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Medical Journal of the Islamic Republic of Iran (MJIRI)
Iran University of Medical Sciences

Preparation of factor VII concentrate using CNBr-activated Sepharose 4B immunoaffinity chromatography

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Received: 9 February 2014 Accepted: 18 November 2014 Published: 28 January 2015

Abstract

Background: Factor VII concentrates are used in patients with congenital or acquired factor VII deficiency or treatment of hemophilia patients with inhibitors. In this research, immunoaffinity chromatography was used to purify factor VII from prothrombin complex (Prothrombin-Proconvertin-Stuart Factor-Antihemophilic Factor B or PPSB) which contains coagulation factors II, VII, IX and X. The aim of this study was to improve purity, safety and tolerability as a highly purified factor VII concentrate.

Methods: PPSB was prepared using DEAE-Sephadex and was used as the starting material for purification of coagulation factor VII. Prothrombin complex was treated by solvent/detergent at 24°C for 6 h with constant stirring. The mixture of PPSB in the PBS buffer was filtered and then chromatographed using CNBr-activated Sepharose 4B coupled with specific antibody. Factors II, IX, VII, X and VIIa were assayed on the fractions. Fractions of 48-50 were pooled and lyophilized as a factor VII concentrate. Agarose gel electrophoresis was performed and Tween 80 was measured in the factor VII concentrate.

Results: Specific activity of factor VII concentrate increased from 0.16 to 55.6 with a 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 Factor VII compared to PPSB; 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 nearly complete elimination of tween 80 (2 µg/ml).

Conclusion: It was concluded that immuonoaffinity chromatography using CNBr-activated Sepharose 4B can be a suitable choice for large-scale production of factor VII concentrate with higher purity, safety and activated factor VII.

Keywords: Factor VII, Activated factor VII, chromatography, Sepharose.

Cite this article as: Mousavi Hosseini K, Nasiri S. Preparation of factor VII concentrate using CNBr-activated Sepharose 4B immunoaffinity chromatography. *Med J Islam Repub Iran* 2015 (28 January). Vol. 29: 170.

Introduction

Human coagulation factor VII (FVII) is a glycoprotein with a molecular mass of 50 KDa. It is synthesized in the liver and 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,

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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 approaches, 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 deficiency 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 to improve tolerance and safety in the treatment of patients with preparing highly purified factor VII from PPSB using immunoaffinity chromatography technique (15-18).

Methods

One thousand mililiter of Fresh Frozen Plasma was thawed overnight at 4°C. Then it was centrifuged for 7 minutes at 4°C (5000 g). The obtained supernatant was treated with DEAE-Sephadex A50 (Pharmacia). First, it was washed by sodium citrate 0.01 M, pH 7.0, and sodium chloride 0.2 M; followed by eluting with sodium citrate 0.01 M, pH 7.0, and sodium chloride 2.0 M. The eluted prothrombin complex was used as starting material for purification of coagulation factor VII. For the purpose of virus inactivation, the solvent/detergent method was applied. Prothrombin complex (50 ml) was treated with a mixture of 0.3% tri-(n-butyl) phosphate (TNBP) as solvent and 1% Tween 80 (detergent) at 24°C for 6 h with continuous stirring.

Antiserum against human FVII (Assera factor VII, Stago) was further purified by ammonium sulphate 50%, after centrifugation dialyzed in coupling buffer (0.1 M NaHCO3, pH 8.3 containing 0.5 M NaCl) for overnight at 4°C.

Preparation of the CN Br-activated Sepharose 4B gel (2 g powder), coupling the ligand (Assera factor VII) with coupling buffer rotating overnight at 4°C, blocking excess remaining groups with Tris buffer (pH:8, 0.1 M) for 2 h and packing of the gel were performed. The ligand to be coupled, was dissolved in coupling buffer, 0.1M $NaHCO_3$ pH8.3 containing 0.5 M NaCl. Five ml of coupling solution was used per gram of CN Br-activated Sepharose 4B. The excess ligand was washed away with 5 volumes of coupling buffer (0.1M $NaHCO_3$ pH8.3). For blocking any remaining active groups, the medium was transferred into the 0.1 M Tris-HCl buffer, pH 8.0 for 2 hours.

The mixture of prepared prothrombin complex (50 ml) in the PBS buffer (pH: 7.4) was filtered (0.22 µ) and then chromatographed (Pharmacia 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.

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 was performed using barbital buffer (pH: 8.6) at 220 V for 35 min with Ciba Corning equipment. Tween 80 was measured spectrophotometrically at 535 nm (19).

Results

Elution pattern of FVII from PPSB is shown in Figure 1. In this pattern the major

