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Review

Preparation of vitamin K-dependent proteins, such as clotting factors II, VII, IX and X and clotting inhibitor Protein C

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Abstract

A review is given of preparative methods for the isolation of the vitamin K-dependent clotting factors II, VII, IX, X and clotting inhibitor protein C, all derived from human plasma. Factor II, activated factor VII and activated protein C are also obtained from recombinant animal cells. The methods for their purification are described. The problem of difference in posttranslational modifications between plasma derived and recombinant protein is discussed with regard to therapeutic proteins.
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1. Introduction

The clotting factors II, VII, IX and X as well as the inhibitors protein C and protein S belong to the group of vitamin K-dependent proteins. This group of proteins plays a key role in blood clotting [1–4].

The most important therapeutic preparations which contain vitamin K-dependent proteins, are clotting factor IX concentrate and prothrombin complex concentrate (PCC) [5,6]. The recombinant factor IX (rFIX) and activated recombinant factor VII (FVIIa) concentrates are also used therapeutically [7,8]. After

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successful clinical trials also activated recombinant protein C (arPC) entered the market [7–10].

All vitamin K-dependent proteins, which play a part in the clotting cascade, are synthesized in the liver [4,11]. Prothrombin or factor II (FII), factor VII (FVII), factor IX (FIX), factor X (FX) and protein C (PC) circulate in blood as zymogen molecules and are converted to active serine proteases during the coagulation cascade [1,2]. Protein S (PS), however, participates as a cofactor to activated PC (aPC), and these two proteins are involved in the regulation of the coagulation pathway by the inactivation of factor Va and factor VIIIa in the presence of phospholipid [1,2].

Each of the six human vitamin K-dependent coagulation factors consists of a series of homologous structural domains. The N-terminal region of each vitamin K-dependent coagulation protein contains from nine to 12 γ-carboxyglutamic acid (Gla) residues. These residues are formed by carboxylation of glutamic acids located within the first 40–45 amino acid residues in the N-terminal region of each protein [3]. The Gla domains are required for the calcium-dependent binding of vitamin K-dependent proteins to phospholipid surfaces [11]. This Ca^{2+}-dependent binding localizes the vitamin K-dependent coagulation factors at the site of vascular injury, where platelet plug formation has been made available.

The Gla domain in prothrombin is followed by two kringle domains [3]. In FVII, FIX, FX, PC and PS the kringle domains are replaced by two to four growth factor domains [3,11]. Both the Gla and the growth factor domains have been implicated in the specific interactions and assembly of the vitamin K-dependent factors and their cofactors on membrane surfaces [3].

The C-terminal region of FII, FVII, FIX, FX and PC contains the catalytic or serine protease domain that includes the active site residues Asp, His and Ser. In contrast to pancreatic serine proteases, the serine proteases generated during blood coagulation have a high degree of substrate specificity [7,12,13].

Vitamin K-dependent proteins are components of blood plasma, where they occur in very low concentrations (cf. Table 1). Until about 10 years ago, these proteins were isolated exclusively from human plasma. Recently, FIX, FVIIa and PC have also been produced with recombinant cells [10,17–19].

Of all vitamin K-dependent proteins, FIX is needed mostly for therapeutical purposes. The absence or a defect of FIX nearly always causes hemophilia B, a genetically inherited disease in which the clotting cascade is disturbed [3,5]. Until now, infusions of FIX are the only way to treat this disease. Preparations of PCC are used for replacement therapy of prothrombin complex factors in order to prevent or arrest bleeding resulting from an overdose of oral anticoagulants or from liver dysfunction [5,6,19]. FVIIa is used for treatment of bleedings in connection with certain complications, where the appearance of inhibitorial antibodies blocks the function of clotting factor VIII (FVIII) or, in rare cases, also of FIX [8]. Extensive clinical trials with aPC have shown that this vitamin K-dependent clotting inhibitor can be used for treatment of severe sepsis [9,20].

The first enriched concentrates of vitamin K-dependent proteins were produced in the late 1950s. The method was adsorption of the protein to barium

Table 1
Properties of vitamin K-dependent proteins[a]

Protein	Molecular mass (Da)	Number of polypeptide chains	Number of of Gla residues	Carbohydrate content (%)	Plasma concentration (μg/ml)
Factor II	72 000	One	10	8	80–90
Factor VII	50 000	One	10	9–10	0.47
Factor IX	57 000	One	12	17	4
Factor X	59 000	Two	11	15	6.4
Protein C	62 000	Two	9	23	3.9–5.9
Protein S	71 000	One	11	7–8	25–35

[a] The data were taken from Refs. [3,14–16].

sulfate from the plasma [21]. The barium sulfate was later replaced by tri-calcium phosphate, a non-toxic salt, while the method remained more or less the same [22]. In both cases the active clotting factor IX, which had adsorbed to the salt precipitate, was eluted with a sodium citrate solution. Other vitamin K-dependent clotting factors were enriched along with FIX. Hence the name was PPSB for such concentrates. They were rich in prothrombin (FII), proconvertin (FVII), Stuart Prower factor (FX) and antihemophilic factor B (FIX) [23]. Such concentrates were successfully used from the 1960s onwards for treatment of hemophilia B. Infusion with PPSB (also called prothrombin complex concentrate, PCC) was for more than 20 years the method of choice for this kind of treatment [5,24,25].

A disadvantage of the precipitation methods was that they could not easily be integrated into the fractionation method of Cohn et al. [26]. Only the introduction of chromatographic methods made it possible to fully harmonize the production of PPSB 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. [27]. While the cryoprecipitate representing the FVIII-rich fraction was separated, the supernatant, the so-called cryopoor plasma, was used for the production of PPSB. The batch adsorption step using anion-exchange resins, causes only a short delay in the main fractionation scheme designed for albumin and IgG. The anion-exchange medium is incubated with the cryopoor plasma, and the unbound fraction is used for further bulk purification. The resin is recovered by filtration, washed usually with a citrate buffer containing 0.2–0.3 *M* NaCl to remove loosely bound proteins, then washed again with up to 2 *M* NaCl to elute the fraction containing the vitamin K-dependent factors. Instead of the cryopoor plasma the Cohn fraction IV can be used also, but with a much higher risk of activating the clotting factors [28].

