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Review

# Chromatography in plasma fractionation: benefits and future trends

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**Abstract**

Industrial-scale chromatographic fractionation and purification methods have been used increasingly in the last few years for plasma fractionation. This has resulted in the development of a new generation of therapeutic plasma derivatives, especially coagulation factors, protease inhibitors and anticoagulants. Implementation and combination of ion-exchange, affinity and size-exclusion chromatography have allowed the development of new therapeutic products with improved purity and safety for treating congenital or acquired plasma protein deficiencies in patients. More recently, the benefit of chromatographic purification of plasma proteins in the removal of plasma-borne viruses has been revealed. Development of packing materials with improved characteristics for industrial applications, including higher capacity and rigidity, should further promote the use of chromatography as an essential plasma fractionation tool and confine more and more the traditional ethanol precipitation methods to the final processing stages used to recover albumin.

## Contents



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## List of abbreviations

| | |
|---|---|
| AAT | Alpha 1-antitrypsin |
| ATIII | Antithrombin III |
| C1-Inh | C1-inhibitor |
| F | Factor |
| HIC | Hydrophobic interaction chromatography |
| HIV | Human immunodeficiency virus |
| IgG | Immunoglobulin G |
| PPSB | Prothrombin complex concentrate |
| PSR | Porcine pseudorabies virus |
| SD | Solvent–detergent |
| SV 40 | Simian virus 40 |
| SEC | Size-exclusion chromatography |
| VSV | Vesicular stomatitis virus |
| vWF | Von Willebrand factor |

## 1. Introduction

Chromatographic methods represent an essential tool for the extraction of biological compounds at the laboratory scale. These methods can offer high specificity and selectivity, permitting the recovery of biological compounds with adequate purity and under conditions preserving their biological features.

Chromatography has also become an established means for the industrial-scale extraction of therapeutic biological products. Many therapeutic products, including recombinant proteins and glycoproteins, are obtained using procedures that include one or several chromatographic purification steps. Chromatography has also been used for the extraction of plasma derivatives since the early seventies and has gained wider acceptance since the early eighties. It has been shown to be of significant benefit for extracting new therapeutic plasma concentrates, for improving their purity and safety, especially for coagulation factors and protease inhibitor products, and for improving their viral safety.

In this paper, the major benefits afforded by chromatographic methods in plasma fractionation are reviewed. Only applications which have led to the manufacture of currently licensed therapeutic concentrates are mentioned. Potential future trends in this field are discussed.

## 2. Background

Human plasma must be regarded as a raw biological material of extreme complexity [1]. It consists of numerous proteins (more than 100) which are present at very different concentrations and which exhibit quite different physiological functions. Of the total protein content of ca. 60 g/l, the major proteins are albumin, present at ca. 45 g/l, immunoglobulin G (IgG) at 8–11 g/l, and fibrinogen at 2–3 g/l. Plasma also contains several protease inhibitors including alpha 1-antitrypsin (AAT; 1.5 g/l), antithrombin III (ATIII; 0.3 g/l), or C1-inhibitor (C1-inh; 0.2 g/l). Coagulation factors, such as Factor IX (FIX), FVII, FX, and von Willebrand factor (vWF), are found at 5–10 mg/l, while FVIII is present at even lower concentrations (<1 mg/l). By definition, one coagulant unit of these coagulation factors is the activity found in one ml of pooled plasma.

Although about 17 million liters of plasma are collected in the world per year for fractionation,

its supply remains limited considering the important therapeutic needs of plasma-derived therapeutic products today, especially albumin (about 300 tons), FVIII (1 kg), and IgG (10 tons). As a consequence, optimal fractionation methods are important to increase the diversity in therapeutic proteins extracted from each plasma pool, and to improve the yields of the leading products.

Plasma fractionation first started in the mid-forties. The original fractionation method developed then [2], based on the use of ethanol as the partitioning agent of the major plasma protein fractions, remains the backbone of the fractionation processes in use today in most fractionation plants. This method leads to the production of albumin and IgG preparations according to the following parameters: ethanol concentration, pH, temperature, ionic strength, and protein content. Precipitates generated during this process are separated by centrifugation or filtration. Prior to the actual ethanol fractionation process, plasma is thawed at 2–4°C to separate the cryoprecipitate fraction which contains FVIII, vWF, fibrinogen, and fibronectin. Advantages of ethanol fractionation include simplicity, low toxicity, bacteriostatic effects, and inactivation/elimination of human immunodeficiency virus (HIV). Significant disadvantages include low specificity in the purification of trace plasma proteins and potential denaturing effects of ethanol or low pH conditions on labile proteins (coagulation factors, protease inhibitors and anticoagulants).

