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

S. NASIRI, M.D., H. REZVAN, Ph.D., K. MOUSAVI, Ph.D., AND M.H. ROOSTAEI,* Ph.D.

*From the Department of Research and Development, Blood Research and Fractionation Co., Tehran, and the *Department of Virology, Tarbiat Modarres University, Tehran, I.R. Iran.*

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.

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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-

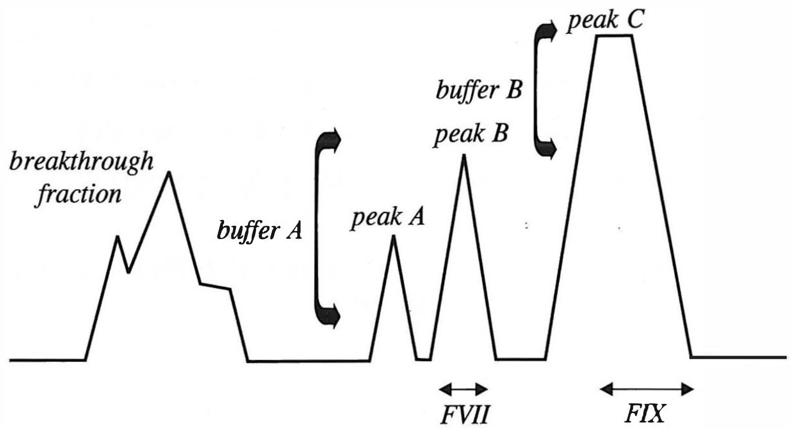

Fig. 1. Chromatogram of preparation of coagulation factor VII and factor IX concentrates from PPSB by DEAE-Sepharose CL-6B chromatography. Buffer A, 5mM phosphate citrate buffer pH= 7 containing 0.25 M NaCl; buffer B, 5mM phosphate citrate buffer pH= 8 containing 0.5 M NaCl.

tor VIIa[8-12] and prothrombin complex concentrates[13-16] have been successfully used. FEIBA (Factor Eight Inhibitor Bypassing Activity, Immuno, Vienna, Austria) is an activated prothrombin complex concentrate which has been widely used in the treatment of hemophilia patients with inhibitors.[7] Factor VII concentrates are used in patients with congenital or acquired factor VII deficiency or treatment of hemophilia patients with inhibitors.[5,8] It was observed that high levels of factor VIIa in prothrombin complex concentrates containing factor VII, may contribute to the thrombogenic potential of these preparations,[17] therefore purifying FVII from PPSB should improve its thrombogenicity. Factor VIIa measurement and its correlation with FVII coagulant activity has been studied previously.[18-19]

Since the technology for the production of PPSB already existed in the Research & Fractionation Center of IBTS, this study was planned so as to improve tolerance and safety in the treatment of patients.[7,20,21] In addition, an extra step of virus inactivation using solvent-detergent treatment was also included to improve virus safety of the products. The most widely used method for virus inativation is the S/D treatment. This procedure involves treating plasma products with a mixture of an organic solvent and a non-ionic detergent in order to destroy enveloped viruses. This procedure was originally developed by the New York Blood Center and is now used by at least 50 manufacturers world-wide.[22,23]

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

	Total Protein FVII (mg)	Total Activity FVII (u)	S.A.* FVII (u/mg)	Purification-fold	Yield FVII (%)	FVIIa (u/dL)	FVII (u/dL)	FVIIa/FVII
PPSB	6000	1000	0.16	1	100	40	250	0.16
DEAE-Sepharose CL-6B (purification step)	200	612	3.06	19.12	61	500	1100	0.45