The PPSB concentrates produced by these methods in the 1970s were not subjected to virus inactivating procedures. At the beginning of the 1980s, pasteurization was introduced as the first method for inactivating viruses. Concentrates of FVIII were the first to be treated in this manner [29]. Virus inactivation of commercially available FIX concentrates was carried out only several years later [30]. By the late 1980s and early 1990s the established methods for virus inactivation of plasma products were, apart from pasteurization, steam treatment at 60 °C for 10 h and at 80 °C for 1 h in a special heating chamber which construction ensures constant product temperature. Other, non-thermal methods are treatment with β-propiolactone, with ultraviolet radiation, and solvent/detergent (S/D) treatment. With the exception of β-propiolactone combined with ultraviolet radiation, these methods are still being used for virus inactivation of FIX concentrates. The method of β-propiolactone combined with ultraviolet radiation was given up, when several cases of HIV transmission occurred through products treated in this way [31].

The introduction of a virus-inactivating step almost always requires the addition of different reagents. In the case of pasteurization, stabilizers have to be added to protect the sensitive proteins against denaturing [32]. In contrast, the other reagents that are added, such as solvent and detergent, destroy the lipid-enveloped viruses [33]. They have to be removed from the preparation in a subsequent step. Chromatography is a frequently used tool for removing solvent/detergent and stabilizers [34]. If anion-exchange chromatography (AEC) is used for this purpose in the production of PPSB, additional purification results. It has been observed that through AEC a large part of the proteases is removed, apart from other proteins [35]. The proteases come from source material, plasma as contaminants, and possibly they are also activated in the purification process [6,36].

At present, the vitamin K-dependent clotting factors IX and VIIa and the clotting inhibitor PC are being produced in different ways. They are either isolated from human plasma or produced with the help of genetically manipulated animal cells [7,8]. These methods are summarized in Table 2. The possible production of FIX and PC from the milk of transgenic animals is still at an experimental stage [37]. The very rare genetic and acquired deficiencies of other vitamin K-dependent clotting factors such as factors VII and X are still treated with PPSB concentrates, derived from plasma [6].

All proteins that have been mentioned here are

Table 2
Production and virus inactivation methods for some therapeutically used vitamin K dependent proteins

Product	Plasma derived	Recombinant	Purification method	Virus inactivation/removal	Specification activity IU/mg protein	Refs.
PCC	Yes	–	SPE/AEC	S/D and nanofiltration	–[a]	[6]
PCC	Yes	–	SPE precipitation	Pasteurization and nanofiltration	–[a]	[56]
FIX conc.	Yes	–	SPE/AEC and heparin affinity chromatography	S/D and nanofiltration	150–180	[35,62]
FIX conc.	Yes	–	SPE/AEC and hydrophbic interaction chromatography	Steam treatment at 60°C/10 h and 80°C/1 h.	107–161	[65]
FIX conc.	Yes	–	SPE and multiple chromatography/ affinity chromatography steps	S/D		[65]
FIX conc.	Yes	–	SPE/metal-chelate chromatography	S/D and chromatography	About 160	[55,66]
FIX conc.	Yes	–	SPE/SPE/AEC/immunoaffinity chromatography	Thiocyanate incubation and nanofiltration	204–273	[65]
FIX conc.	–	Yes	Pseudo-affinity chromatography/ affinity chromatography/hydroxylapatite and metal-chelating chromatography	Chromatography and nanofiltration	240–30	[74]
FVII	Yes	–	SPE and $Al(OH)_3$	Steam treatment at 60°C/10 h and 80°C/1 h	~1[b]	[104]
FVIIa	–	Yes	AEC, pseudo-affinity and immunoaffinity chromatography	?	High	[17]
Activated PC	–	Yes	AEC/immunoaffinity chromatography	Chromatography (?)	High	[120,121]

S/D, solvent/detergent.
[a] Specific activity of prothrombin complex concentrates is about 1 IU FIX/mg protein.
[b] ‘‘Medium–purity’’ concentrate.

middle-sized, with molecular masses between 40 000 and 80 000 kDa (see also Table 1). However, with γ-carboxylation, phosphorylation and glycosylation they have without exception rather complex post-translational modifications [4]. This means, firstly, that during their biosynthesis in recombinant animal cells there is a danger of these modifications not being copied with precision in such systems. For this phenomenon, rFIX has been an example over the last 2–3 years [38]. Secondly, there is a considerable risk that the proteins are activated, proteolytically cleaved or chemically modified during production, down-stream processing or storage [39]. This does not necessarily result in a loss of biological activity. However, other adverse side-effects may occur such as immunogenicity or poor recovery in vivo, if such substances are used as therapeutics [40].

2. Prothrombin complex concentrate

Although the increasing use of highly purified FIX preparations reduces the application of PPSB (and of ‘‘three-factors’’ PCC) for this indication, a demand for concentrates from vitamin K-dependent factors especially PPSB still remains. The most activated prothrombin-complex concentrates are used in the treatment of haemophilia A patients, who have developed inhibitors against FVIII [41]. The PPSB concentrate consisting of FII, VII, IX and X is rather widely used for treating a deficiency of vitamin K-dependent clotting factors, of PC and PS, as in cases of phenprocoumon or warfarin overdose and liver disease [6,19]. These preparations are preferred to fresh frozen plasma in life-threatening situations, since they reverse the respective coagulopathy more rapidly without causing volume overload [19].

Starting material for the production of PCC (or PPSB, see above) is cryopoor plasma. This is plasma from which the cryoglobulins have been removed by cryoprecipitation [27]. In the older process the vitamin K-dependent proteins were isolated in a first purification step from the other plasma components through adsorption to an anorganic salt [21,22]. This method is no longer used, since the resulting super-natant cannot be fractionated further, making it

impossible to obtain other plasma proteins, above all human serum albumin and immunoglobulins.