## 3. Chromatographic methods in plasma fractionation: rationale

The rationale for using chromatography to prepare therapeutic plasma derivatives is important. New plasma products are necessary to allow selective hemotherapy, making it possible to treat patients with standardized, biologically active purified products instead of crude plasma fractions. Plasma products with higher purity and safety, to limit side-effects in patients, had to be developed through the implementation of selective purification methods. The cost-effectiveness of plasma fractionation could be improved by more effective chromatographic purification methods designed to improve yields and extract new products. Finally, in the developing world, implementation of chromatographic fractionation schemes is often regarded as being easier, more flexible, and less costly than ethanol-based technologies.

## 4. Chromatography in plasma fractionation: strategy and constraints

The manufacturing process of plasma derivatives, as for any pharmaceutical, must ensure consistency in product specifications, stability, and safety. As human plasma is a highly complex material made of components exhibiting various physiological functions (coagulation factors, protease inhibitors, proteases, complement components, growth factors, etc.), establishing a purification method requires appropriate validations. In addition, due to the scarcity of plasma, several therapeutic products must be extracted simultaneously from the same plasma pool. This requires compatibility between the purification processes for several proteins, under conditions not impairing the quality and recovery of the leading products (presently, albumin and FVIII). Thus, in already-established plants using ethanol fractionation to extract albumin and IgG, chromatography must be implemented in a way which is compatible with the pre-existing bulk fractionation process [3]. In addition, new designed processes must, within pharmaceutical compliance, ensure appropriate flexibility to adjust to potentially changing therapeutic needs [4].

A further difficulty in implementing chromatography in established fractionation plants is the daily processing volume of several thousand liters of plasma; such large volumes require appropriate equipment and chromatographic materials exhibiting high capacity and good flow-rate properties.

## 5. Chromatographic techniques in plasma fractionation

Because of limitations due to the instability of plasma proteins under extreme conditions of pH, ionic strength, or pressure, and their sensibility to most organic solvents, a limited range of chromatographic principles have been applied in the production of plasma protein concentrates. Table 1 indicates the major chromatographic principles used in the production of several plasma derivatives.

### 5.1. Ion-exchange chromatography

Ion-exchange chromatography has many applications in plasma fractionation and is well adapted to the extraction of proteins from complex mixtures. Because of their diversity (soft, semi-rigid or rigid), the relative flexibility of use, their relatively low cost, ion exchangers have been introduced both upstream of a purification scheme, to extract the protein(s) of interest from a relatively crude plasma fraction, or as a polishing step to eliminate unwanted proteins.

As many plasma proteins carry a negative charge at the almost neutral pH required to protect biological activity, anion-exchangers are often used. They are helpful to bind the targeted protein(s) and elute them subsequently under a more purified and concentrated form by modifying the pH or ionic strength of the chromatographic buffer. More common anion exchangers used to extract various therapeutic coagulation factors (FVIII, FVII, FIX, vWF), anticoagulants (protein C), or protease inhibitor (AAT, C1-Inh) concentrates include DEAE-Sephadex A-50, DEAE-Toyopearl 650 M [5,6], DEAE-Fractogel EMD, DEAE-Sepharose [7–13], and Q-Sepharose. DEAE-trisacryl has been used for the purification of IgG [14].

Cation exchangers have been less employed until now although applications using sulfate-Sepharose and sulfate-Fractogel EMD 650 M have been described for FXI and C1-inh [15,16] and CM-Sepharose for albumin [17]. Negatively charged sulfated polymers (heparin, dextran sulfate) are used in the purification of FIX therapeutic concentrates [9–12].

### 5.2. Size-exclusion chromatography (SEC)

SEC can be helpful in plasma fractionation. However, the resolution of SEC may not be convenient for upstream use when protein mixtures are complex; in addition, SEC is difficult to handle when dealing with large volumes. Consequently, SEC is used rather as a terminal polishing step to eliminate protein contaminants from a concentrated and pre-purified protein mixture, as applied for AAT [8] or albumin [17].

### 5.3. Affinity chromatography

Affinity chromatography is used in plasma fractionation either upstream, to capture a protein from a complex plasma fraction, or downstream, as a polishing step. The ligand most currently used is heparin, a negatively charged polymer, which is used especially in the purifica-

Table 1
Chromatographic methods in plasma fractionation

| | Ion-exchange | Affinity | Size-exclusion | Immuno-affinity | Hydrophobic interaction |
|---|---|---|---|---|---|
| Factor VIII | ++[5] | + | | +[22,23,28] | |
| Factor IX | ++[9–12] | +[9–12] | | +[24,25] | |
| Antithrombin III | | ++[18–20] | | | |
| Von Willebrand Factor | +[6] | +[6] | | | |
| Alpha 1-Antitrypsin | +[7,8] | | +[8] | | |
| Albumin | +[17] | | +[16] | | |
| IgG | +[14] | | | | |
| C1-inhibitor | +[16] | | | | +[27] |

tion of ATIII (heparin co-factor) [18–20]. Other ligands used include gelatin to remove fibronectin from a purified vWF concentrate [6], and lysine to purify or eliminate plasminogen [21]. Metal chelate affinity chromatography with copper ions immobilized on Sepharose Fast Flow [13] is used to purify a therapeutic IX concentrate presently under clinical evaluation. Immobilized murine monoclonal antibodies are used for the purification of FVIII [22,23], FIX [24,25], and protein C concentrates.

