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Preparation of Factor VII concentrate by immunoaffinity chromatography

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ABSTRACT

In this research, immunoaffinity chromatography was used to purify factor VII from prothrombin complex (PPSB), which contains coagulation factors II, VII, IX and X. For this purpose DEAE-Sephadex and CNBr-activated Sepharose 4B gels were used. Specific activity of factor VII concentrate increased from 0.16 to 55.6 with purification-fold of 347.5 and the amount of activated factor VII (FVIIa) was found higher than PPSB (4.4-fold). Results of electrophoresis on agarose gel indicated higher purity of FVII compared to PPSB and these finding revealed that factor VII migrated as alpha-2 proteins. In order to improve viral safety, solvent-detergent treatment was applied prior to further purification and elimination of tween 80 was nearly complete (2 µg/ml). Factor VII concentrate is used for patients with factor VII deficiency and also for hemophilia patients with inhibitors.

Introduction

Human coagulation factor VII (FVII) is a glycoprotein with a molecular mass of 50 kDa, is synthesized in the liver and it circulates in the blood at a plasma concentration of 0.5µg/ml[1]. 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[2]. In 1973, it was concluded that DEAE-Sephadex was more suitable than DEAE-Cellulose for routine large-scale production of the prothrombin complex[3]. 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[4]. In 2003, an activated Factor VII (FVIIa) concentrate, prepared from human plasma on a large scale became available for clinical use for haemophiliacs with antibodies against FVIII and FIX[5]. The management of bleeding episodes in patients with inhibitors may require different therapeutic approches, among which factor VIIa[6-10] and prothrombin complex concentrates [11-12] 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[13]. Factor VII concentrates are used in patients with congenital or acquired factor VII defeciency or treatment of hemophilia patients with inhibitors[4-6]. It was shown that high levels of FVIIa in prothrombin complex concentrates containing factor VII, may contribute to the thrombogenic potential of these preparations[14], therefore purifying FVII from PPSB should improve its thrombogenecity. This study was planned so as to improve tolerance and safety in the treatment of patients with preparing highly purified factor VII from PPSB by using immunoaffinity chromatography technique[15-18].

Material and Methods

Prothrombin complex preparation:

PPSB was prepared from plasma conventionally by DEAE-Sephadex method (Na citrate 0.01 M, pH:7.0, NaCl 2M) and was used as the starting material for purification of coagulation factor VII . Prothrombin complex was treated with a mixture of 0.3% tri-(n-butyl) phosphate (TNBP) and 1% Tween 80 at 24°C for 6 h with constant stirring.

Preparation of specific antibody aginst human factor VII

Antiserum against human FVII (Assera factor VII , Stago) was further purified by ammonium sulphate 50% , after centrifugation dialysed in coupling buffer (0.1 M NaHCO3 , pH 8.3 containing 0.5 M NaCl).

Preparation and packing the gel

Preparation of the gel (2 g powder), coupling the ligand (Assera factor VII) with coupling buffer for one night, blocking excess remaining groups with Tris buffer (pH:8, 0.1 M) for 2 h and packing of the gel were performed according to the brochure of the kit.

Immunoaffinity chromatography

The mixture of PPSB (50 ml) in the PBS buffer (pH: 7.4) was filtered (0.22 µ) and then chromatographed (Pharmacie LKB Fraction Collector 2210) on a column (K 9/15 Pharmacia) containing CNBr-activated Sepharose 4B coupled with specific antibody. Flow rate was adjusted to 0.75 ml/min. After washing step, elution was performed by glycine buffer (0.1 M, pH:2.5) and FVII collected in collection phosphate buffer (1.0 M, pH:8) fractions.

Clotting assays

Factors II, IX, VII, X and VIIa were assayed on the fractions by one stage clotting assay method using Stago kits. Fractions of 48-50 were pooled and lyophilized as a factor VII concentrate.

Agarose gel electrophoresis

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

Determination of Tween 80

Tween 80 was measured spectrophotometrically at 535 nm[19].

Results and Discussion

Elution pattern of FVII from PPSB is shown in Fig.1. In this pattern one major peak of FVII (Fractions 48-50) and the minor peak of FIX (Fractions 46-51) has been shown, because the activity of other coagulation factors was lower. Other unadsorbed or unwanted proteins have been removed in breakthrough and other fractions.

