Strategy for Purification of Coagulation Factor Concentrates

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ABSTRACT

Advances in biotechnology are permitting significant changes in traditional plasma fractionation schemes. Increases in product purity, safety, and recovery are possible, and new products are being developed. Applications of technology to the purification of factor IX, factor X, protein C, antithrombin III, thrombin, and fibrin glue are described.

Introduction

Although the biotechnology revolution is more commonly associated with the products of recombinant deoxyribonucleic (DNA) technology, it is also having a great impact in the area of plasma fractionation. New products are being developed and current products are being improved in numbers and at rates unprecedented in the fractionation industry. Major advances that have been made in separation process technology, especially liquid chromatography, are making it possible to increase product purity, safety, and recovery while in many cases maintaining or even reducing product cost. New products which were previously not feasible because of costly, complex purification requirements are now being developed. Advances in viral inactivation techniques have also made a great contribution to improvements in the safety of plasma products.

Many of the new or improved products of plasma fractionation are proteins of the coagulation system, both procoagulants such as Factors VIII and IX, and anticoagulants such as antithrombin III and protein C. This has been a traditional area of specialization in the industry and continues to be a major focus of research and development.

A strategy for plasma fractionation has evolved since the 1940s as a means of making the most efficient use of plasma
for the production of various derivatives. Development of this strategy has been most strongly influenced by the Cohn method for producing albumin and immune globulins and by the cryoprecipitate method for isolating factor VIII. This paper will describe a strategy as it applies to coagulation products by showing how current processes have evolved and how future products are being developed. Factor IX, factor X, protein C, antithrombin III, thrombin and fibrin glue will serve as examples.

Development of Factor IX Concentrates

For a number of years, the development of improved factor IX (FIX) concentrates for the treatment of hemophilia B has been an area of interest to the American Red Cross. The genesis of new FIX products in our laboratories provides a good example of the strategy followed in the development of new and improved products.

The driving force for process development that form a basis of this strategy are listed in table I. The first, and sometimes most problematic, is the identification of a need for a new derivative. Although seemingly trivial, of primary importance is determining which plasma products might be clinically beneficial. It is possible to purify almost any component of plasma, but in many cases the function or perhaps even the existence of a potentially useful protein is unknown. Often the discovery that blood or plasma contains a therapeutic component is either serendipitous or, as in the case of hemophilia, logically postulated without knowledge of the identity of the actual agent. The distinction between hemophilia A and B, let alone the identity of factors VIII and IX, was unknown when it was first shown that transfusion of whole blood could be used to curtail bleeding in hemophilia patients.  

The second important point addressed in table I concerns increased product concentration. Although blood and later plasma were used to treat bleeding disorders, they were only marginally effective because of low concentrations of Factor IX. The first major improvement in therapy was the switch from whole blood to plasma which represented a two-fold increase in FIX concentration. Even so, it was not always possible to raise a patient's FIX to an effective level because the large volume of plasma necessary to achieve hemostasis caused hazardous fluid and protein overloads.

The first true FIX concentrate was PPSB (an acronym for Prothrombin or Factor II, Proconvertin or Factor VII, Stuart Factor or Factor X, and anti-hemophilic factor B or Factor IX) which was developed in France. This PPSB was produced by adsorption of factor IX and the other vitamin K-dependent clotting factors from plasma using calcium phosphate. In terms of FIX activity, PPSB was 20 to 25 times more concentrated (units per ml) and 85 times more pure (units per mg of protein) than plasma. Although successfully used to treat hemophilia B for 10 years, the original PPSB had disadvantages which eventually led to its discontinuance. In the PPSB process, FIX was adsorbed onto calcium phosphate directly from non-citrated plasma. This precluded the pro-
duction of factor VIII from that plasma and did not fit with the evolving strategy of using compatible processes to produce multiple products from the same batch of plasma. In addition, the process had a relatively low FIX recovery of 20 to 25 percent.\textsuperscript{1,4} Nevertheless, the PPSB method led to the development of a number of other processes based on calcium phosphate adsorption. These involved adsorption of FIX from downstream fractions such as Cohn fraction IV-1 where the non-citrate requirement can be met without interfering with factor VIII production. One such process still used by a U.S. fractionator is shown in figure 1.

