at 27°C. The S/D reagents are then removed, and separation of FIX, mainly from FX, is performed by means of affinity chromatography on heparin–Sepharose CL 6B (Amersham–Pharmacia). The eluate of the affinity column undergoes virus filtration [16]. After this, the appropriate concentration is adjusted by means of ultra/diafiltration (UF/DF). A sterile filtration follows, and after lyophilisation the final product is obtained.

In the new production scheme, the capture step is modified. The cryopoor plasma is pumped through a fractogel EMD Amino 650M column (Merck, Darmstadt, Germany). In this step a radial column ($h=35$ mm, ICT-Sepragen, Vienna, Austria) was used. A total of 23 l cryopoor plasma per 1000 ml gel could be applied. The column is subsequently rinsed with 20 m*M* sodium citrate buffer, pH 7.0, containing 100 m*M* NaCl and the enriched FIX fraction is eluted with 20 m*M* sodium citrate buffer, pH 7.0, containing 1 *M* NaCl. Flow-rate during this step was 100 ml/min. The eluate from the first column (about 2000 IU FIX) is pumped directly on an octyl-Sepharose CL4B column (XK 26, 40×26 mm I.D., Amersham–Pharmacia). This column is subsequently rinsed with the aforementioned elution buffer, and the enriched FIX fraction is eluted with 10 m*M* sodium citrate buffer, pH 7.0, at flow-rate 10 ml/min and 1% (w/w) detergent Renex® (Triton X-100®). 0.3% TnBP (w/w) is subsequently added to the eluate. It is virus inactivated for 6 h at 27°C. The next purification step, affinity chromatography, is carried out with heparin–Sepharose CL6B. Column dimensions were 100×16 mm I.D. (Amersham–Pharmacia), flow-rate 2 ml/min. Application buffer was 20 m*M* sodium citrate, pH 7.0, washing buffer was 20 m*M* sodium citrate, pH 7.0, containing 215 m*M* NaCl. FIX was subsequently eluted with a 20 m*M* sodium citrate buffer, pH 7.0, containing 430 m*M* NaCl.

Equally the appropriate concentration is adjusted by means of ultra/diafiltration followed by a final sterile filtration.

2.2. Analytical techniques

Protein measurements were performed according to Bradford [23] with a Hitachi U2000 spectrophotometer (INULA, Vienna, Austria). The chemicals were purchased from Bio-Rad (Vienna, Austria). Radial immune diffusion (Behring, Marburg, Germany) 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 (AVL, Vienna, Austria) or automatically with an ACL 300 apparatus from Instrumentation Laboratory (IL, Vienna, Austria). Coagulation factor-deficient plasmas, calibration plasmas and other substances were either from Behring or Instrumentation Laboratory.

Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed in order to monitor the additional purification that occurs during the filtration process. In general the method of Laemmli [24] was followed. Gradient gels contained 4.5–15% polyacrylamide (Bio-Rad). A Bio-Rad 3000xi power supply and a Hoefer Scientific Instruments (Amersham–Pharmacia) electrophoretic chamber were used. Gels were run for approximately 3 h at 200–240 V at 12°C. Staining was carried out

with Coomassie Brilliant Blue G (Sigma, St. Louis, MO, USA).

For analytical size-exclusion chromatography a modular system was used containing autosampler, pump, variable-wavelength detector and HPLC software/hardware (data acquisition and control station called HP ChemStation LC 1100), all belonging to HP 1100 series (Hewlett-Packard, Vienna, Austria).

For semi-preparative and preparative chromatography in process development a microprocessor controlled Bio Logic System (Bio-Rad) containing a Biologic Controller, fraction collector, injection valve, two pumps and Biologic Optic Module (detector) connected to a Bio Logic Workstation.

Other chromatographic conditions are listed in figure legends.