### 5.4. *Hydrophobic interaction chromatography (HIC)*

Plasma protein concentrate production rarely relies on HIC. However, phenyl-Sepharose and hexyl-Sepharose have been described for the purification of C1-Inh [26]. More recently, reversed-phase packings have been introduced [27] or described [28] for the binding of virus sterilizing agents after SD treatment of plasma, allowing the recovery of most, if not all, plasma proteins in the column filtrate.

## 6. Chromatography: a fractionation tool of plasma

A convenient approach in using chromatography has been to combine it with traditional precipitation methods of plasma (a) by making an appropriate use of discarded side-fractions containing concentrated, semi-purified plasma proteins or (b) by performing batch extraction of plasma under conditions not interfering significantly with the bulk fractionation process [3,4,29]. Fig. 1 shows an example of a fractionation scheme for plasma integrating chromatography and ethanol precipitation steps. Significant fractionation procedures are described below.

### 6.1. *Chromatographic fractionation of cryopreciptate*

Cryoprecipitate is the fraction generated when thawing plasma at ca. 2°C. It contains FVIII, vWF and fibrinogen, all of which have established therapeutic indications in coagulation disorders. Until the early eighties, and still today in some countries including the USA, crude cryoprecipitate has been used in therapeutics, thus exposing the patients (e.g. hemophilia A patients) to unnecessary, potentially harmful, overloads of allogenic plasma proteins. By developing a large-scale chromatographic fractionation scheme of cryoprecipitate, it has been possible to separate these major proteins. As shown in Fig. 2, cryoprecipitate is solubilized, subjected to aluminium hydroxide adsorption and to a precipitation step to eliminate residual vitamin K-dependent coagulation factors, and fractionated on a Toyopearl DEAE 650 M column. Fibrinogen and other proteins (residual IgG, IgM, albumin, etc.) are recovered in the filtrate (Fig. 3; peak a), while part of the fibronectin, as well as vWF and FVIII, are bound on the gel. By successively increasing the ionic strength of the buffer used to equilibrate the gel, fibronectin and vWF (peak b) and then FVIII (peak c) elute [5]. vWF and fibronectin can be concentrated and further purified by a second chromatographic step on a Toyopearl DEAE 650 M column and separated on immobilized gelatin [6]. Thus, such a simple process allows the simultaneous production of four different highly-purified therapeutic concentrates from the same batch of cryoprecipitate.

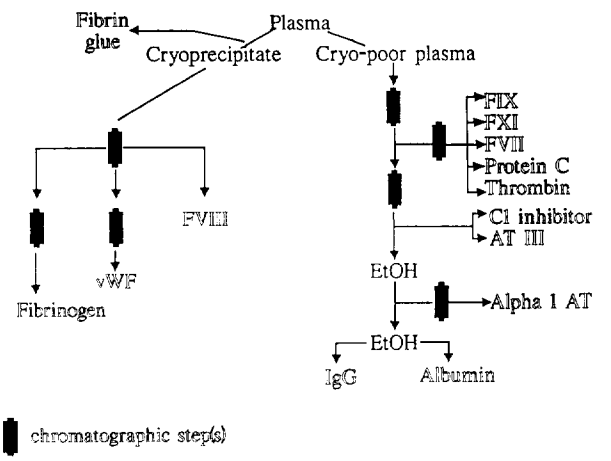


Fig. 1. A modern fractionation scheme of human plasma.