A flow diagram for a typical plasma fractionation scheme is shown in figure 1. This scheme incorporates both cryoprecipitation for the production of a fraction rich in factor VIII, and the Cohn fractionation process\textsuperscript{15} for the isolation of a number of useful plasma constituents. The scheme has been labeled "traditional" for this discussion, but there are
many variations in use. In this system, factor IX complex, the generic name in the United States for the mixture of clotting factors first named PPSB, is produced by calcium phosphate adsorption from Cohn fraction IV-1, an otherwise unused fraction.

The next major development in factor IX concentrates was the use of ion exchange chromatography on di-ethylaminoethyl (DEAE)-cellulose or DEAE-Sephadex resins to recover the factor IX complex. This represents one of the first major uses of chromatography in plasma.

**PLASMA FRACTIONATION SCHEME**

*Factor IX Complex by Ion Exchange Chromatography*

![Flow diagram for plasma fractionation scheme to produce factor IX complex concentrate by adsorption on di-ethylaminoethyl (DEAE) Sephadex ion exchange resin.](image-url)
fractionation. As shown in figure 2, cryo-poor plasma is contacted with DEAE-Sephadex to remove the FIX complex proteins. The supernatant plasma containing the unadsorbed components is further fractionated by the Cohn procedure, while the resin-bound proteins are eluted to produce a FIX Complex, often with additional purification steps.

Although the DEAE-purified FIX complex was developed more recently than PPSB, it is a similar product produced by a different method, not necessarily an improved product. The DEAE-purified FIX complex contains much less factor VII which is a disadvantage for some indications but makes little difference for treatment of hemophilia B. The concentration and purity of FIX in the two products are comparable. There are undoubtably reasons for the existence of the two different processes, but these are known only to the respective manufacturers.

Figure 2 represents a current, basic model of the strategy for plasma fractionation. The first step is always purification of factor VIII because it is rapidly inactivated in liquid plasma. Likewise, Cohn fractionation is usually the final process in the scheme although additional methods may be used for further purification of desired proteins from the Cohn fractions. At present, Cohn fractionation is the most efficient method for production of albumin on a large scale. Any process for a new derivative is usually designed to fit somewhere in the stream between the factor VIII and Cohn processes as is the case with DEAE Sephadex-purified FIX, or else to utilize a waste fraction of an existing process as with FIX purified from Cohn fraction IV-1. The new process should have little or no effect on the purification of the already existing products.

Currently FIX complex is the primary product for the treatment of hemophilia B. However, FIX complex has been associated with the occurrence of thromboembolic complications, especially when used in large amounts for extended periods of time, for instance during surgery. One hypothesis is that these complications are due to zymogen overload, that is, the administration of large, unneeded doses of factors II, VII, and X which are present in FIX complex. On that premise the American Red Cross has developed a new FIX concentrate, coagulation factor IX (CFIX), that is much purer and is essentially free of the other vitamin K-dependent clotting factors. This CFIX is non-thrombogenic compared to FIX complex in several animal models and in limited clinical experience.

This CFIX is produced by a two step process, shown in figure 3. An intermediate, FIX complex-like material is isolated from cryo-poor plasma by DEAE Sephadex ion exchange chromatography.
This intermediate is further purified to remove the other vitamin K-dependent clotting factors as well as other contaminants, by chromatography on sulfated dextran resin. The product, still only 10 percent to 15 percent active FIX in terms of total protein, has a specific activity 15 to 20 times higher than FIX Complex, as shown in table II. The sodium dodecyl sulfate-polyacrylamide gels electrophoresis (SDS-PAGE) gels in figure 4 demonstrate that CFIX has fewer protein contaminants than a generic FIX Complex product here represented by the DEAE Sephadex eluate intermediate.

The higher purity of CFIX comes at some cost. The CFIX process is a longer, more complicated process with a lower recovery than the FIX Complex process. Such trade-offs frequently must be made among the goals listed in table I. In this case, the reduction in thromboembolic side effects clearly justifies the additional production costs, but in many cases the choices are not so clear-cut.

The American Red Cross is also developing an immunoaffinity purified Coagulation FIX concentrate (CFIX-M) which would be essentially free of any other contaminating proteins. Large scale processing using immunoaffinity methods has recently become a reality with the introduction of monoclonal antibody purified Factor VIII products. The process envisioned for the production of CFIX-M is shown in figure 5. Here the sulfated dextran column of the CFIX process is replaced by a column containing a resin which has a FIX-specific monoclonal antibody bound to it. The DEAE eluate is passed through this highly selective column which binds only FIX while contaminants fail to bind. After washing the resin to remove non-specifically bound proteins, the FIX is eluted yielding a homogeneous product. Typical results from laboratory-scale
Purification by Immunoaffinity Chromatography

**Figure 5.** Flow diagram for production of immunoaffinity purified coagulation factor IX concentrate from di-ethylaminoethyl (DEAE) Sephadex eluate.