Proteolytic activity caused by proteases sensitive to arginin can be measured by the chromogenic substrate S-2288 (Chromogenix, Mölndal, Sweden)

H–D–Ile–Pro–Arg–pNA +

$H_2O \rightarrow$ proteolytic act. $\rightarrow$ H–D–Ile–Pro–Arg–OH + pNA

The amount of *p*-nitroaniline (*p*-NA) release is an indicator of the proteolytic activity exhibited by many serin proteases and can be measured spectrophotometrically at 405 nm, according to the manufacturers' manual.

3. Results and discussion

3.1. Capture step

The first step plays a key role in the subsequent production process of a FIX concentrate. It was therefore important to find a separation medium, which allows the binding of FIX in flow-through (replacing the batch procedure). This would also speed up subsequent fractionation in the Cohn process [13,16]. The DEAE–Sephadex A-50, which is being used for binding three-factor PCC from plasma, allows adsorption in the batch procedure only [15,16]. However, the newly developed supports DEAE–Sepharose fast-flow, Q-XL-Sepharose and Fractogel EMD-Amino (HAP), allow the use of a chromatographic column in the first step also. The results of investigations comparing the four supports are shown in Table 1. When a column is used, the capture step is reduced from 6 h (batch procedure) to 1 h. The HAP material showed the best FIX enrichment of all the investigated supports. It is 4–5 times higher when compared to DEAE–Sepharose and –Sephadex and 2.5 times higher when compared to Q-XL-Sepharose. Proteolytic activity in the eluate was very low both with the HAP material and the DEAE–Sepharose fast-flow, whereas the use of Q-XL-Sepharose resulted in surprisingly high proteolytic activity (see Table 1). High proteolytic activity is linked to the activation of clotting factors, in most cases of FVII. This in turn implies the risk of proteolytic degradation [25]. Therefore DEAE–Sepharose fast-flow was excluded from further investigations because of its rather poor performance in terms of purification, Q-XL-Sepharose was excluded because of the aforementioned risk of proteolytic degradation.

The use of hydroxy-amino supports on a glycidyl-polymethacrylate basis (Toyopearl) for the purification of vitamin K-dependent proteins from the clotting cascade has been described elsewhere [26]. The method requires dilution of the plasma before its application to the column, since binding of FIX to the support is not guaranteed at a physiological salt concentration. This excludes the further use of the non-bound fraction for the production of albumin in Cohn fractionation.

The hydroxyaminopropyl ligand, which is bound to the support through a hydrophilic spacer, allows

Table 1
Comparison of different chromatographic supports used in capture step (first step in FIX production process)

	HAP	DEAE A-50	DEAE FF	Q-XL
Purification factor	250	50	50	100
FIX-yield (%)	>80	60	70	>80
p. A. S2288 (U/I)	<10	<10	<10	700

the binding of FIX directly from the undiluted plasma. While the flow-through from the column can be fractionated further, the fraction containing the FIX is eluted in a parallel step by a salt gradient (see Fig. 1). The material is adequately stable and allows repeated regeneration and sanitation with 0.5 *M* sodium hydroxide, up to 20 times.

3.2. Hydrophobic interaction chromatography (HIC)

The object of this chromatographic step is to remove further contaminations, especially vitamin K-dependent proteins (FII, FVII and FX). The chromatographic support, octyl-Sepharose CL4B, is so hydrophobic that the elution buffer from the first step, the capture step, can serve as application buffer for the HIC column. Consequently ultra/diafiltration, which would usually be carried out for reaching the necessary ionic strength, is not necessary between these two steps. As is seen in Table 2A, clotting factors II and X are removed by HIC to a large extent. In the process shown here, this step enriches FVII as well as FIX (cf. Table 2A). In alternative processes where DEAE–Sepharose is used as a second purification step, FVII is almost completely removed, whereas the amount of FX in relation to FIX is relatively high (cf. Table 2B). The binding of FVII to heparin Sepharose is much weaker than that of FX. Therefore the subsequent purification of FIX from the eluate of the second production step (HIC or anion-exchange chromatography) by means of affinity chromatography with heparin-Sepharose, which also implies the removal of FVII, is much easier in the case of the HIC eluate. Elution of the enriched FIX is achieved by a Triton X-100®