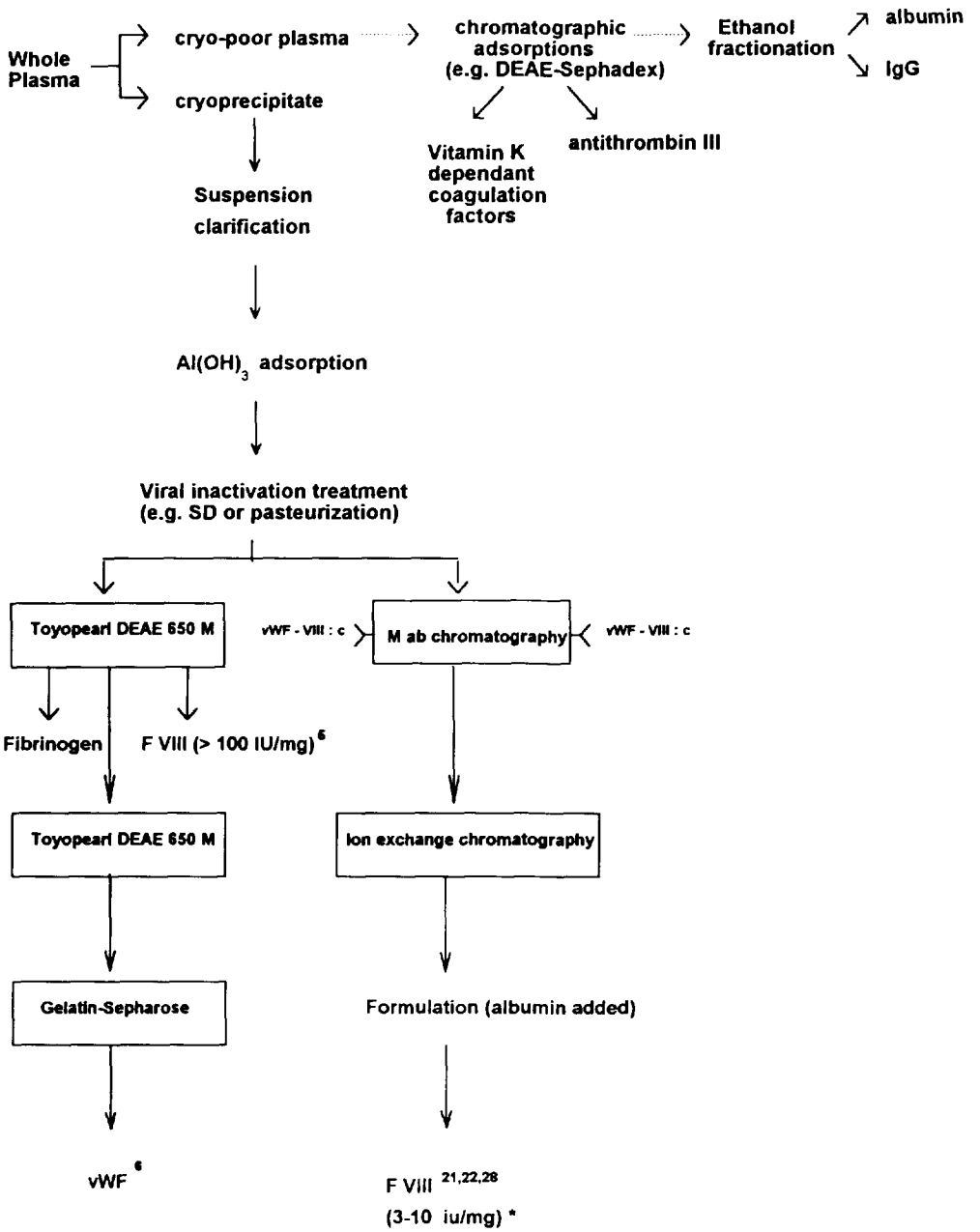


Fig. 2. Integrated chromatographic fractionation process of cryoprecipitate allowing simultaneous production of FVIII, vWF, fibrinogen, and fibronectin therapeutic concentrates (from Refs. [5,6]), and immunopurification methods of FVIII from cryoprecipitate using either anti-FVIII and anti-vWF murine antibodies, followed by ion-exchange chromatography (from Ref. [30]).

### 6.2. Chromatographic fractionation of vitamin K-dependent plasma proteins

Vitamin K-dependent proteins (Factors II, VII, IX, X, and proteins C and S, etc.) share common biochemical properties, including the presence of gamma-carboxyglutamic residues involved in calcium binding, which are exploited for their extraction from plasma. The crude extract containing these factors is known as the prothrombin complex concentrate (PPSB or PCC). PPSB has been obtained by batch extraction of cryo-poor plasma or COHN supernatants I or II using DEAE- groups attached to stable support matrices such as Sephadex or cellulose [29]; such batch processes have no significant impact on the purification of unbound plasma proteins (albumin, IgG, AAT, or ATIII, etc.,) [3]. The ratio of ion exchanger to plasma fraction, the pH and the ionic strength must be determined carefully to ensure reproducible adsorption of weaker binding proteins (FVII, protein C and protein S). PPSB has been used to treat complex bleeding disorders for many years but, due to its complex composition, carries thromboembolic risks when used at high doses in hemophilia B patients (these patients lack only FIX). Fractionation of PPSB into its components has been made possible by the use of chromatographic techniques using media with good large-scale performance. An example of such a process [10] is in Fig. 4 where PPSB is first fractionated on a DEAE-Sepharose FF column to successively separate FVII, protein C, and a fraction containing factors II, IX and X. These three coagulation factors are then separated on a heparin-Sepharose CL-6B column.

