SEPARATION OF ANTIHEMOPHILIC FACTOR VII FROM HUMAN PLASMA BY COLUMN CHROMATOGRAPHY

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KEY WORDS

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ABSTRACT

TMAE-Fractogel 650 (M) (Trimethylaminoethyl) is an ion exchange medium can be used to capture factor VIII (F VIII) directly from plasma. Previous reports have focused on the use of DEAE-Fractogel 650 (M) (Dimethylaminoethyl) ion exchange medium to capture F VIII from cryoprecipitate and plasma. Our main objectives were (I) to standardise the purification of FVIII from human plasma by column chromatographic technique. (II) to study the recovery of FVIII activity in purified fraction at 18 - 20°C process condition. (III) to study the effect of virucidal step on recovery of FVIII activity and (IV) to study the effect of lyophilisation on FVIII activity. In this report, Citrate Phosphate Dextrose (CPD) plasma was batch stirred with dry DEAE-Sephadex A50, filtered, diluted, loaded on to a column packed with TMAE-Fractogel and chromatographed. Most of the unwanted proteins flowed through the gel unadsorbed. Bound F VIII was eluted by increasing the ionic strength of the buffer. This purification step gave an overall 80% recovery from the plasma with a specific activity of 0.97 IU FVIII/mg protein. The purified F VIII fraction was made virus safe by employing the virucidal technique developed by New York Blood Centre (NYBC). There was 48.43% loss of FVIII activity in Virus inactivation treatment and the loss of FVIII activity in lyophilisation was 8.45% which is acceptable. This method of purification gave a higher yield of FVIII than cryoprecipitation, and is a promising alternative method to cryoprecipitation of F VIII.

INTRODUCTION

Blood coagulation is a sequential process of chemical reactions involving plasma proteins, phospholipids and calcium ions. Absence of any of the coagulation factor leads to bleeding disorders. Blood coagulation factors are designated by Roman numerals. The FVIII is one of the blood coagulation factor deficient in "Haemophilia A". This occurs due to defective synthesis of coagulation FVIII. Bleeding problem in Haemophilia A patients are managed by plasma, cryoprecipitate or factor concentrate. Factor VIII rich source prepared by cryoprecipitation of fresh frozen plasma is used to replace FVIII to control bleeding.

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As cryoprecipitate preparation is the major source of FVIII, it is still carried out by the original method of Pool and Shannon (1). Although the production of cryoprecipitate is a simple procedure, it has two major disadvantages, the recovery of FVIII is low about 40% of that in normal plasma, and the purity is poor (2, 3). To overcome these disadvantages, the cryoprecipitate is further purified using various techniques such as adsorption (4), gel filtration (5) and ion exchange chromatography (6, 7, 8). The application of ion exchange chromatography for the isolation of FVIII from cryoprecipitate has been demonstrated in a number of reports using DEAE-Fractogel (Dimethylaminoethyl) and Q Sepharose Fast Flow resin (6, 7, 8). Our haemophilia patients have to depend on imported product of F VIII at enormous cost. Thus our aim was to purify FVIII and develop the indigenous technology for producing this factor. TMAE-Fractogel (Trimethylaminoethyl) was studied to assess its suitability for adsorption of factor VIII directly from plasma, and also the study

was carried out to use solvent detergent techniques developed by New York Blood Centre (NYBC) for virus inactivation using Tri-N-Butyl Phosphate (TNBP) and Triton X-100 as a solvent and detergent respectively. This paper presents the results of the investigation.

MATERIALS AND METHODS

Plasma

Whole blood (350-450ml) was collected from regular voluntary or replacement donors into anticoagulant Citrate Phosphate Dextrose (CPD) using triple blood bag (Baxter, Penpol, Terumo). The plasma was separated within 6 hrs of collection by using refrigerated centrifuge. The separated plasma was snap frozen called Fresh Frozen Plasma (FFP), stored at -30°C until ready for use. All subsequent experiments were carried out at 18-20°C unless otherwise stated.