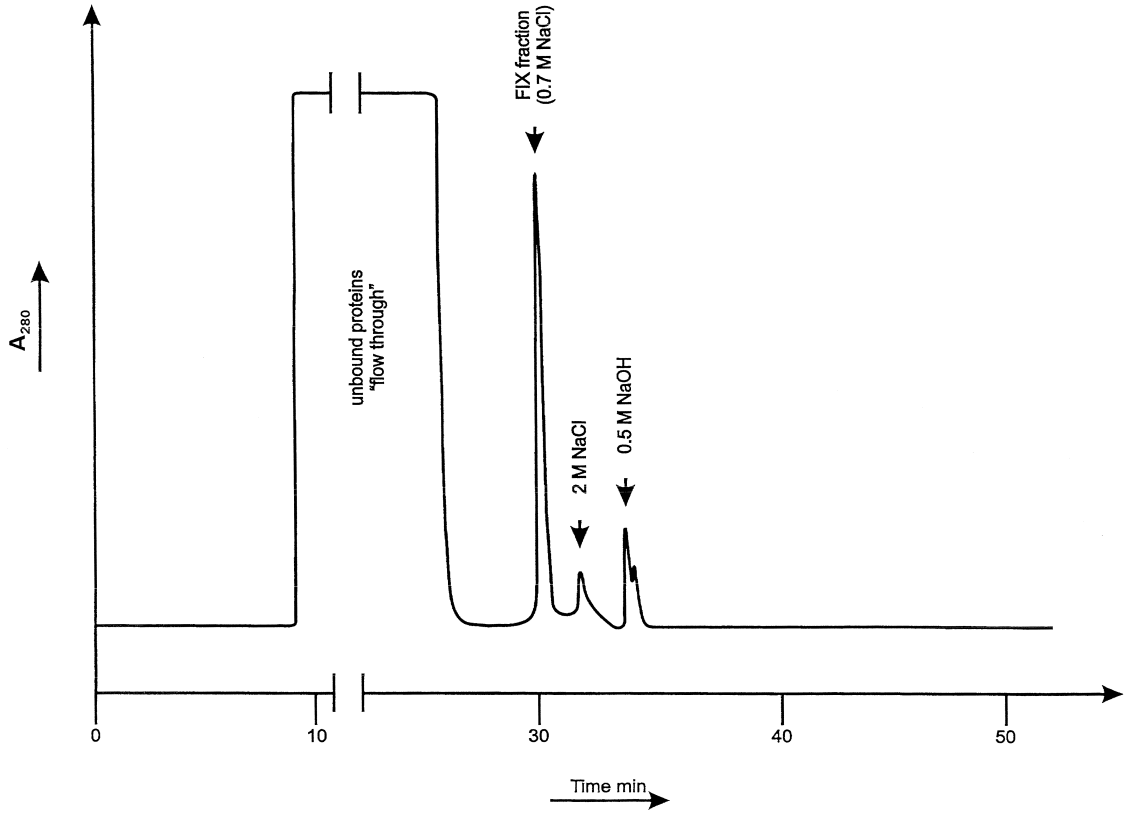

Fig. 1. First chromatographic step in production of clotting factor IX concentrate–adsorbtion chromatography on Fractogel EMD-Amino (HAP). Human plasma (2.3 l) after cryoprecipitation was pumped through a HAP column. FIX was eluted with 0.7 *M* NaCl-step gradient. The column was regenerated with 2 *M* NaCl and sanitized with 0.5 *M* NaOH. For additional data see Experimental.