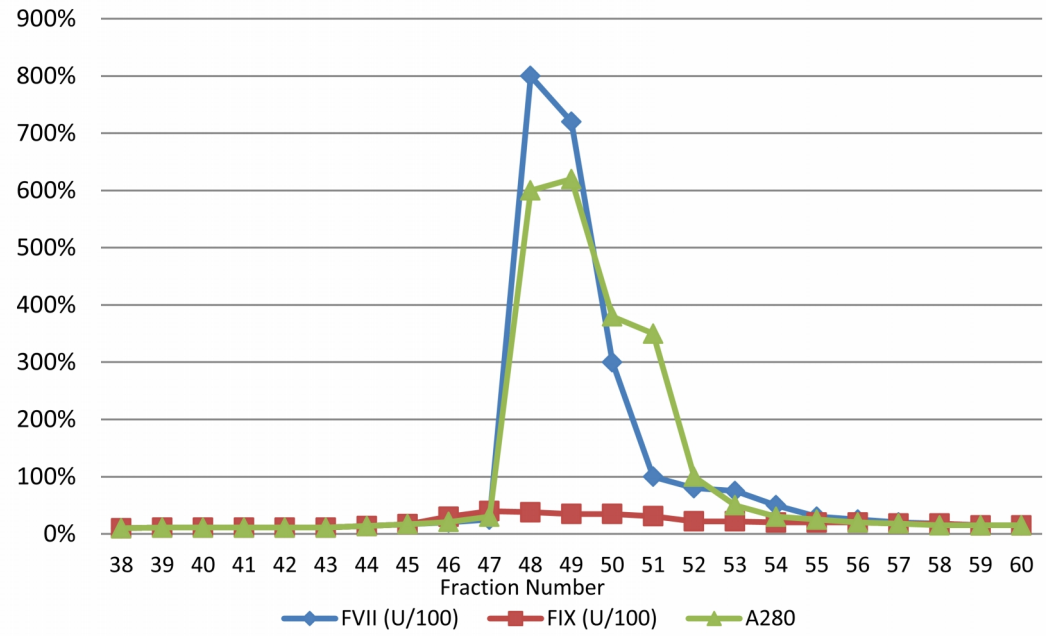

Fig.1. Elution pattern of factor VII from prothrombin complex concentrate (PPSB) using immunoaffinity chromatography.

peak of FVII (Fractions 48-50) and the minor peak of FIX (Fractions 46-51) has been shown; activity of other coagulation factors was lower. Other unabsorbed or unwanted proteins have been removed in breakthrough and other fractions.

Table 1 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 55.6 and 3971, respectively. Table 1 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 higher than PPSB (4.4-fold) and the yield was 62%.

Table 2 shows the characteristics of coagulation factor VII and PPSB concentrates and confirms higher purity of factor VII concentrate.

The result of gel electrophoresis (Figure

Table 1. Results of preparation of coagulation factor VII concentrate from PPSB using 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	55.60	3971	62	175

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

Table 2. Characteristics of factor VII concentrate prepared 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

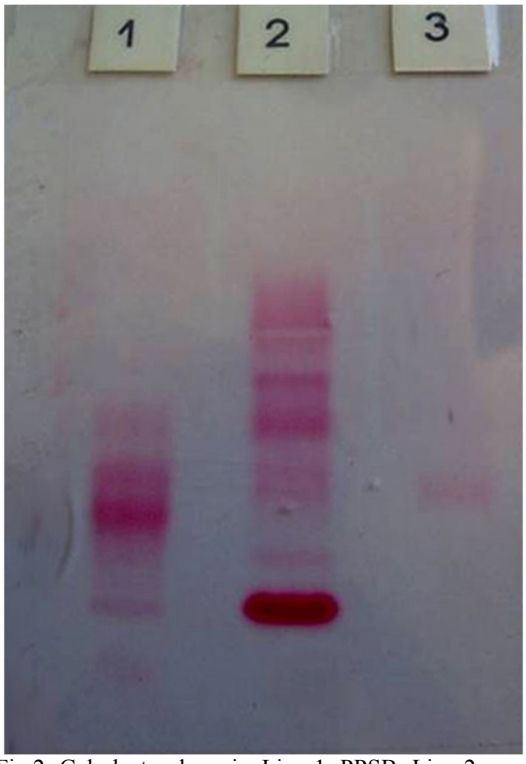

Fig.2. Gel electrophoresis. Line 1, PPSB; Line 2, normal plasma; Line 3 highly purified FVII concentrate (fractions 48-50).

2) indicated that factor VII concentrate contained approximately 89% alpha-2 proteins, providing evidence of its improved purity as compared with PPSB which contains three major bands of alpha-1, alpha-2 and beta proteins. These findings revealed that factor IX migrated as alpha-1 and factor VII as alpha-2 proteins.

Discussion

Apart from human plasma fractionation (20-26), the affinity chromatography is usually used as one of the final steps in the purification process of proteins. It combines advantages of time-saving and high capacity of selection and concentration of the target protein from a complex mixture of contaminating substances in a large sample volume (27). In this study we demonstrated that factor VII concentrate, essentially free of factors II, IX and X, can be further purified from prothrombin complex using immunoaffinity chromatography; and a virus inactivation step of solvent-detergent treatment could also be included. 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 62%. Activity of activated factor VII (FVIIa) in our purified concentrate and PPSB were 250% and 555% respectively. It indicates that factor VII concentrate similar to recombinant activated factor VII, may be used for treatment or prevention from 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 viral safety of the product (28-36). The potential ability of chromatography for eliminating viruses has been reviewed and up to more than 3-8 $\log_{10}$ removal of various viruses used as models during some chromatographic steps (37-39). On the other hand, in this method lower pH: 2.5 conditions within the elution buffer may lead to further viral inactivation (40). It seems that application of both solvent-detergent method and immunoaffinity chromatography can increase viral safety efficiently in comparison with other purification methods.

It may be concluded that CNBr-activated Sepharose 4B immuonoaffinity chromatography can be a suitable choice for large-scale production of factor VII concentrate with higher purity, safety tolerability, activated factor VII and may be used for treatment or prevention of bleeding in patients with factor IX inhibitors.

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