The most widely used method for the first, the ''catching step'' in the isolation process of vitamin K-dependent proteins from human plasma is solid-phase extraction with an anion-exchange resin. In most cases Sephadex is used, with DEAE or quaternary ammonium (Q) groups as ligands. Sometimes poly-sulfated cellulose supports have been used. FVII binds only weakly to DEAE Sephadex and poly-sulfated cellulose [5,42,43]. Therefore the use of these supports yields the so-called ''three-factor PCC'' containing FII, FIX and FX [44]. ''Three-factor'' PCC has been favoured for preventing and treating bleeding in hemophilia B [31]. However, PCC has been associated with thromboembolic complications, including acute myocardial infarction [44–46] and disseminated intravascular coagulation (DIC) [47]. Its thrombogenicity has mainly been attributed to activated clotting factors [24,48] or procoagulant phospholipid [49]. ''Three-factor'' PCC has now been replaced by high purity FIX concentrates [50].

To the strong anion-exchange resin Q-Sephadex binds the clotting inhibitors PC and PS, along with all four clotting factors. Whereas ''three-factor PCC'' for treatment of haemophilia B is increasingly replaced by highly purified FIX concentrates [49], the ''four-factor PCC'' or PPSB is still constantly used for clinical purposes (see above and Ref. [6]). The flow-chart for production of such a four-factor PCC is shown in Fig. 1. In this concentrate FII, FVII, FIX and FX should be evenly distributed, the optimum being a ratio of 1:1:1:1 [6]. Together with the four clotting factors, protein C and protein S should be present in therapeutically effective concentrations of about 1 IU per 1 IU of FIX [6]. Apart from these active components, other plasma proteins are found in PPSB such as ceruloplasmin, inter-α-trypsin inhibitor (ITI), high-molecular mass (HMW) kininogen, C_4, C_5, C_9 and vitronectin [32,51]. As shown by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) in Fig. 2, this concentrate contains additional, still non-identified proteins [6].

In the case of concentrates of PPSB, similar to ''three-factors'' PCC, their thrombogenicity has been a recurring problem [6,46,47]. The above-mentioned complications such as thrombotic disorders and DIC are associated with the high-dose use of PPSB possibly because of an overload of factors II:c, X:c, activated FIX, VII or X and/or coagulant active phospholipids [6]. Also, the relatively high proteolytic activity in some PPSB concentrates may contribute to their thrombogenicity [6,35].

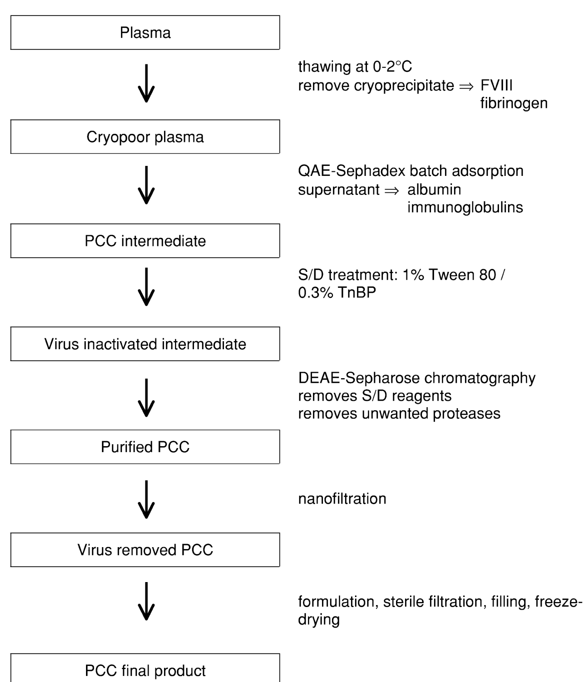

Fig. 1. Manufacturing process for PPSB concentrate (four-factor prothrombin complex concentrate).

As is seen in Fig. 1, the intermediate product is treated with solvent/detergent (S/D) in the following step. This inactivates the lipid-coated viruses [33]. Subsequent anion-exchange chromatography is mainly applied to remove the S/D reagents, but also for the additional removal of contaminating proteases [6].

In the case of another, commercially available PPSB concentrate virus inactivation is carried out by pasteurization in the presence of stabilizers, chiefly glycine and saccharose. The removal of the stabilizers and of possible contaminating proteins is achieved by precipitation with ammonium sulfate and calcium-phosphate adsorption. In order to increase virus safety, both concentrates are subsequently nanofiltered [6,52].

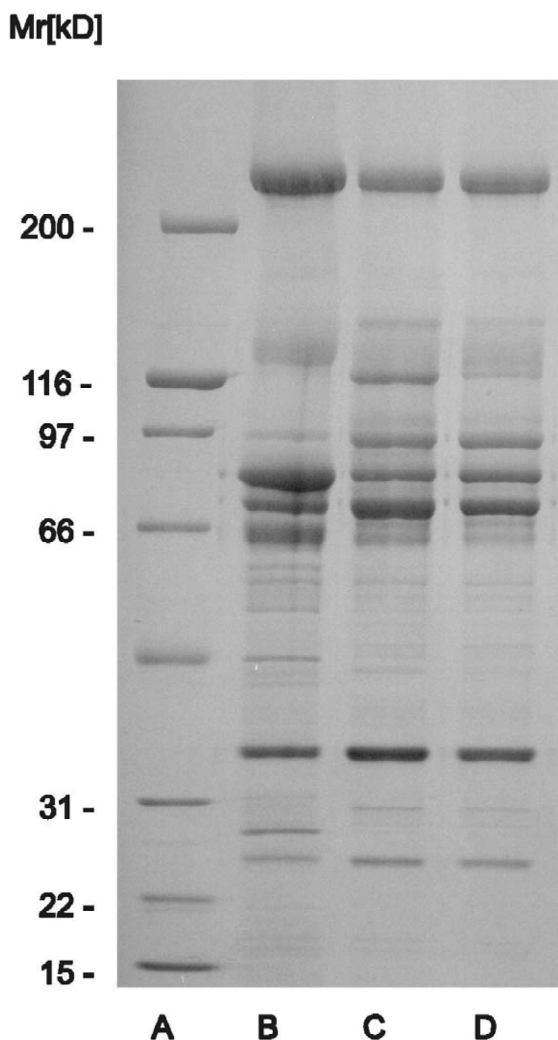

Fig. 2. Sodium dodecylsulfate–polyaorylamide gel electrophoresis of different PPSB concentrates. Line A, standard proteins; lines B–D, PPSB concentrates. Reprinted from Ref. [6] with permission.