*Specific activity

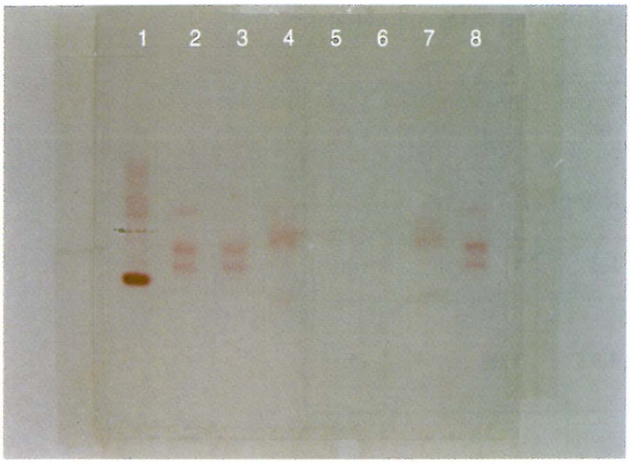

Fig. 2. Gel electrophoresis. From left to right: Gel 1, normal plasma; Gels 2 & 8, PPSB; Gel 3, factor IX concentrate; Gels 4 & 7, factor VII concentrate; Gel 5, recombinant activated factor VII (Novo); Gel 6, highly purified factor IX (fraction 94).

brated with 5 mM phosphate citrate buffer, pH 6.0 at a flow rate of 150 mL/h. During eluting the breakthrough fraction, the column was washed with 5mM phosphate citrate buffer pH 7 containing 0.25 M NaCl and subsequently with 5mM phosphate citrate buffer, pH 8.0 containing 0.5 M NaCl to elute factors VII and IX, respectively. Fractions containing the activities of factors VII (38-44) or IX (73-107) were pooled, sterile-filtered, dispensed into vials and lyophylized. The gel was regenerated by washing with 2 column volumes of 2 M NaCl solution followed by 0.5 N NaOH. The gel was stored in 0.1 N NaOH.

Clotting assays

Factor VII and factor IX were assayed by one stage clotting assay methods.[24,25] Activated factor VII was assayed by one stage clotting using Stago kit.[26]

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.[6]

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

Table II. Results of preparation of coagulation factor IX concentrates from prothrombin complex (PPSB).

	Total Protein FIX (mg)	Total Activity FIX (u)	S.A.* FIX (u/mg)	Purification-fold	Yield FIX (%)
PPSB	6000	7200	1.2	1	100
DEAE-Sepharose CL-6B (purification step)	1100	5425	4.93	4.08	75

*Specific activity

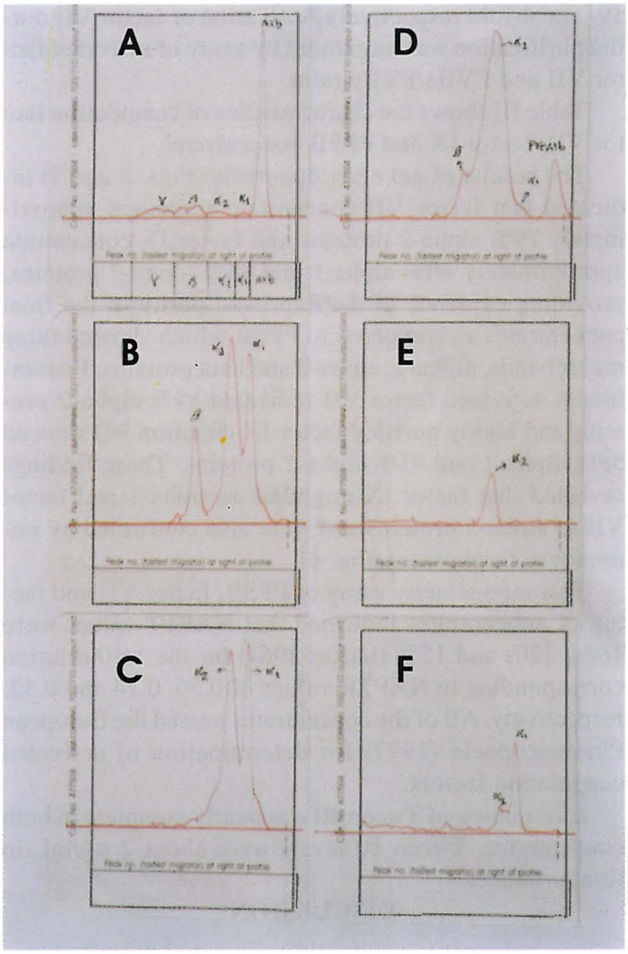

Fig. 3. Patterns of gel electrophoresis. Normal plasma (a), PPSB (b), factor IX concentrate (c), factor VII concentrates (d), recombinant activated factor VII (e), Highly purified factor IX of fraction 94 (f).