Table 2
Distribution of coagulation factors as FIX purification proceeds along the process (A) EMD-amino (HAP)–octyl-Sepharose CL 4B (HIC)–heparin-Sepharose CL 6B (HEP)[a] and (B) DEAE–Sephadex A-50–DEAE-Sepharose FF–heparin-Sepharose CL 6B[b]

		FII (IU/ml)	FVII (IU/ml)	FIX (IU/ml)	FX (IU/ml)	Protein (mg/ml)	Purification (fold)
(A)							
HAP eluate	Mean	8	7.6	12	8	1.8	333
HIC eluate	Mean	1	61	14	1	0.9	778
Heparin-Sepharose eluate	Mean	0	0	15	0	0.06	12 500
(B)							
Sephadex eluate	Mean	17	4.2	28	18	16	88
DEAE Sepharose eluate	Mean	8	0.1	14	9	2.3	304
Heparin–Sepharose eluate	Mean	0	0	55	0	0.7	3900

[a] Data obtained from five consecutive batches of FIX-production.
[b] Data obtained from nine consecutive batches of FIX-production.

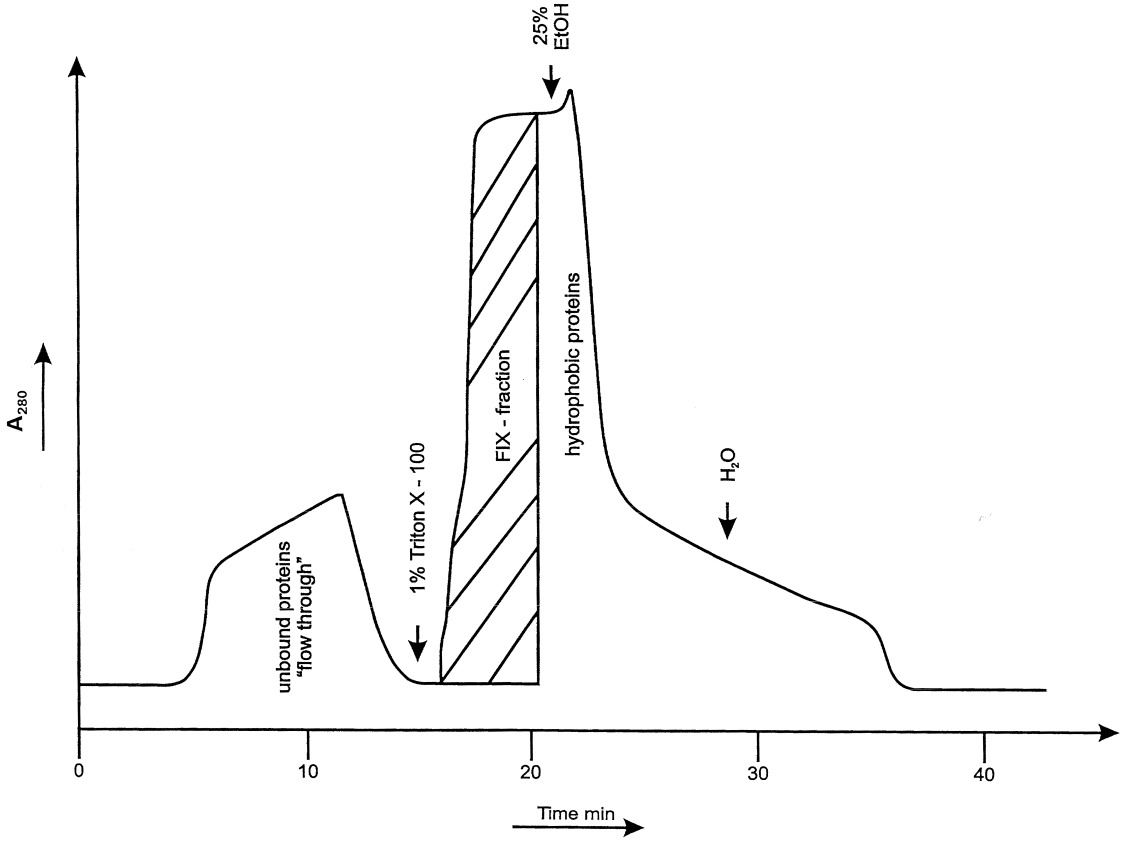

Fig. 2. Second step in production of clotting factor IX–hydrophobic interaction chromatography (HIC). The eluate from HAP columns was applied to a octyl-Sepharose CL4B column. FIX was eluted with 1% Triton X-100®. The column was subsequently washed with water and sanitized with 0.5 *M* NaOH. For additional data see Experimental.

solution (cf. Fig. 2). The remaining proteins, which bind even stronger to the column, are subsequently eluted with 25% ethanol. The column can easily be sanitized with sodium hydroxide. After this chromatographic step a specific activity of 20 IU of FIX/mg of protein is reached. This means that the enrichment after this step is up to 1500-fold. The yield of FIX is high, about 80%.