The administration of PPSB can lead to complications caused either by contaminations or by activated vitamin K-dependent clotting factors (see above and Refs. [24,47,48]). Their activation, above all the forming of activated FVII, may occur during production. In order to suppress the activation of the clotting factors, heparin may be added at the beginning of the production process. Anion-exchange chromatography is further optimized by replacing the axial column with a radial one [6]. This speeds up the production process considerably, significantly reducing the risk of activation of PCC factors. The PPSB concentrate produced in this way has low thrombogenicity. The respective activities of the four vitamin K-dependent clotting factors have a ration of roughly 1:1:1:1. The other vitamin K-dependent proteins (inhibitors), PC and PS, are contained in the concentrate in therapeutically effective concentrations [6].

Solid-phase extraction as the first purification step is easy to perform and to validate, since the resin is used only once and then discarded. However, it is this step which is time-consuming and requires a lot of work. As it is performed in batch procedure, automation is virtually impossible. Besides, the starting material of this step is a very complex mixture with a rather high risk of activation of both clotting factors and other zymogens. Therefore several attempts have been made to carry out this capture step not in batch procedure but on a column. However, the experiments with promising results, involving supports with hydroxyaminopropyl groups as ligands, have not yet, to the best of our knowledge, left the pilot-scale. Whereas the initial experiments required the dilution of the starting material in order to achieve optimal binding of the vitamin K-dependent proteins [53], the introduction of appropriate chromatographic conditions allowed binding of undiluted cryopoor plasma. This was achieved by optimization of ligand presentation using appropriate spacer and ligand density [54].

3. Clotting factor IX

3.1. Plasma-derived clotting factor IX

Over the last years different strategies have been developed for the production of single vitamin K-dependent plasma proteins. Based on modern chromatographic methods, highly purified FIX concentrates were produced, e.g., by a combination of anion-exchange and affinity chromatography [32,51,55] or by immunoaffinity chromatography [56]. When such highly purified FIX concentrates were used for treatment of haemophilia, the above mentioned problems concerning thrombogenicity were no longer reported [48,50].

The first steps in the production of FIX from human plasma are similar to those performed for the purification of PPSB. Fig. 3 shows the flow-chart for the production of a highly purified, plasma-derived FIX concentrate. Again, the starting material is cryopoor plasma. In the first step, the catching-step, the weak anion-exchange resin DEAE-Sephadex is used instead of the strong anion-exchange resin Q-Sephadex. This choice reduces from the very beginning binding of FVII to the resin, and the process yields "three-factor PCC" as desired. Consequently, FVII as a possible contaminant is removed at this early stage. It may easily be activated in subsequent steps.

The next step, chromatography on DEAE-Sepharose Fast Flow, is applied chiefly to remove residual FVII and contaminating proteases, which might otherwise cleave FIX at the later stages of the production process, especially during S/D virus inactivation, thereby reducing yield. This is followed by affinity chromatography with heparin Sepharose to accomplish a 2-fold task. At first the S/D reagents are removed, then the vitamin K-dependent clotting factors II and X are totally removed. The FIX purified in this way has on the average a specific activity around 100 IU/mg of protein [51,57].

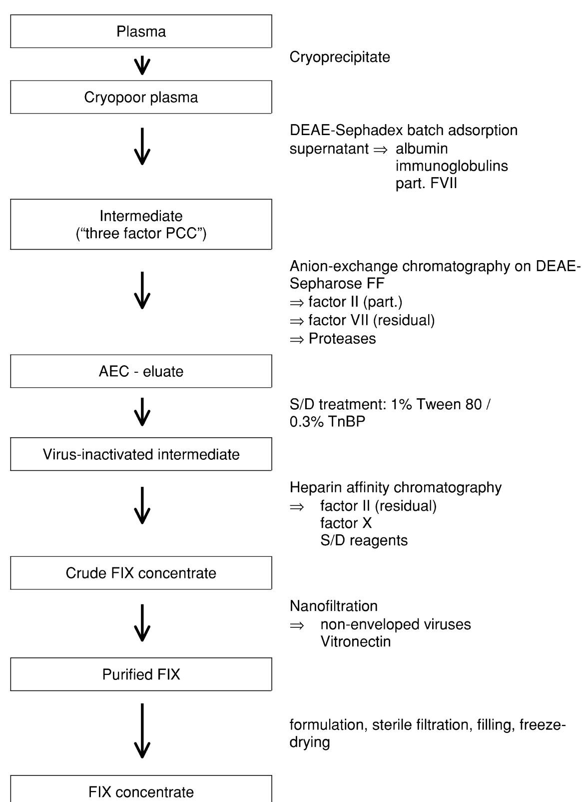

Fig. 3. Manufacturing process for highly purified clotting factor IX concentrate.

This means that the preparation contains up to 50% of proteins other than FIX [32,57,58]. It has been found that these substances have as a rule molecular masses which are higher than that of FIX. This allows size-exclusion chromatography (SEC) to be applied for their quantitative separation [57,59]. Then vitronectin (VN) was identified as an accompanying protein, which occurs in large quantities in FIX preparations produced in this way [32,58]. VN is an adhesion protein, which is found in human plasma in rather high concentrations between 0.2 and 0.4 mg/ml. It is not related to vitamin K-dependent factors [60]. The almost 100% increase of specific activity in a FIX concentrate (from about 100 to about 200 IU/mg of protein), which at first came as a surprise, could subsequently be attributed to selective removal of VN by nanofiltration (cf. Fig. 4). The chief object of nanofiltration was again the removal of eventually present, residual viruses [52,61–63].