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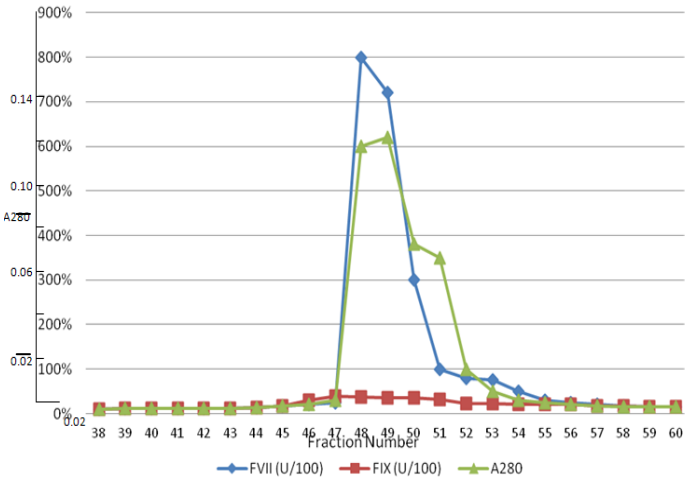

Fig. 1. Elution pattern of factor VII from prothrombin complex concentrate (PPSB) by immunoaffinity chromatography

Table I indicates the results of preparation of human coagulation FVII concentrate from PPSB by immunoaffinity chromatography step. Specific activity and purification-fold of FVII concentrate were found 55.6 and 3971 respectively.

Table I. Results of preparation of coagulation factor VII concentrate from PPSB by immunoaffinity chromatography

Purification step	Total Protein FVII (mg)	Total Activity FVII (u)	Specific Activity FVII (u/mg)	Purification-fold*	Yield FVII (%)	FVIIa (u/dl)
PPSB	780	125	0.16	12.3	100	40
CNBr-activated Sepharose 4B	0.72	40	55.6	3971	32	175

* Purification-fold was determined with regard to the specific activity of FVII in plasma (0.013).

Table I shows higher purity of factor VII concentrate with lower coagulation activities of FII, FIX and FX during its preparation by immunoaffinity chromatography. The amount of activated factor VII (FVIIa) in FVII concentrate was found more than PPSB (4.4-fold).

Table III shows the characteristics of coagulation Factor VII and PPSB concentrates.

Table II. Characteristics of prepared-factor VII concentrate by ---------- chromatography. concentrate by immunoaffinity chromatography.

		PPSB	Factor VII concentrate
Total protein	mg/dl	1500	10
Factor II	u/dl	2800	11
Factor IX	u/dl	1800	12
Factor VII	u/dl	250	555
Factor VIIa	u/dl	40	175
Factor X	u/dl	4800	9
Tween 80	µg/ml	-	2

The result of gel electrophoresis (Fig. II) indicated that factor VII concentrate that contained approximately 89% 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. These finding revealed that factor IX migrated as alpha-1 and factor VII as alpha-2 proteins.

Our study demonstrated that factor VII concentrate, essentially free of factors II, IX and X, can be further purified from prothrombin complex by immunoaffinity chromatography, and a virus inactivation step of solvent-detergent treatment could also be included.

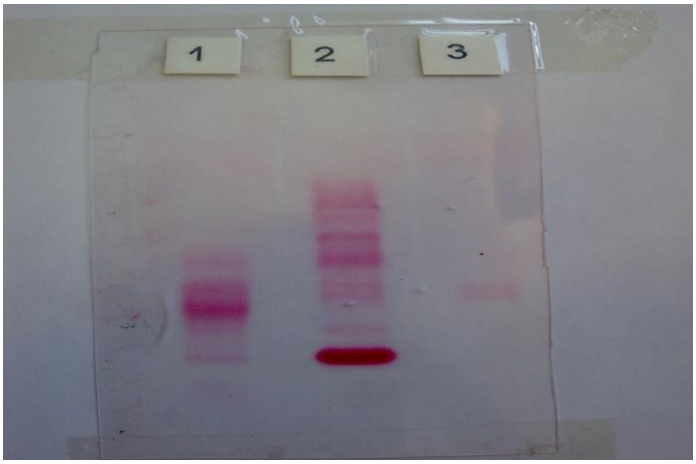

Fig. II. Gel electrophoresis. Line 1, PPSB; Line 2, normal plasma; Line 3 highly purified FVII plasma; Line 3 highly purified FVII concentrate (fractions 48-50).

The specific activity of FVII in our procedure was increased from 0.16 to 55.6 with purification-fold of 3975 and the yield was 32%. Activity of activated factor VII (FVIIa) in our purified concentrate and PPSB were 250% and 555% respectively, indicating that factor VII concentrate similar to recombinant activated factor VII, may be used for the treatment or prevention of bleeding in patients with factor IX inhibitors.

Viral inactivation and chromatographic methods are being used increasingly in the preparation of high purity plasma or blood component products and can also contribute to improve virus safety of the product[20-25]. 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[26]. It may be concluded that immuonoaffinity chromatography can be a suitable choice for large-scale production of factor VII concentrate with higher purity and safety.

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