3.3. Virus inactivation and heparin affinity chromatography

The Triton X-100® eluate is virus inactivated with solvent/detergent in another step. For this, the Triton X-100 concentration is calibrated at 1% (w/v), then 0.3% TnBP (w/v) is added. The subsequent step, affinity chromatography with heparin-Sepharose, serves as the polishing step. It also removes solvent and detergent (Fig. 3). The enrichment after this step is more than 10 000-fold. The specific activity of the product is over 200 IU/mg of protein, corresponding to the specific activity of the product, which was isolated by immunoaffinity chromatography [22]. The purity of the product was also confirmed by the size-exclusion chromatography (cf. Fig. 4). The total yield is over 50%.

3.4. Characterization of the production process and the final product

Tables 2–4 show a comparison between two production processes for FIX. One is based on

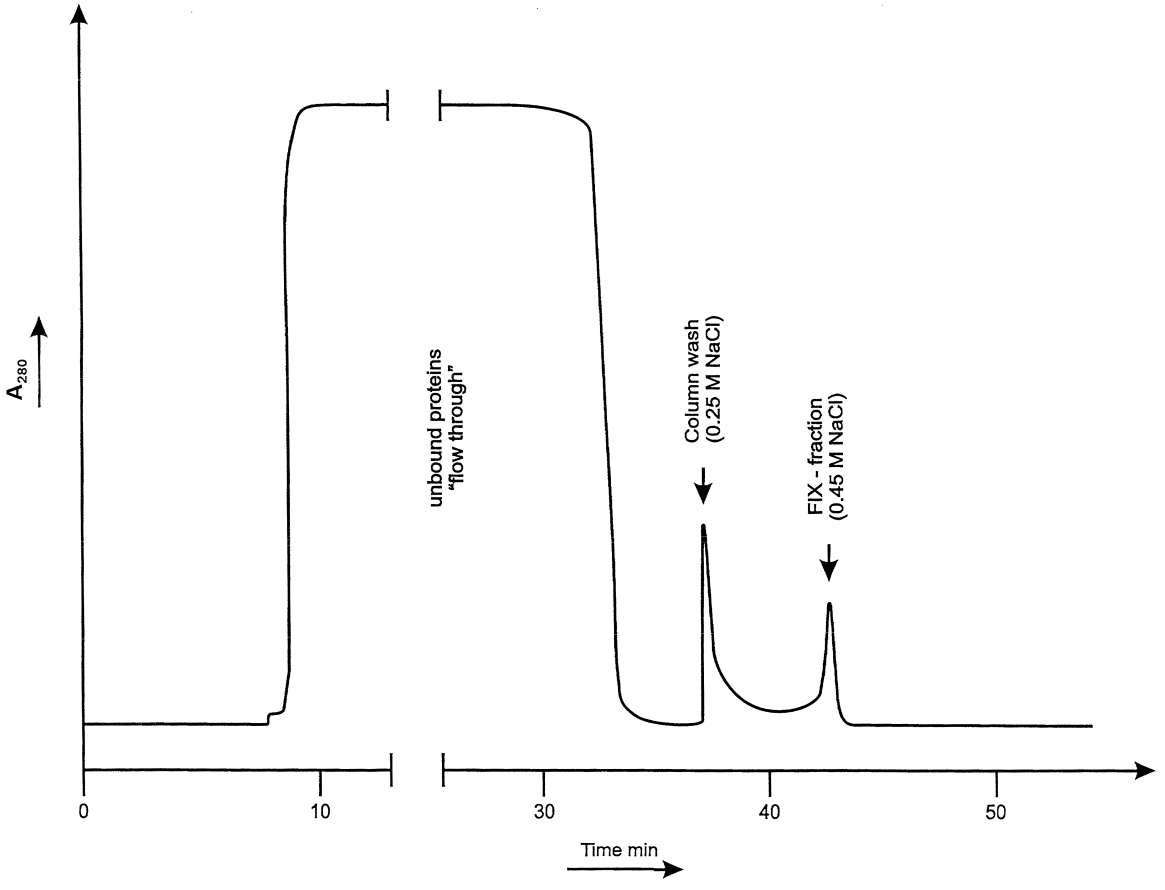

Fig. 3. Third step in production of clotting factor IX–heparin affinity chromatography. Eluate from HIC was first virus inactivated with solvent/detergent and applied to a heparin-Sepharose column. Contaminating proteins were removed with 0.25 *M* NaCl (step gradient), the fraction containing FIX eluted with 0.45 *M* NaCl. For additional data see Experimental.

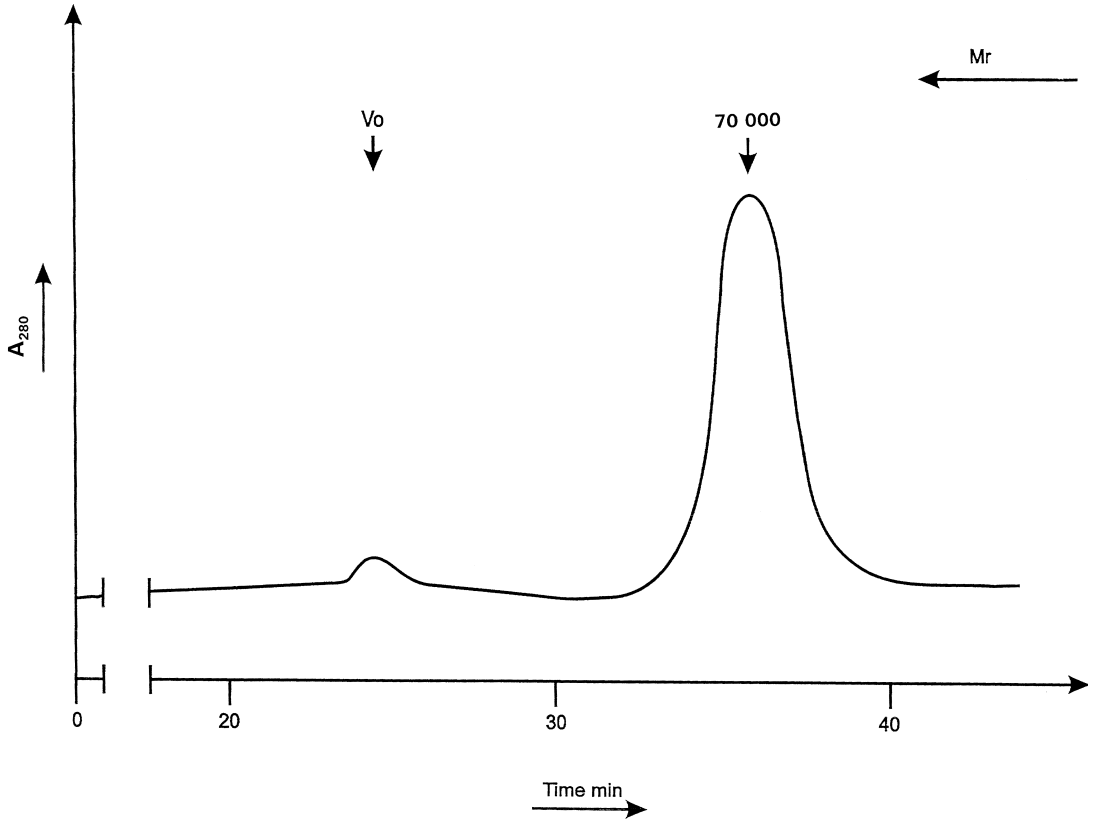

Fig. 4. Analytical size-exclusion chromatography of purified clotting factor IX concentrate. Conditions: A TSK 3000 SW column (600×8.0 mm) was used, mobile phase was phosphate-buffered saline, pH 7.2, flow-rate 0.5 ml/min, pressure 8 bar, room temperature. Injection volume was 10 μl (about 1 IU FIX).