In other procedures the second anion-exchange chromatography is replaced with ammonium sulfate precipitation, followed by hydrophobic interaction chromatography (HIC). The advantage is that the HIC step removes a major part of the VN [34,64,65].

Sometimes sulfated dextran is used instead of heparin Sepharose [42], thereby avoiding the use of any animal material (heparin) in the production process. The disadvantage of this method is that the selectivity of the support is poorer than that of heparin Sepharose, making it more difficult to separate other vitamin K-dependent factors.

For the isolation of FIX, metal-chelate affinity chromatography with copper-charged chelating Sepharose can be used as an alternative to either anion-exchange chromatography or heparin affinity chromatography [55,66]. The catching-step with a weak anion-exchange resin is the same. The FIX concentrate is S/D virus inactivated. The authors claim that metal-chelate chromatography constitutes a significant step of virus removal [55]. The resulting concentrate has a relatively high specific activity of

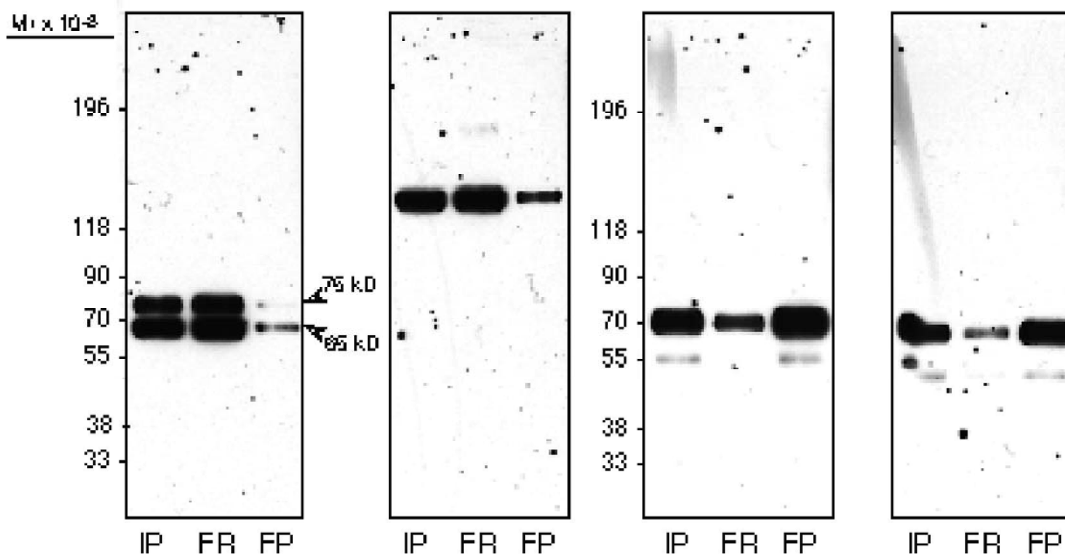

Fig. 4. Separation of vitronectin (VN) by Viresolve nanofiltration, which is carried out for virus removal in an FIX concentrate. VN was detected by immunoblot with monoclonal antibodies after SDS–PAGE under reducing (A) and non-reducing (B) conditions. As a control, immunoblot with anti-FIX monoclonal antibodies was performed under reducing (C) and non-reducing (D) conditions. The initial product (IP), the final retentate (FR) and the final product (FP) were applied to the gel. Reprinted from Ref. [32] with permission.

over 160 IU/mg of protein. The only accompanying proteins that occur in significant quantities are PC and inter-α-trypsin inhibitor, about 20% of the total amount of protein. The concentration of copper from the chromatographic column is 0.01 μg/IU of FIX in the final product and therefore quite low [55].

The advantage of this method is that one chromatographic step is made redundant. However, the quoted yield of about 200 IU/kg of plasma (20%) is rather low when compared with the yield obtained through a combination of anion-exchange chromatography and heparin affinity chromatography, which is twice as high (see above).

Instead of the combinations of anion-exchange and hydrophobic interaction chromatography described above, the eluate resulting from solid-phase extraction with a weak anion-exchange resin can also be purified in a subsequent step with immunoaffinity chromatography with immobilized monoclonal antibodies [67–69].

Some monoclonal antibodies bind to FIX only in the presence of divalent cations. Elution of FIX is effected simply by using a chelating agent such as EDTA or citrate. Such divalent cation-dependent monoclonal antibodies are used for affinity purification of FIX. The column with immobilized antibodies is loaded with FIX containig material in the presence of divalent cation (calcium or magnesium) and washed with high concentrated salt solutions. FIX is subsequently eluted under gentle conditions using a buffer containing chelating agent [67–69].

Smith [67] describes the use of immobilized metal ion-dependent anti-FIX antibodies for the isolation of FIX from commercial FIX concentrates with low purity. The additionally purified, S/D virus inactivated FIX concentrate had a specific activity of between 134 and 155 IU/mg of protein.

Tharakan et al. [68] have used similar metal ion-dependent antibodies for further purification of FIX from the eluate after extraction with DEAE-Sephadex. The eluate from DEAE-Sephadex was applied to an immunoaffinity column with immobilized antibodies (mentioned above). After rinsing the column with high salt concentrations in the presence of magnesium ions, FIX was eluted with a buffer containing either citrate or EDTA. The purified FIX had a specific activity of over 200 IU/mg of protein, the yield in this chromatographic step was 95%. The S/D treatment was incorporated in the process. The authors claim that there was no detectable leakage of

monoclonal antibodies from the column. Therefore no specific steps were taken for their removal, not even as a precaution in case leakage occurred after all.