Studies are shown in table II, and gels showing the high purity are pictured in figure 4.

A tactic often employed in process improvement is the grouping of a number of process changes. The rationale is that all significant process changes must be submitted for regulatory approval before they can be implemented. The studies necessary to support such changes can be both costly and time consuming. Consequently, it is reasonable to make a number of changes at once rather than carrying out individual studies for each process change as it is developed. Thus, the CFIX-M process will incorporate improvements in addition to immunoaffinity chromatography.

The CFIX-M data shown in table II and figure 4 are based on studies with several new, higher performance DEAE resins in place of DEAE Sephadex for the initial adsorption from plasma. Because of enhanced flow properties of the new resins, cryo-poor plasma can be pumped directly through a packed column at high flow rates instead of stirring the resin and plasma together in a tank. As has been shown previously, this change in operating mode can significantly increase FIX recovery. Instead of the approximately 50 percent yield of FIX shown for the DEAE Sephadex eluate in table II, contacting resin and plasma in a packed bed can give 80 percent to 100 percent recovery in the DEAE eluate.

**Other Products from Factor IX Complex**

The discussion thus far has focused on process strategies for a single product, coagulation factor IX concentrate. As important is optimizing the number of products from each unit of plasma processed. The FIX Complex has been used to treat deficiencies of its other components such as factor X and protein C. During the development of the CFIX process (figure 3), it became obvious that the discarded wash streams containing factors II, VII, X, and protein C might themselves be sources of useful products. These "waste" streams were already proving valuable as sources of relatively pure factors for laboratory research. It became apparent that such factors could be easily processed into concentrates for clinical use.

The first area of interest was factor X (FX). Based on a survey of hemophilia treatment centers, there are an estimated 65 homozygous FX deficient patients in the United States. However, the fact that minimal additional processing could turn a waste stream that was essentially already 50 percent pure FX into a useful product made this a perfect example of an orphan drug that could be provided by the Red Cross. Interest in protein C (PC) and activated protein C (APC) as new products and in thrombin made by activation of factor II
(as a possible reagent for the activation of protein C and as an ingredient in a fibrin glue) has led to work on the process shown in figure 6. Here a number of products can be produced by immunoaffinity chromatography from a common plasma adsorption step. The initial eluate is passed through a succession of monoclonal antibody columns each of which removes one component from the process stream. The SDS-PAGE gels demonstrating the purity of the products compared to the starting eluate are shown in figure 7.

Antithrombin III

Another important goal is development of new products made by processes which do not interfere with existing production schemes. In addition to normal manufacturing concerns, this is a regulatory consideration since changes in the other processes would require studies to show that the safety and effectiveness of the existing product were not compromised. In the case of FIX, for instance, it was necessary to show that adsorption of the cryo-poor plasma with DEAE resin did not affect the downstream Cohn fractionation process or its products. Similar considerations had to be addressed for the development of antithrombin III (AT-III), another product being developed in our laboratory. Antithrombin-III is an anticoagulant which has been used prophylactically to prevent thrombosis in patients with congenital AT-III deficiencies.

As shown in figure 8, AT-III is adsorbed from plasma using heparin Sepharose, an affinity resin. The heparin Sepharose adsorption again does not affect the downstream Cohn process, but it would affect the DEAE adsorption step since heparin Sepharose binds factor IX and the other vitamin K-dependent clotting factors. Heparin Sepharose adsorption therefore must be placed downstream of the DEAE adsorption step in the overall scheme. Antithrombin-III is also a product which probably would not benefit from the use of immunoaffinity chromatography because heparin itself has a high, specific affinity for AT-III. As shown by SDS-PAGE gels

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**MONOCLONAL ANTIBODY PURIFICATION OF SELECTED VITAMIN K-DEPENDENT PROTEINS**

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**Figure 6.** Flow diagram for production of concentrates of selected vitamin K-dependent clotting factors by immunoaffinity chromatography from di-ethylaminoethyl (DEAE) Sepharose fast flow eluate.
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**Figure 7.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (non-reduced) of (1) factor IX complex (FIX-complex) (2) immunoaffinity-purified concentrates of protein C (PC-M), (3) coagulation factor X (CFX-M), and (4) coagulation factor IX (CFIX-M).