Table 3
Optimisation of FIX production: comparison of different chromatographic steps between new and old process (cf. [14])

Step	Old process	Enrichment factor	Time (h)	New process	Enrichment factor	Time (h)
FIX capture	DEAE–Sephadex A-50	50		Fractogel EMD Amino 650M	250	
pH of plasma	7.2–7.8			7.0		
Performance	Batch		6	Column		1
Preparation for next step	UF/DF			Not necessary		
FIX purification	DEAE–Sepharose FF	200	6	Octyl-Sepharose CL4B	1500	2
	UF/DF			Not necessary		
Virus inactivation	1% Tween 80®		6	1% Triton X-100®		6
	0.3% TnBP			0,3% TnBP		
FIX polishing	Heparin–Sepharose CL 6B	2500	2	Heparin-Sepharose CL 6B	10 000	2
Second virus treatment	Nanofiltration		4	Dry heat		2
Formulation	No change					
Process time	24 h			13		

Table 4
Comparison of different production steps in new developed and existing production process (cf. [16])

Step	Parameter	Old process	New process	Consequence/comment
FIX capture	Resin	DEAE–Sephadex A-50	Fractogel EMD Amino 650M	Yield ↑ (60→80%) Spec. act. ↑ (1→5) Proteolytic act. ↓
	pH of cryo-poor plasma	7.2–7.8	7.0	Variation of yield ↓
	Absorption/elution	Batch	Column	Process time ↓ Reproducibility ↑
	Reduction of salt	UF/DF	Not necessary	Process time ↓ Activation ↓
FIX purification	Resin	DEAE–Sepharose FF	Octyl-Sepharose CL4B	Yield ↑ (70%→80%) Spec. act. ↑ (5→20) FX, contents ↓
	Reduction of salt	UF/DF	Not necessary	Process time ↓
Virus inactivation	S/D treatment 6 h/27°C	1% Tween 80® 0.3% TnBP	1% Triton X 100® 0.3% TnBP	Inactivation efficiency ↑
FIX polishing	Resin	Heparin-Sepharose CL 6B	Heparin-Sepharose CL 6B	Removal of S/D reagents Overall yield ↑ (40%→50%) Spec. act. ↑ (50→200)
Second virus treatment		None	Dry heat	Virus safety ↑ (at least 4–5 orders of magnitude)
Formulation	Sterile filtration filling lyophilisation	No change		

'classical' column chromatography, that is without the use of immunoaffinity chromatography with monoclonal anti FIX-antibodies. The quoted process based on batch adsorption in capture step has been described previously [16]. The period of time required for the newly developed process is reduced from 24 to 13 h (see Table 3). Besides, the introduction of column chromatography instead of batch adsorption in the capture step, allows a comfortable integration of the new process into the Cohn-fractionation (see above). The replacement of Tween 80® by Triton X-100® for S/D virus inactivation provides a more efficient destruction of viruses with lipid envelopes (cf. Table 4). Moreover, the production process of FIX presented here has a much higher yield (more than 50% compared to a maximum of 40%, Table 4), and FIX is more highly enriched (more than 10 000-fold, cf. Table 2). The specific activity of the product is over 200 IU of FIX/mg of protein, and therefore close to the theoretical figure of this clotting factor [2]. As seen in Fig. 4, the result was confirmed by size-exclusion chromatography. In the old product, between 20 and 50% of impurities with high molecular masses are found [16]. In the new product such contaminants are hardly detected at all.

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