The above-described methods of immunoaffinity chromatography for the isolation of FIX have hardly surpassed the pilot-scale. The production process of the only FIX concentrate that is used world-wide and which uses immunoaffinity chromatography as a method of purification, is slightly different from the protocols cited above. The eluate from DEAE-Sephadex is applied to a column with immobilized anti-FIX antibodies. After removal of weakly bound accompanying proteins, FIX is eluted with a concentrated NaSCN solution. Since NaSCN is used for virus inactivation as well as for elution, FIX is incubated in this solution for some more time. Removal of NaSCN is followed by so-called viral-retentive filtration (nanofiltration) of the relatively diluted FIX solution. Any murine antibodies resulting from leakage of the immunoaffinity column that may still be in the product, are removed chromatographically with an amino hexyl-Sepharose column [70,71]. The FIX purified in this way has a specific activity of over 200 IU/mg of protein (usually of 250 IU/mg of protein and more). It is practically free of cleavage products [65].

3.2. Recombinant clotting factor IX

As early as 1986 Kaufmann et al. [72] succeeded in synthesizing human, recombinant clotting factor IX (rFIX) in Chinese hamster ovary (CHO) cells, with subsequent purification by a combination of anion-exchange chromatography and immunoaffinity chromatography. In terms of glycosylation and γ-carboxylation, the isolated rFIX had posttranslational modifications that were according to authors similar to those found in plasma-derived FIX (pd FIX). However, the differences were still too significant, and therefore until about 1993 the development of rFIX was considered to be questionable [73].

Several investigations have followed, which were aimed at synthesizing rFIX with posttranslational modifications still more similar to those of pd FIX, especially with regard to glycosylation and γ-carboxylation. Again, the methods used for purification were a combination of anion-exchange chromatography and affinity chromatographic methods [72,74]. Also in the mid-1990s, the interesting attempt was made to modify (''tailor'') in vitro the glycosylation of rFIX, which had been produced in recombinant cells, with the help of glycosyl transferases. The aim was to bring the glycosylation patterns of the recombinant product as close as possible to the point of identity with the patterns of pd FIX [75]. This idea, which originally was looked upon as purely academic, has recently been taken up again. Similar systems were used to modify glycosylation in recombinantly produced proteins in vitro in order to reach identical with the glycosylation patterns found in the corresponding natural glycoprotein [76].

The only commercially available rFIX preparation was not registered before the late 1990s, almost 10 years later than rFVIII [74,77]. A CHO cell line is used for its synthesis. This cell line does not only contain a DNA segment encoding FIX, but is also transfected with a plasmid encoding human PACE-SOL, a truncated, soluble form of the serine protease PACE (paired basic amino acid cleaving enzyme). Expression of this protease has substantially increased the capacity of CHO cells to remove the amino-terminal peptide from rFIX [74]. If this protease is not co-transfected, the secreted rFIX still contains approximately 20–30% of the product with non-cleaved 18-amino acid propeptide [78]. The culture medium used for cell growth does not contain any proteins from either blood or plasma [74].

Consequently, isolation is accomplished without using any substances of animal origin. This means that in chromatography neither immobilized, monoclonal antibodies nor immobilized heparin are used. The following chromatographic steps are carried out [74]:

(1) Pseudo affinity chromatography on Q-Sepharose FF; FIX is eluted with a diluted buffer to which 10 m*M* $CaCl_2$ have been added.

(2) Cellufine sulfate chromatography.

(3) Ceramic hydroxylapatite chromatography.

(4) Metal-chelate chromatography with immobilized copper.

The chromatographic steps are coordinated in such a way that the eluate from one column can be applied to the next column with a minimum of additional operations such as dilution, ultra/diafiltration, etc. [74]. The last purification step is nanofiltra-

tion using Viresolve 70 membranes. Along with lipid-coated and non-coated viruses that may still be present, this step also removes any larger proteins and protein aggregates [62,74]. The final product, the rFIX concentrate, has a very high specific activity (between 265 and 286 IU/mg of protein) and is practically free of any contaminating proteins [74].

The case of rFIX has shown that the tiniest differences in posttranslational modifications between pdFIX and rFIX can have a bigger impact in clinical use than originally expected. Recombinant FIX differs marginally from pdFIX in its γ-carboxylation and glycosylation. Additionally, one sulfation site and one phosphorylation site are not occupied in the recombinant protein [77]. Although these differences are negligible for clotting activity in vitro, it was soon detected that in the case of some patients, especially younger ones, the in vivo recovery of rFIX is significantly lower compared with pdFIX [38,79,80]. This was one of the reasons why the European regulatory authorities have recently put the product under close scrutiny, demanding additional clinical trials [81].

This case demonstrates yet again how important it is to pay attention to any changes that may occur in such therapeutics, during the fermentation process as well as during isolation and storage [39,82,83].

3.3. New methods for preparative isolation of clotting factor IX

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. An additional demand for the isolation process of therapeutic proteins, among them FIX from human plasma, is its integration into the fractionation schema [28,84]. It has to be ensured that the option to purify other plasma proteins such as albumin and IgG and also of those proteins, which occur in much lower concentrations, such as antithrombin III, α_1-antitrypsin, etc., is reduced as little as possible, if at all.

In designing a production process, the most important factors are its speed and the ability for fast in-process analysis [85,86]. As shown here already, the use of modern filtration and chromatographic techniques allow the production of highly purified concentrates of therapeutic proteins such as PPSB and/or isolation of single plasma proteins [28,87]. However, chromatographic techniques in particular have serious limitations, especially in terms of speed and capacity [85]. Although radial columns have reduced considerably the period of time required for the production of PPSB concentrate (see above and Ref. [88]), these methods still carry the risk of unwelcome changes of proteins and loss of activity.

In an interesting publication, pointing the way forward, Iberer et al. [89] have shown that separation of FIX and VN from an FIX concentrate can be performed by continuous annular chromatography in anion-exchange mode. Subsequent sanitation, regeneration and equilibration of the column were carried out in the same cycle, without interrupting the process. In the investigated case the productivity of the optimal batch process was higher than that of the process with preparative continuous annular chromatography. However, it was shown that this was mainly due to technical and constructive shortcomings of the continuous chromatographic device used in the experiments. Therefore it can be expected that once the construction of the equipment has been improved, especially in terms of sample application and detection, such continuous chromatography will be used for said purpose on a preparative scale. The same group has shown that also continuous size exclusion chromatography of plasma proteins can be carried out [90]. In this case, however, the productivity of the continuous process was higher than the productivity of the optimal batch process.