F VIII Chromatography

Preparation of Column : TMAE - Fractogel (Merck, German), preserved in 20% ethanol packed in 11.5 X 100 mm column, (10 ml column from Affinity Chromatography Limited, UK) with flow rate 5 ml/min. One unit of FFP approximately 100 ml was thawed in a 37°C waterbath, pH was adjusted to 7.0 with IM HCl and treated with dry DEAE-Sephadex A50 gel (Pharmacia, Uppsala, Sweden) at 0.5g/l plasma and stirred for 30 minutes at room temperature to remove prothrombin complex (9). After 30 minutes the plasma was filtered through nylon net (40 µm mesh). The filtrate was diluted with an equal volume of pure sterile D/W and loaded at a linear flow rate of 60 cm/hr on to a column containing fractogel by a peristaltic pump (Electrolab, India). The column was pre-equilibrated with **buffer A** (10mM tri-Na-Citrate, 16mM lysine HCl, 1mM CaCl₂, 120mM glycine, 45mM NaCl, pH 7.0). The column was then washed with washing **buffer B** (10mM tri-Na-Citrate, 16mM lysine HCl, 1mM CaCl₂, 120 mM glycine, 80mM NaCl, pH 7.0), and eluted with same buffer containing, 250mM NaCl, pH 7.0 **buffer C** at 60 cm/hr flow rate.

The protein fractions eluting out were monitored by absorbance at 280nm by UV monitor (Pharmacia, Sweden) and the fractions eluted were recorded on recorder (REC-482, Pharmacia, Sweden). The column was sanitised by 0.5 M NaOH followed by 0.01 M NaOH.

Virus Inactivation

The eluted purified fractions of FVIII were treated for virus inactivation by solvent detergent treatment (10, 11). Calculated amount of TNBP and Triton X-100 (to give final concentration 0.3% & 1% respectively) were added in small quantity of pyrogen free distilled water, well stirred and the emulsified mixture was then added to FVIII fraction in a beaker kept at 4°C on magnetic stirrer to be stirred continuously for 4 hrs. The virucidal treated FVIII was reloaded at linear flow rate of 60 cm/hr on the same column to remove solvent and detergent, and eluted with the same conditions described above. The purified FVIII eluted in elution **buffer C** after Virus Inactivation was filled in USP type 1 glass vials (2 ml/vial). The vials were frozen at - 40°C in Freeze-Driver (Virtis model: SL 600, USA) overnight and lyophilisation was completed with primary and secondary drying.

Analytical Methods

Total protein was determined by Biuret method (12). FVIII activity was determined by one stage clotting assay (13), using reference plasma calibrated against the 17th British standard plasma for factor VIII (NIBSC Cat. No: 89/550). Purity of the factor VIII was analysed by gel permeation chromatography (GPC) on Superose 12HR column 10 mm X 300 mm using Fast Protein Liquid Chromatography (FPLC) system (Pharmacia, Sweden) as follows: 100 µl of the samples were automatically injected in the column previously equilibrated in phosphate buffer pH 7.2 at a flow rate of 0.4 ml/ minute, the same quantity of commercial FVIII concentrate with same procedure was analyzed by GPC. The Native Polyacrylamide gel electrophoresis (Native-PAGE) was performed using 8-25% gradient of gel on Phastsystem (Pharmacia, Sweden), the run conditions for electrophoresis were 200V, 10mA, at 15°C, for 268 volt hours. Standards of purified human IgG and albumin were run simultaneously with the sample.

RESULTS

Figure 1 shows the elution profile of TMAE-Fractogel chromatography. The washed fraction in washing buffer 'B' contains less than 5% of the total FVIII loaded, the majority of FVIII is eluted in the elution buffer 'C' fraction. Average FVIII activity in starting material i.e. FFP is 0.56 IU FVIII/ml (n=10) with a specific activity of 0.0096 IU FVIII/mg protein (Table 1).