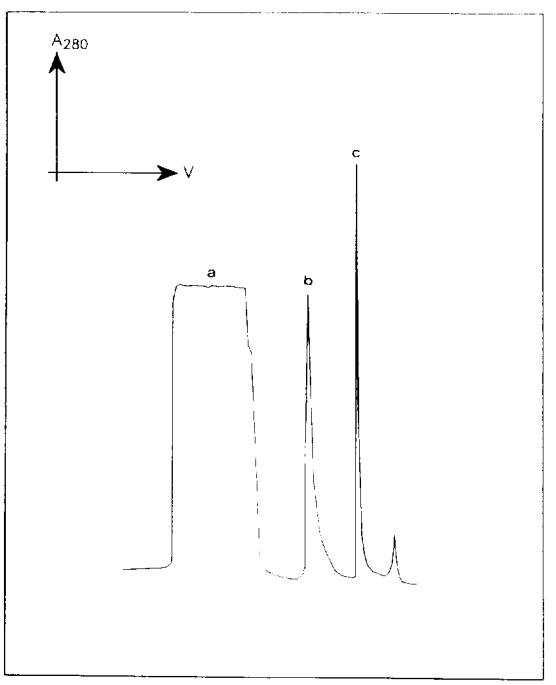


Fig. 3. Elution profile of a cryoprecipitate extract chromatographed on DEAE-Toyopearl 650M. a = effluent containing fibrinogen, IgG and the viral sterilizing agents; b = 0.15 *M* NaCl wash containing vWF and fibronectin; c = 0.25 *M* NaCl wash containing FVIII; d = 2 *M* NaCl wash (from Ref. [5]). The sensitivity of the detection unit ($A_{280nm}$) has been increased 10 times to detect peaks b and c.

## 7. Chromatography: a purification tool

Until the mid to late eighties, hemophilia A and B patients have been treated by plasma fractions in which the active proteins, FVIII and FIX, respectively, were purified only 50- to 100-fold, respectively, from the starting plasma. These fractions were obtained either by precipitation methods and/or by relatively unselective adsorption phases. Since then, the implementation of chromatographic purification procedures has allowed the development of a new generation of protein products with improved purity. Conventional chromatographic adsorption techniques, mainly based on anion exchange, and immunoaffinity purification procedures using murine monoclonal antibodies, have been used to obtain therapeutic concentrates of higher purity.

### 7.1. Factor VIII

Chromatographic purification of FVIII has long been considered as being difficult due to potential risks of activation and instability in the presence of plasma proteases coeluting with FVIII. Amino-hexyl agarose gels have failed to allow purification and stability of FVIII from

cryoprecipitate extracts. The choice of chromatographic resins ensuring sufficient specific binding and purification of FVIII, or its complex with vWF, has thus required significant development.

In the purification process described before (Figs. 2 and 3), DEAE-Toyopearl TSK 650M is used to produce a highly purified FVIII concentrate, essentially free of fibrinogen, IgG and fibronectin [5]. This concentrate exhibits an average final specific activity of about 200 IU/mg [5], which corresponds to a purification factor of more than 10 000 from plasma. The average purification factor of FVIII achieved during the DEAE-Toyopearl chromatographic step itself, as compared to the cryoprecipitate extract prior to chromatography, exceeds 200-fold and suggests interactions other than ionic between the FVIII–vWF complex and this specific packing material. The FVIII concentrate does not require the addition of extraneous protein stabilizers (such as albumin), probably due to partial chromatographic co-elution of vWF (the carrier and stabilizer of FVIII in plasma) which represents a major protein in this preparation.

Extensive purification of FVIII from cryoprecipitate can also be achieved by immunoaffinity methods using murine anti-human FVIII or human vWF monoclonal antibodies (mAb) (Fig. 2). Subsequent chromatography on either aminohexyl agarose or on a QAE column is performed to remove murine antibodies leaching from the immunoadsorption column [22,23,30]. To limit loss of FVIII during production steps, albumin is added as a stabilizer, thus decreasing the specific activity from ca. 2000–4000 FVIII:c IU/mg to 3–10. The products are essentially devoid of fibrinogen or immunoglobulins G.

### 7.2. Factor IX

PPSB, a complex mixture of coagulation factors, has long been used to treat FIX deficiency. However, improved purification of FIX has been made necessary to avoid thrombogenic side-effects associated with the clinical use of PPSB in hemophilia B patients.