Josic and Strancar [91] reported about chromatography on monolithic supports and its use for fast in-process analysis in the case of FIX isolation from human plasma. Branovic et al. [92] report the use of such monolithic columns for downstream processing of different FIX concentrates in anion-exchange mode. Separation of FIX and VN was first accomplished by using a disk-shaped monolithic column on a laboratory scale. As shown in Fig. 5, the same applications were carried out with identical results also on up-scaled, tube-shaped monoliths. A total of 8000 IU of FIX were applied to an 8-ml tube-shaped column, an amount that comes close to separation on

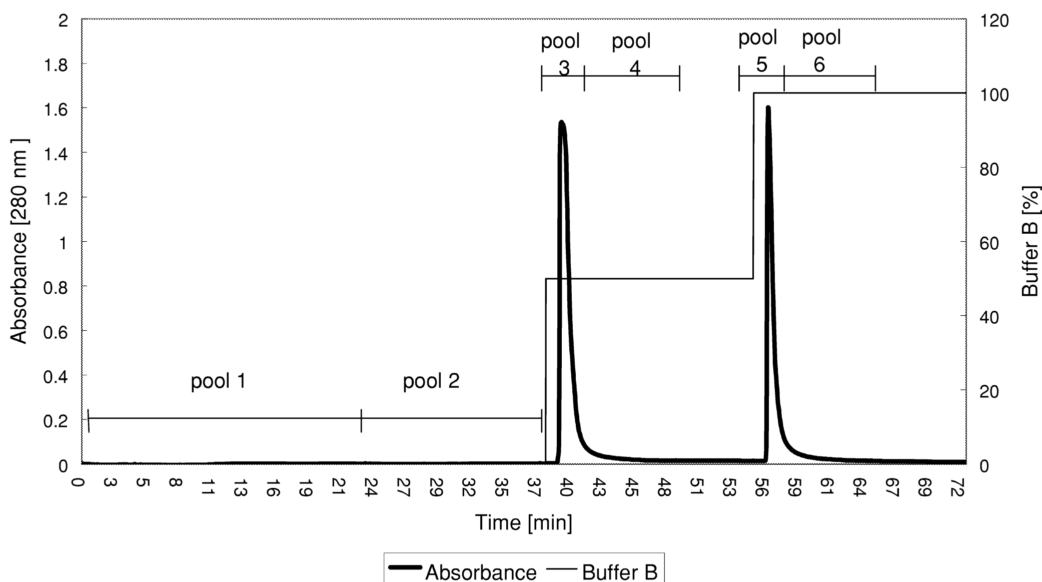

Fig. 5. Separation of FIX from VN on a DEAE monolithic tube. 8000 IU of a FIX preparation containing VN were applied to a DEAE monolithic tube. Column volume was 8 ml/min. Flow rate was 8 ml/min. Buffer A 20 m*M* Tris–HCl, pH 7.4. Buffer B 20 m*M* Tris–HCl, pH 7.4, containing 1 *M* sodium chloride. Pool 3, FIX; Pool 5, VN. Reprinted from Ref. [92] with permission.

a semi-preparative scale. In this way it was shown that the use of these media, which allow very fast separations of proteins, is not limited to in-process control, but can be extended to preparative chromatography of FIX, after appropriate up-scaling. The latest up-scaling experiments using a tube-shaped monolithic column with a bed volume of 500 ml have been successful (see the report in this volume and Ref. [93]). This time not only a sample with rather highly enriched FIX was applied, but also the eluate resulting from DEAE-Sephadex extraction. This product appears at a very early stage and shows rather low specific activity. About 50 000 IU of FIX were applied, corresponding to about 10% of the entire batch from the production. This means that it was possible to work on a large pilot-scale.

Whereas the isolation of clotting factor VIII and to some extent also the isolation of FVIIa is characterized by the introduction of tailor-made gels with immobilized combinatorial ligands [94–96], no similar efforts are known to be going on in the field of FIX isolation. However, such ligands, especially in combination with monolithic supports, can be expected in the future to contribute significantly to improvements in the purification process of FIX also.

4. Prothrombin, thrombin and clotting factor X

Prothrombin (FII) and FX can be isolated by further purification of ‘‘three-factor PCC’’. Bajaj et al. [97] have separated, by affinity chromatography on heparin agarose, single vitamin K-dependent factors, FII, FIX and FX, using this starting material. The yield of each factor was between 30 and 40%. However, the process was developed only on a pilot-scale.

During heparin affinity chromatography, the last step in the isolation of FIX, a rather pure FX fraction is obtained from the column during its rinsing with diluted NaCl solution. This ‘‘waste’’ or ‘‘side’’ fraction can be used for subsequent purification and the production of an FX concentrate [28,98]. However, FX deficiency is a very rare disease. In the USA, in the late 1980s, only 65 FX-deficient patients

were described. This means that the FX concentrate can be used in these cases as an orphan drug [98].

Husi and Walkinshaw [99] used "three-factor PCC" as starting material for the separation of vitamin K-dependent clotting factors. They compare the performance of different supports such as heparin Sepharose, Affigel Blue Sepharose and of hydrophobic supports such as phenyl Sepharose HP. The best results were obtained with phenyl Sepharose, where FII, FX and FIX can be separated from one another in a single step. The subsequent polishing step for single, isolated factors is carried out with anion-exchange chromatography.

The authors maintain that this strategy, which employs hydrophobic interaction chromatography for blood macromolecules, could be of immense value for purifying the human vitamin K-dependent proteins and represents a considerable simplification over other purification schemes. Besides, they say that this process can be readily up-scaled and is a cost-efficient alternative. However, until now the method has been used only on a laboratory scale or at best on a pilot scale.

