PREPARATION OF HIGHLY PURIFIED SOLVENT-DETERGENT COAGULATION FACTOR VII AND FACTOR IX CONCENTRATES FROM PROTHROMBIN COMPLEX (PPSB)

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ABSTRACT

In this study, anion-exchange chromatography was used to purify factor VII and factor IX from prothrombin complex (PPSB), which contains coagulation factors II, VII, IX and X. For this purpose, DEAE-Sepharose CL-6B gel, Pharmacia column XK-26, high flow rate and two stepwise gradients with phosphate citrate buffer were used. The yield of the two lyophylized products, factor VII and factor IX concentrates, was 61% and 75%, respectively. Specific activity of factor VII increased from 0.16 to 3.06 (purification-fold= 19.1) and specific activity of factor IX increased from 1.2 to 4.9 (purification-fold= 4.1). Results of electrophoresis on agarose gel—as well as immunoelectrophoresis—indicated higher purity of factor VII and factor IX compared to PPSB. Thrombogenicity of the two products were within the normal range defined for PPSB. In order to improve viral safety, solvent-detergent treatment was performed prior to further purification. Factor VII concentrate is used for patients with factor VII deficiency and also for hemophilia patients with inhibitors. Factor IX concentrate is used for treatment of hemophilia B patients.


Keywords: Factor VII, Factor VIIa, Factor IX, Hemophilia, PPSB, Viral inactivation.

INTRODUCTION

Factors VII and IX are plasma proteins involved in blood coagulation which require vitamin K for their complete synthesis.1 The first reliable therapeutic concentrate of factor IX was prepared by a method requiring specially collected blood.2 In 1972 Dike, Bidwell and Rizza reported the preparation and clinical use of a concentrate of factor VII as a by-product of the preparation of a therapeutic concentrate of factors II, IX and X by adsorption on DEAE-Cellulose.3 In 1973, it was concluded that DEAE-Sephadex was more suitable than DEAE-Cellulose for routine large-scale production of the prothrombin complex.4 In 1980, batch adsorption on DEAE-Sepharose CL-6B followed by elution on a chromatographic column, concentrated factor VII about 25-fold without a need for further dialysis or concentration steps.5 A human solvent-detergent-treated factor IX concentrate was produced by DEAE-Sepharose CL-6B chromatography in 1988.6

The treatment of congenital hemophilia A and B may be dramatically affected by the occurrence of high-titer alloantibodies to factor VIII and less frequently to factor IX molecules. Similar to autoantibodies in acquired hemophilia, some of these alloantibodies are produced against the procoagulant part of the molecule, which confers an inhibitory effect to therapy using coagulation factor VIII or factor IX concentrates.7 The management of bleeding episodes in patients with inhibitors may require different therapeutic approaches, among which fac-
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buffer A

III - FIX

Fig. 1. Chromatogram of preparation of coagulation factor VII and factor IX concentrates from PPSB by DEAE-Sepharose CL-6B chromatography. Buffer A, 5mM phosphate citrate buffer pH= 7 containing 0.25 M NaCl; buffer B, 5mM phosphate citrate buffer pH= 8 containing 0.5 M NaCl.

MATERIAL AND METHODS

About 400 mL of prothrombin complex concentrate (PPSB) used as the starting material for purification of factor VII and factor IX was kindly supplied by the Iranian Blood Fractionation Research Co. Prothrombin complex was treated with a mixture of 0.3% tri-(n-butyl) phosphate (TNBP) and 1% Tween 80 (final concentration) at 24°C for 6 hours with constant stirring. The mixture was then chromatographed on an XK-26 column (Pharmacia) containing DEAE-Sepharose CL-6B equili-

Table 1. Results of preparation of coagulation factor VII concentrates from prothrombin complex (PPSB).

<table>
<thead>
<tr>
<th></th>
<th>Total Protein FVII (mg)</th>
<th>Total Activity FVII (u)</th>
<th>S.A.* FVII (u/mg)</th>
<th>Purification-fold</th>
<th>Yield FVII (%)</th>
<th>FVIIa FVII (u/dL)</th>
<th>FVII (u/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPSB</td>
<td>6000</td>
<td>1000</td>
<td>0.16</td>
<td>1</td>
<td>100</td>
<td>40</td>
<td>250</td>
</tr>
<tr>
<td>DEAE-Sepharose CL-6B (purification step)</td>
<td>200</td>
<td>612</td>
<td>3.06</td>
<td>19.12</td>
<td>61</td>
<td>500</td>
<td>1100</td>
</tr>
</tbody>
</table>

*Specific activity
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Fig. 2. Gel electrophoresis. From left to right: Gel 1, normal plasma; Gels 2 & 8, PPSB; Gel 3, factor IX concentrate; Gels 4 & 7, factor VII concentrate; Gel 5, recombinant activated factor VII (Novo); Gel 6, highly purified factor IX (fraction 94).

RESULTS

The chromatogram for the preparation of coagulation factors VII and IX concentrates from prothrombin complex is shown in Fig. 1. In this chromatogram four major peaks are seen. The first peak is breakthrough fraction which contains unadsorbed proteins, TNBP and Tween 80. Assays of factor VII and factor IX activity were performed and then fractions 38-44 (peak B) were pooled for factor VII and fractions 73-107 (peak C) were pooled for factor IX.