First purification step shows average 80% FVIII recovery (n=10) with a specific activity of 0.97 IU FVIII/mg protein before Virus Inactivation. The specific activity has increased by 100 fold compared to starting plasma (Table 1).

Figure 2 shows the elution profile after Virus Inactivation, the solvent and detergent is eluted as a flow through fraction and pure FVIII is eluted in same elution buffer 'C' containing 250 mM NaCl. There is significant loss of FVIII activity in virus inactivation step, which is almost 48% with a recovery of 51.57% (Table 1).

The samples before and after lyophilisation were preserved and analysed for FVIII activity. About 8.45% loss of FVIII activity was seen (n=5) after freeze-drying (Table 2). The step by step average FVIII activity recovery is given in table 3.

DISCUSSION

TMAE-Fractogel is a tentacle type strong anion exchanger, copolymers of oligoethyleneglycol dimethacrylate, glycidemethacrylate and pentaerythritoldimethacrylate with a hydrophilic surface. TMAE-Fractogel provides the highest binding capacity independent of the sample pH as compared to DEAE-Fractogel. DEAE-Fractogel is a tentacle type weak anion exchanger which has previously been used in the purification of FVIII and vWF (6,8,14). However, in two cases the investigators used cryoprecipitate as a source material (6,14) Tech *et al.* (8) used DEAE-Fractogel ion exchange chromatography for purification of FVIII from plasma, but they have not studied the effect of Virus Inactivation and lyophilisation on the activity of FVIII. This work is a modification of the method of Tech *et al.* (8) using TMAE-Fractogel instead of DEAE-Fractogel. This study reveals the direct capture of FVIII from plasma using TMAE-Fractogel instead of DEAE-Fractogel, but we have observed that the FVIII recovery is the same as using DEAE-Fractogel. A procedure to capture FVIII directly from plasma with a high recovery is highly desirable as it would eliminate the disadvantages of cryoprecipitate such as low yield and poor purity.

The result from figure 1 show that FVIII can be readily and directly adsorbed from plasma with TMAE-Fractogel. The adsorbed FVIII can be eluted from the gel using buffer containing sodium chloride.

Despite progress in donor selection and blood screening methods, infectious viruses can still be

transmitted by single donor blood products and large pool plasma derivatives. Genomic Amplification Techniques (GAT) testing of plasma pool is currently available. The beneficial effect of GAT testing can be seen in the reduction in the risk of window-period donation for hepatitis C and HIV (15). Viral safety provided the impetus for two significant changes in the products used to treat haemophilia: (i) Techniques were developed to inactivate or remove viruses during the manufacture of human plasma-derived coagulation factors and other proteins and (ii) Alternative source of human plasma-derived protein were developed by means of recombinant DNA technology (16). In last 10-15 years manufacturers of blood products have improved fractionation methods, allowing greater removal of viruses during purification, they have also developed virucidal methods compatible with the preservation of the biological activity of such labile proteins as coagulation factors. The solvent detergent technology is widely accepted all over the world, It provides high virucidal activity without apparent protein denaturation for a wide range of proteins (17) and its clinical safety is well documented (18).

If the plasma is to be used for separation of other plasma proteins after Virus Inactivation, the Virus Inactivation treatment to plasma is not recommended by NYBC (10,11). We have given the Virus Inactivation treatment after purification of FVIII, so that the FVIII deficient plasma can be used for other plasma proteins separation like albumin, IgG. The recovery of FVIII after virucidal treatment is reported to be 80-88% (19,20). Present study shows 51.57% recovery which is less by 28.43 - 36.43% as compared to the available data. In this study the virus inactivation treatment was given on purified fraction of FVII and this may be the possible reason for more loss, because from all the data available, the virus inactivation treatment was given at cryoprecipitate level and cryoprecipitate is used as a source material for FVIII purification (20).