As one of the main components of two-component collagen-based or fibrin-based glue, the activated factor II (FIIa) or thrombin has a rather wide clinical application [100–102]. If the isolated thrombin comes into contact with a plasma fraction, which chiefly consists of fibrinogen, fibrinogen is cleft into fibrin. When the resulting mixture is applied to the wound, it causes faster and more complete hemostasis [100]. Human thrombin can be enriched directly from plasma by calcium ions-mediated pseudo affinity chromatography. The PPSB factors are first adsorbed to a 2-hydroxyaminopropyl support. Prothrombin (FII) is activated by applying calcium to the gel. Subsequently, the resulting thrombin (FIIa) is eluted with 25 m*M* $CaCl_2$. Polishing is carried out at the end of the process by cation-exchange chromatography [53]. Kraus and Möller have simplified the process further by replacing the 2-hydroxyaminopropyl with a weak anion-exchange resin (Fractogel DEAE). In a way similar as described above, prothrombin is activated with Ca^{2+}-ions, and the resulting thrombin is eluted with $CaCl_2$. The polishing step consists of cation-exchange chromatography with a Fractogel Sulphate support [103]. This allows an S/D virus inactivation step to be included in the isolation schema. The detergents do not bind and are removed from the product by the last cation-exchange step [103].

5. Clotting factor VII and factor VIIa

Clotting factor VII is usually isolated either from the side-fraction resulting from three-factor PCC or in connection with the production of FIX [104]. In order to isolate FVII for its protein biochemical characterization [17] and cDNA sequencing [105], anion-exchange chromatography is used repeatedly, starting with the side-fraction of PCC (after adsorption with a weak anion-exchange resin, see below). A buffer containing $CaCl_2$ is used for elution. This step is followed by immunoaffinity chromatography on a Sepharose 4B column with immobilized monoclonal antibodies against human FVII. The antibody binds FVII only in the presence of Ca^{2+}, which means that FVII can be eluted from the column by buffers that contain EDTA. Final purification is carried out with two steps of anion-exchange chromatography, in which FVIIa is eluted with buffers containing $CaCl_2$ and NaCl, respectively. When the procedure described above is used, FVII is activated into FVIIa during purification [17].

Shapiro et al. [104] describe the production of a pd FVII concentrate, which is virus inactivated by vapor-heating and which has been used clinically for treatment of FVII-deficient patients. In the process, the plasma fraction constituting the prothrombin complex is combined with DEAE Sephadex beads and then subjected to centrifugation. The supernatant is processed further by $Al(OH)_3$ adsorption and elution.

Chabbat et al. [106] further purify the eluate that results from this process, and which is FVII-enriched, with an additional step on anion-exchanger Q-Sepharose. However, this leads to the activation of FVII. The resulting FVIIa concentrate contains only small quantities of other vitamin K-dependent clotting factors, but is enriched in protein C and S.

Factor VII deficiency is a rather rare disease. However, it has been known for quite some time that FVIIa can be used for treating and stopping bleedings of patients with hemophilia A and B [107,108]. The option of using FVIIa for this purpose is

particularly important in the case of patients who have developed antibodies against FVIII or FIX, so-called inhibitors [107–111]. With such patients the application of clotting factors has little or no effect, since the antibodies in their bloodstream soon inactivate the active protein [112]. This use of FVIIa has opened the way for the industrial production of recombinant FVIIa, since corresponding experiments in the laboratory were completed in the 1980s [113].

Recombinant FVIIa is produced in transfected baby hamster kidney (BHK) cells [17]. After filtration, the cell supernatant is diluted and pH is adjusted to 8.6. The solution is pumped on to a Mono Q anion-exchange column. Activated rFVII is then eluted with a Tris–HCl-buffered $CaCl_2$ solution. The immunoaffinity chromatographic step using immobilized Ca^{2+}-dependent antibodies and the remaining part of the purification procedure are identical with the ones described above for the purification of FVIIa on a laboratory scale (see above). Here too, the activation of rFVII to rFVIIa occurs during the purification process [17].

Whereas rFVIIa appears to be for the time being the drug of choice for patients with antibodies (inhibitors) against FVIII and FIX, the FVII-deficient population seems to be a risk group. Recently, it has been found that in this group anti-FVII antibodies may form. In this case it is recommended to use other substances such as PPC or, eventually, pdFVII concentrates [114].

6. Protein C and activated Protein C

All vitamin K-dependent proteins described so far have an activity towards clotting. In contrast, PC is a clotting inhibitor. Human PC circulates in plasma as a zymogen. It is converted to activated PC (aPC) via specific cleavage by thrombin bound to thrombomodulin on the membranes of endothelial cells [115,116].

As mentioned above, PC is present in PPSB preparations, but also in some enriched FVII and FIX concentrates [6,55,106,117]. Plasma-derived PC (pdPC) is usually obtained from PPSB concentrate, after barium sulfate adsorption or Q-Sephadex extraction. The eluate is subsequently precipitated with 35 to 37% ammonium sulfate and then purified with immobilized monoclonal antibodies against PC. It is possible to convert PC to aPC with immobilized thrombin [118]. In other procedures the ammonium sulfate step is omitted, and the eluate from the anion-exchange gel is applied directly to the column with immobilized anti-PC antibodies [119].

Wu and Bruley [66] have shown that PC can be separated from prothrombin through immobilized metal affinity chromatography (IMAC) with complexed copper. Similar as in the case of FIX purification (see above and Ref. [55]), the IMAC column with immobilized copper should also be used for purification of pdPC from human plasma, above all that contained in the Q-Sephadex eluate. Purification from the Cohn Fraction IVa, as proposed by the authors, appears to be an unlikely option [120].

Recombinant PC can be synthesized in different transfected mammalian cells [120] or in the milk of transgenic animals [121,122]. Its production became commercially promising, when it was found that aPC can be used for treatment of patients with severe sepsis [9,123]. Recombinant aPC is purified with a combination of anion-exchange chromatography and immunoaffinity chromatography [120]. Subsequent activation is carried out with immobilized thrombin or some other protease [121].

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