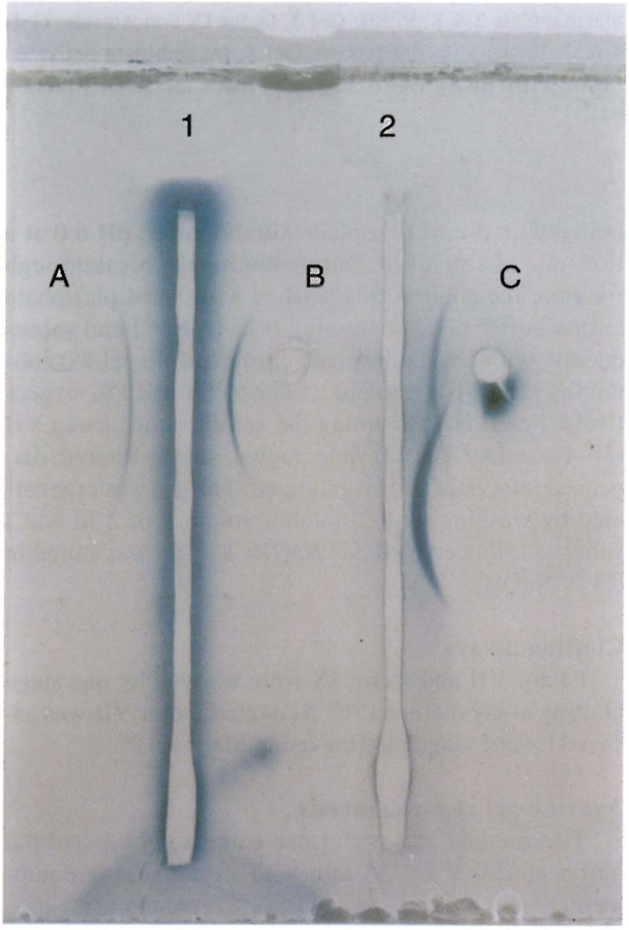

Fig. 4. Immunoelectrophoresis. Sample (A) factor VII concentrate and sample (B) recombinant activated factor VII (Novo) against specific anti-human factor VII (1); Sample (B) and sample (C), normal human plasma against antihuman serum (2). Factor VII was concentrated 100-fold.

factor VII concentrate similar to recombinant acivated 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

Table III. The characteristics of concentrates.

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

*Not done

can be prepared in a single manufacturing process using DEAE-Sepharose CL-6B chromatography. The purification method used differs significantly in the type of chromatographic gels. We used semirigid DEAE gels as the first adsorbant instead of soft DEAE-Sephadex A50, to take advantage of the better mechanical resistance of sepharose which make it more suitable for industrial production. It also allows better control of the various elution steps, and more reproducible recovery of intermediate chromatographic plasma fractions that can be used to prepare α_1-antitrypsin and protein C. A large volume of plasma could be adsorbed to DEAE-Sepharose CL-6B, binding a maximum amount of trace proteins. The filtrate containing albumin and immunoglobulins could be concentrated and adjusted to an ionic strength suitable for cold ethanol precipitation.[5,6]

Chromatographic methods are being used increasingly in the preparation of high purity plasma products and can also contribute to the virus safety of the product.[27] The potential of chromatography for eliminating viruses has been reviewed and up to more than 3-5 $\log_{10}$ removal of various viruses used as models can be achieved during some chromatographic steps.[28] This chromatography method can be used after virus inactivation to bind the proteins of interest and can easily eliminate the chemicals (Tween 80, TNBP) in the breakthrough fraction through extensive washing of the gel (Table III).

Our study therefore confirms that conventional adsorption chromatography can be used to further purify factor VII and IX concentrates from PPSB on a bench scale, which theoretically can be easily modified for large scale production. In addition SD treatment can 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.

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