By combining chromatography of cryo-poor plasma on DEAE-Sephadex A-50, followed by DEAE-Sepharose fast flow and heparin-Sepharose (see Fig. 3), a FIX concentrate with a specific activity higher than 100 IU/mg could be obtained with a recovery of 300–350 IU/l of plasma [10,31]. An identical purification strategy has been followed by others [12]. FIX can also be extracted from cryo-poor plasma by combining DEAE-Sepharose, followed by heparin-Sepharose and a cation exchanger [11].

A FIX concentrate, presently under clinical investigation in the United Kingdom, has recently been obtained by DEAE-Sepharose purification of the prothrombin complex, followed by metal chelate affinity chromatography using copper ions (Fig. 4). Prothrombin does not bind to the Cu-Sepharose column used; other contaminant proteins from PPSB are removed by buffer washes at low salt and low pH. FIX is sub-

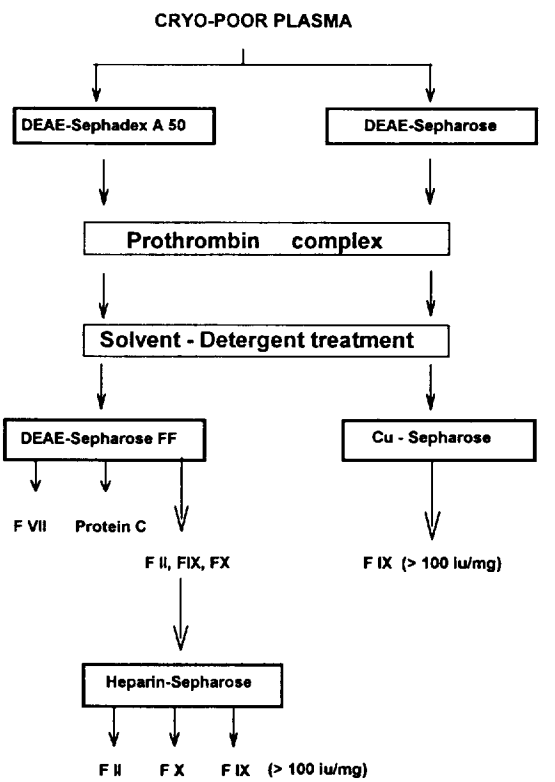


Fig. 4. Chromatographic fractionation process of vitamin K-dependent proteins, including FIX, from cryo-poor plasma using ion-exchange chromatography combined with immobilized heparin adsorption (from Refs. [10,31]) or with metal chelate (copper ions) affinity chromatography (from Ref. [13]).

sequently eluted from the gel by a buffer containing glycine [13].

A FIX concentrate purified using murine anti-human FIX monoclonal antibodies has also been developed for clinical use [24]. Its purification scheme involves adsorption of the cryo-poor plasma on DEAE-Sephadex, followed by immunoaffinity of FIX and aminohexyl-Sepharose to decrease the contamination by murine antibodies.

Chromatographic high-purity FIX preparations are essentially free of other coagulation factors. Their use is associated with a lower risk of thromboembolic complications [32,33], as compared to PPSB [34].

### *7.3. Other proteins*

As reviewed recently [3,4,29], many other plasma concentrates, mostly coagulation factors and protease inhibitors, are obtained by procedures in which chromatography is essential to ensure sufficient purity and safety and to provide an improved and more selective treatment for patients. These products include vWF [6], ATIII [19,35], AAT [7,8], FXI [15,29,36], thrombin [29,37] and C1-inh [16,38]. Fig. 5 shows the elution profile obtained during S-Sepharose chromatography of a cryoprecipitate-poor plasma fraction allowing to increase the specific activity of FXI (peak b) from 12 to 150 U/mg [15]. Similarly, Fig. 6 presents the elution profile achieved during purification of C1- inhibitor (peak a) on $SO_3$- Fractogel EMD [15]. Extensive purification or polishing of albumin by anion-exchange chromatography to remove protein contaminants, polymers, vasoactive substances or endotoxins, has also been performed on an industrial scale [17, 39–43].

## 8. Chromatography: a means to improve viral safety

### *8.1. Elimination of virus inactivating agents*

One major industrial technique used to inactivate plasma-borne viruses consists of incubating plasma fractions with a combination of an or-