The samples before and after lyophilisation indicated about 8.45% loss of FVIII activity which is acceptable. The data available from other investigators show an average 8.0% loss of FVIII activity in lyophilisation (21,22).

FPLC Analysis

Human albumin was added as a stabiliser in the purified FVIII. The chromatogram of gel permeation chromatography on Superose 12HR column for

purified FVIII shows only two peaks, first one is of FVIII and second one is of human albumin which is added as a stabiliser (figure 4), and the chromatogram of gel permeation chromatography on Superose 12HR column for commercial FVIII shows three peaks, first FVIII, second human albumin and third extra peak of contaminant protein (figure 3). It is concluded that, the purified FVIII is more pure than commercial plasma derived factor VIII from Koate-HP, Miles Inc, USA

Native-PAGE Electrophoresis

The electrophoresis was performed using gradient gel 8-25% Native-PAGE on PhastSystem. The result indicate that, the purified FVIII is more pure as

compared to imported commercial plasma derived FVIII, (Figure 5).

This laboratory study reveals, that, virus treated FVIII concentrate production can be easily adopted for pilot or large scale separation. The yield and purity of FVIII is on par with those reported by other investigators.

If this laboratory scale study on purification of FVIII is adopted for regular production of FVIII concentrate, the purified FVIII can be available at very low cost. Thus this indigenously manufactured factor VIII concentrate can be available for management of bleeding problem in Haemophilia A patients in India to begin with.

TABLE I. STEP BY STEP AVERAGE FVIII RECOVERY (N* = 10)

Steps of purification	Volume (ml)	FVIII activity IU/ml	Total FVIII Units	Protein mg/ml	FVIII specific activity IU/ml	% Recovery
Starting plasma	98.5 ± 3.17	0.56 ± 0.13	55.16 ± 13.62	58.1 ± 2.75	0.0096 ± 0.002	100
Purified FVIII	50.5	0.88	44.44	0.905	0.97	80.56
Before Virus Inactivation	± 7.99	± 0.123	± 10.43	± 0.17	± 0.21	± 9.46
Purified FVIII	31.4	0.73	22.92	0.64	1.14	51.57
After Virus Inactivation	± 4.27	± 0.61	± 4.74	± 0.10	± 0.12	5.42

n* = No. of experiments

Table 2. Effect of lyophilisation on F VIII activity (n* = 5)

Factor VIII activity before Freeze-drying IU/ml	Factor VIII activity after Freeze-drying IU/ml	% Recovery	% Loss
0.71	0.65	91.55	8.45
SD $\pm$ 0.06	SD $\pm$ 0.08	SD $\pm$ 2.01	SD $\pm$ 2.03

Table 3. Average F VIII recovery step by step (n* = 10)

Steps	Total FVIII Units	% Recovery	% Loss
Starting Plasma	55.16	100	—
Purified F VIII (Before Virus Inactivation)	44.44	80.56	19.44
Purified F VIII (After Virus Inactivation)	22.92	51.57	48.43

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Column Chromatographic separation of FVIII

Fig.1 Elution profile of TMAE -Fractogel before Virus Inactivation

Fig.2 Elution profile of TMAE -Fractogel after Virus Inactivation

Fig.3 FPLC analysis of commercial FVIII on GPC column

Fig.4 FPLC analysis of purified FVIII on GPC column

Fig.5 Native -PAGE electrophoresis

- Lane 1:- Pooled Plasma
- Lane 2:- Reference standard factor VIII
- Lane 3:- Commercial factor VIII concentrate
- Lane 4:- Purified factor VIII without human albumin as stabilizer
- Lane 5:- Purified factor VIII with human albumin as stabilizer
- Lane 6:- Virus inactivated purified factor VIII without albumin
- Lane 7:- Human albumin standard
- Lane 8:- Human IgG+ albumin standard

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