In figure 9, AT-III produced by this method already has a very high degree of purity. Typical characteristics of the product are a specific activity of 6.0 units per mg total protein, a purity in terms of AT-III antigen of 98 percent, and a recovery from plasma of 15 to 20 percent.

**Fibrin Glue**

In contrast to the purity sought for CFIX, fibrin glue involves a combination of several clotting factors which interact to form a “natural” adhesive. The idea for such a product is old, but its development has not been feasible because of the problems of viral infectivity. However, new, more effective methods of viral inactivation have changed this. Several apparently safe fibrin glue products are currently available in Europe.

As shown in figure 10, fibrin glue is produced from the cold-insoluble precipitate, a waste product of factor VIII purification which is rich in fibrinogen and fibronectin. Fibrin glue is a two-component product, a fibrinogen-rich base which is mixed with thrombin at the site of application. Thrombin cleaves the fibrinogen to fibrin which polymerizes to form a clot, bonding the tissues together. The fibrinogen-rich fraction also contains factor XIII which crosslinks the fibrin clot to increase its adhesive strength. A bovine source of thrombin has been used successfully in most of the current products. The use of human thrombin from the process shown in figure 6 might also be feasible.

**Viral Inactivation**

Improved viral inactivation methods have provided important advances in product safety. Although a complete discussion of this area is well beyond the scope of this paper, some consideration of these new methods as they relate to process development is warranted. All of the products described previously employ one of three viral inactivation methods. The original CFIX concentrate, CFIX-HT, is heat treated as a lyophilized powder at 60°C for 144 hours. Antithrombin-III stabilized with 0.5 M sodium citrate is pasteurized in the liquid state at 60°C for 10 hours. All of the remaining products, including a second-generation CFIX concentrate, CFIX-SD, will be treated by the sol-
PLASMA FRACTIONATION SCHEME
Preparation of AHF-M, AT-III, and Fibrin Glue

FROZEN PLASMA

CRYOPRECIPITATE
Thaw at 4°C
Centrifuge

Processed to AHF-M and Fibrin Glue

CRYO-POOR PLASMA
Mass Capture
DEAE Sepharose

Antithrombin III
Heparin Sepharose

Mass Capture
ELUATE

Processed to CFIX-M, CFX-M, PC and APC

To Downstream Fractionation

FIGURE 8. Flow diagram for production of several coagulation factors from plasma.

vent/detergent method (s/d) using 0.3 percent tri-n-butyl phosphate and one percent Triton X-100.3,7

Although this method for dry heat treatment has been shown in in vitro studies to inactivate HIV3 in ClFX, it is considered less effective in inactivating other viruses than either the s/d or pasteurization methods. Solvent/detergent (s/d) treated factor VIII and FIX concentrates have shown no evidence of HIV, hepatitis B (HBV), or non-A non-B hep-

FIGURE 9. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (reduced) of cryo-poor plasma (2 and 3) and antithrombin III concentrates (4 and 5) produced by heparin-Sepharose adsorption from cryo-poor plasma. Molecular weight markers are shown in lane 1.
titis (NANB) transmission in a number of clinical studies.\textsuperscript{5,6,13} In more limited clinical trials, pasteurized AT-III produced by the Red Cross method has produced no evidence of transmission of HBV or NANB.\textsuperscript{11}

Viral inactivation methods can affect process development in several ways. A method such as dry heat treatment in which the product is treated in the final container puts minimal constraints on the process. However, the other two methods require that components of the treatment system, the citrate stabilizer in the case of pasteurized AT-III and the solvent and detergent inactivation agents in the case of the s/d products, subsequently be removed during the manufacturing process. Also, once treated, the products must be protected from recontamination in the process plant environment.

For solvent/detergent treated CFIX, it is advantageous to treat the DEAE eluate and then remove the solvent and detergent during the washing of the sulfated dextran column that also removes factors II and X. Fortunately, these additives do not interfere with the binding or elution properties of the column. Thus, a separate step to remove the additives is not necessary. The performance of the monoclonal antibody columns used in the multiproduct scheme of figure 6 also does not appear to be affected by the s/d additives. Thus, a single treatment of the DEAE eluate suffices to inactivate virus in all of the subsequent products.

Conclusion

There is no single strategy for the production of plasma derivatives. This paper has presented a view of the relevant parameters that must be considered. Examples of new strategies and new and improved products have been given. Plasma remains a limited, valuable, economical, and increasingly safe resource for therapeutic materials.

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