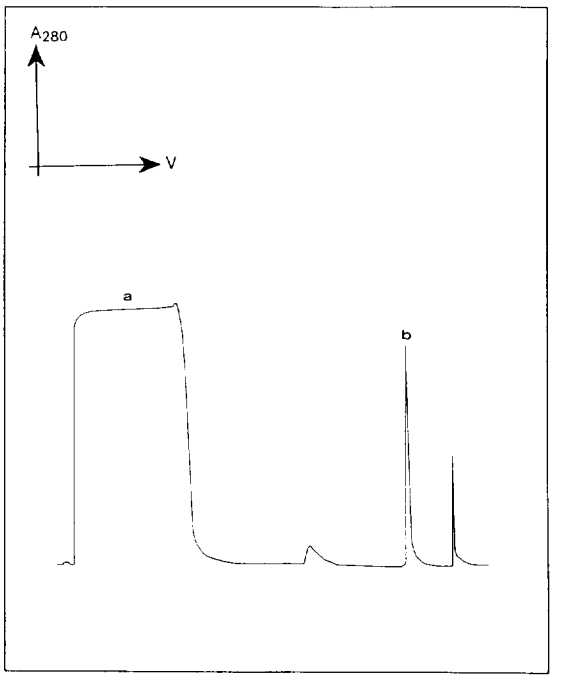


Fig. 5. S-Sepharose chromatography of purified cryo-precipitate-poor plasma allowing to purify FXI (peak b); peak a = breakthrough fraction; V = volume (from Ref. [15]).

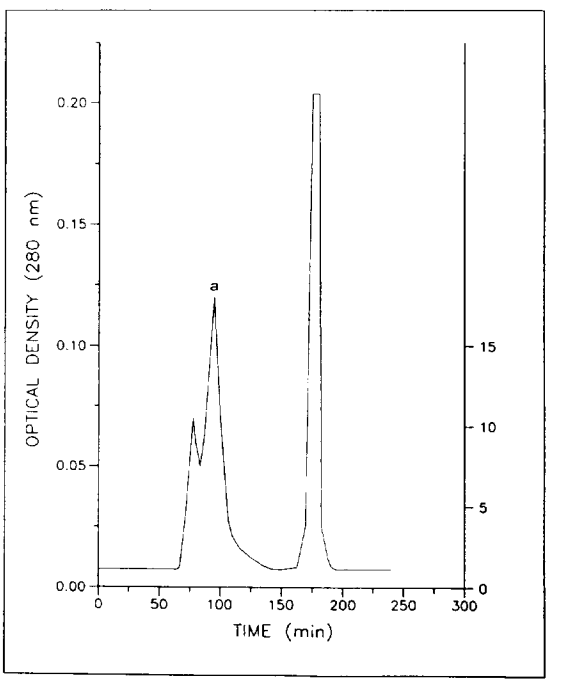


Fig. 6. Chromatographic purification profile ($A_{280nm}$) of C1-inhibitor (peak a) on $SO_3$-Fractogel EMD (from Ref. [16]).

ganic solvent [tri(*n*-butyl) phosphate, TnBP] and a detergent (generally Tween-80 or Triton X-100) [44]. Following this step, the chemicals must be eliminated. One common approach is to use a selected chromatographic resin to bind the protein(s) of interest while the solvent–detergent (SD) mixture is eliminated in the breakthrough fraction following extensive washing. Depending upon the product to be made, another strategy consists of binding the chemicals on a reversed-phase chromatographic support [27,28], the protein(s) of interest being recovered in the breakthrough fraction.

### *8.2. Elimination of viruses*

Ensuring the optimal viral safety of plasma derivatives is critical, considering that plasma can be contaminated by several infectious agents [45]. The most pathogenic plasma-borne viruses are the enveloped human immunodeficiency virus (HIV-1 or -2), and hepatitis B and C viruses, and the delta agent. Other viruses include the non-enveloped parvovirus B19 and, to a lesser extent, hepatitis A, AIDS and hepatitis B and C have been transmitted to hemophilia A and B patients receiving low-purity, nonvirally inactivated FVIII and PPSB products, respectively. However, the viral safety of coagulation factor concentrates has dramatically improved thanks to the implementation of specific viral inactivation methods such as SD and various heat treatments [46,47] during the manufacture of plasma derivatives.

The various safety aspects in the manufacturing of plasma derivatives have been reviewed [45] and the purification steps have been assigned a major safety role. The absence of transmission of infectious agents by albumin and most immunoglobulin concentrates is explained partly by their manufacturing process which included non-specific viral inactivating or eliminating steps. Viral safety of current coagulation factor concentrates is also associated with the improved performance of their purification method. The purification factor of, for example, new chromatographic FVIII, FIX, and vWF concentrates is 100- to 1000-fold higher than that of the former products. New chromatographic concentrates (e.g. protein C or FXI) are also purified about 10 000 to 15 000-fold from plasma. This increased purity has led to lower potential risks of contamination with plasma-borne viruses, as demonstrated by carefully controlled viral validation studies [48].