Tables I and II indicate the results of preparation of coagulation factor VII and factor IX concentrates from PPSB after DEAE-Sepharose CL-6B purification step, respectively. Factor VII and factor IX were purified about 19- and 4-fold respectively. Activation of factor VII during purification was monitored by assay of activated factor VII and FVIIa/FVIIc ratio.

Table III shows the characteristics of coagulation factor VII, factor IX and PPSB concentrates.

The results of gel electrophoresis (Figs. 2 and 3) indicated that factor VII concentrate contained approximately 79% alpha-2 proteins and factor IX concentrate approximately 40% alpha-1 and 45% alpha-2 proteins, providing evidence of the improved purity of the final concentrates as compared to PPSB which showed three major bands, alpha-1, alpha-2 and beta proteins. Recombinant activated factor VII indicated 89% alpha-2 proteins and highly purified factor IX (fraction 94) showed 58% alpha-1 and 41% alpha-2 proteins. These findings revealed that factor IX migrated as alpha-1 and factor VII as alpha-2 proteins and were also confirmed by immunoelectrophoresis (Fig. 4).

Thrombogenicity assay of PPSB, factor VII and factor IX concentrates indicated that NAPTT values were 166s, 220s and 153s (buffer 296s) for the 1:10 dilution corresponding to NAPTR values of 0.56, 0.74 and 0.52, respectively. All of the concentrates passed the European Pharmacopoeia (1997) for determination of activated coagulation factors.

Elimination of Tween 80 was nearly complete in both concentrates. Tween 80 levels were about 2 μg/mL in final products.

DISCUSSION

Our study demonstrated that factor VII concentrate, essentially free of factors II, IX and X, can be further purified from prothrombin complex by anion-exchange chromatography, and a virus inactivation step using solvent and detergent could also be included. The specific activity of FVII in our procedure was increased from 0.16 to 3.06 and the yield was 61%. Activity of activated factor VII (FVIIa) in our purified concentrate and PPSB were 500U% and 40U%, respectively, indicating that

Clotting assays

Factor VII and factor IX were assayed by one stage clotting assay methods. Activated factor VII was assayed by one stage clotting using Stago kit.

Agarose gel electrophoresis

This method was performed using a pH 8.6 barbital buffer at 220 V for 35 min with Ciba Corning equipment.

Immunoelectrophoresis

Immunoelectrophoresis was performed on 2% agarose plates in barbital buffer, pH 8.8. Samples were analyzed against antihuman serum and specific antihuman factor VII serum for purified proteins.

Determination of Tween 80

Tween 80 was measured spectrophotometrically at 535 nm.
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Table II. Results of preparation of coagulation factor IX concentrates from prothrombin complex (PPSB).

<table>
<thead>
<tr>
<th></th>
<th>Total Protein</th>
<th>Total Activity</th>
<th>S.A.*</th>
<th>Purification</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FIX (mg)</td>
<td>FIX (u)</td>
<td></td>
<td>fold (%)</td>
<td>(%)</td>
</tr>
<tr>
<td>PPSB</td>
<td>6000</td>
<td>7200</td>
<td>1.2</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>DEAE-Sepharose</td>
<td>1100</td>
<td>5425</td>
<td>4.93</td>
<td>4.08</td>
<td>75</td>
</tr>
</tbody>
</table>

*Specific activity

Fig. 3. Patterns of gel electrophoresis. Normal plasma (a), PPSB (b), factor IX concentrate (c), factor VII concentrates (d), recombinant activated factor VII (e), Highly purified factor IX of fraction 94 (f).

factor VII concentrate similar to recombinant activated factor VII, can be used for the treatment or prevention of bleeding in patients with factor IX inhibitors without causing thromboembolism according to NAPTT test.

The purity of factor IX which is used in the treatment of hemophilia B was also improved 4.1 times compared to PPSB, by addition of this chromatography step. Therefore it was demonstrated that both factors VII and IX
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Table III. The characteristics of concentrates.

<table>
<thead>
<tr>
<th></th>
<th>PPSB</th>
<th>Factor VII concentrate</th>
<th>Factor IX concentrate</th>
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<tbody>
<tr>
<td>Total protein g/dL</td>
<td>1.5</td>
<td>1.1</td>
<td>0.2</td>
</tr>
<tr>
<td>Factor II u/dL</td>
<td>2800</td>
<td>10</td>
<td>1080</td>
</tr>
<tr>
<td>Factor IX u/dL</td>
<td>1800</td>
<td>2</td>
<td>2500</td>
</tr>
<tr>
<td>Factor VII u/dL</td>
<td>250</td>
<td>1100</td>
<td>30</td>
</tr>
<tr>
<td>Factor VIIa u/dL</td>
<td>40</td>
<td>500</td>
<td>n.d. *</td>
</tr>
<tr>
<td>Factor X u/dL</td>
<td>4800</td>
<td>1</td>
<td>1080</td>
</tr>
<tr>
<td>Tween 80 mg/mL</td>
<td>—</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>NAPTR (dilution 1/10)</td>
<td>0.56</td>
<td>0.74</td>
<td>0.52</td>
</tr>
</tbody>
</table>

*Not done

Hilgartner MW, Knatterud GL, The FEIBA Study Group: Validation of this treatment is also being through extensive washing of the gel (Table III). Be added to the purification procedure without any loss of coagulation activity of factor VII and IX. However, validation of this treatment is also being carried out using appropriate viral models, which will be reported in due course.

REFERENCES

15. Hilgartner MW, Knatterud GL, The FEIBA Study Group:
Preparation of Purified Factors VII and IX