The potential of chromatography for eliminating viruses has been reviewed recently [49,50]. Ion-exchange, immobilized-heparin and immunoaffinity chromatographic steps have been shown to potentially remove viruses [11,25,31,43,51]. Table 2 presents the logarithmic reduction factors achieved during chromatographic purification of three different FIX concentrates [11,25,31]. Up to more than 3–5 $\log_{10}$ removal of various viruses used as models can be achieved during some chromatographic steps. Similar reduction factors have been reported during DEAE-Toyopearl 650 M chromatography of vWF, DEAE-Sephadex A-50 and DEAE-Sepharose Fast Flow purification of FVII,

Table 2
Reduction factors ($\log_{10}$) during fix purification steps

| | DEAE Sephadex | DEAE Sepharose | DEAE Spherodex | mAb Sepharose | Heparin Sepharose | Cation-exchange |
|---|---|---|---|---|---|---|
| HIV-1 | 2.24 [31] | 3.10 [31] | | | 2 [31] | |
| | | ≥2.1 [24] | 1.5 [25] | >4.5 [25] | | |
| | | 2.1 [11] | | | 0.4 [11] | 1.7 [11] |
| VSV | 2.14 [31] | 1.89 [31] | | | 1.92 [31] | |
| | | 3.0 [11] | | | 0.4 [11] | 1.5 [11] |
| PSR | 3.37 [31] | 2.84 [31] | | | 5.51 [31] | |
| SINDBIS | | 3.1 [11] | | | 0.7 [11] | 2.0 [11] |
| SV 40 | 1.68 [31] | 2.21 [31] | | | 2.45 [31] | |
| Avianreovirus | | 0 [24] | 1.9 [25] | 3.4 [25] | | |

DEAE and CM-trisacryl purification of IgG [49], immunopurification of FVIII on immobilized anti-vWF antibodies [50], immobilized heparin purification of ATIII [49], and anion-exchange and cation-exchange chromatography of albumin [43,51].

There are less data on viral reduction factors during size-exclusion chromatography. However, we have validated the elimination of HIV-1, yellow fever virus, Theiler virus, and porcine pseudorabies during the chromatography of AAT on Sephacryl S-200 HR designed to eliminate HMW protein contaminants. The reduction factors achieved were from 1.47 $\log_{10}$ (Theiler virus) to more than 4.5 $\log_{10}$ (HIV-1). Data revealed that the Yellow fever virus and the Theiler virus eluted with the discarded HMW fraction.

Although chromatography can reproducibly reduce virus load, the chromatographic behaviour of viruses is not yet fully understood and requires further study. One can speculate that a number of parameters may influence the ionic or hydrophobic interactions between viruses and chromatographic materials, including the size and shape of the virion, the biochemical nature and structure of the virus membrane or envelope, or the propensity of some viruses to make aggregates under certain physico-chemical conditions [49]. In addition, the overall design of each chromatographic purification step, especially the type and number of washing steps (if any) which may remove viruses from the gel prior to the elution of the targeted protein(s), may have a significant impact on the viral reduction factor achieved.

Chromatography should probably only be considered as a useful contributor to viral safety, since, due to its limitations, it cannot replace specific viral inactivation treatments which are easier to standardize and control. This might be especially true for viruses which can be present at a very high titer in the starting plasma, e.g. 10–12 $\log_{10}$ for parvovirus B19. When chromatographic gels are reused (which is commonplace at the industrial scale), it should be validated that, in case of strong interactions of viruses with the chromatographic material, washing and regeneration conditions of the column permit their elution or inactivation and avoid potential leakage during subsequent run(s).

## 9. Future trends

Chromatography will probably play an ever-increasing role in plasma fractionation in the future. However, this should be largely dependent upon the answers to a number of issues which include potential changes in the leading plasma-derived proteins, requiring an adjustment of fractionation methods to cover evolving therapeutic needs, as well as the impact of recombinant proteins (FVIII, albumin?, etc.) on the clinical demand for their plasmatic analogs. From such answers, especially regarding albumin, will depend whether ethanol fractionation will continue to be regarded as the method of reference to perform final processing stages of bulk plasma proteins [52] or, by contrast, will be replaced by processes exclusively using chromatography.

The development of new chromatographic supports will further contribute to their use in plasma fractionation. Improved characteristics for large-scale use are important factors; these include:

- enhanced binding capacity, allowing to miniaturize the separation step and improve the productivity,
- improved selectivity and specificity to further use chromatography to yield products with high purity and devoid of unwanted biological contaminants,
- stability to sterilizing solutions to allow more thorough microbiological destruction,
- improved bead rigidity and porosity to allow high linear flow-rates with low back pressure and to limit delays in purification.

Implementation of new chromatographic media requires proper validations to demonstrate that the quality and safety of the final purified protein products fulfill established requirements (e.g. viral safety, absence of leakage products, stability, etc.) and that the overall plasma fractionation scheme is not affected.

## 10. Conclusion

Chromatography has already brought about a significant impact in the large-scale fractionation process of human plasma and the purification scheme of several essential therapeutic plasma products. The major benefits of chromatography have been seen in the design of new purification methods yielding plasma products with significantly improved purity and safety.

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