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ISOLATION OF HUMAN FACTOR VIII:C BY PREPARATIVE HIGH-PERFORMANCE SIZE-EXCLUSION CHROMATOGRAPHY

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SUMMARY

Human Factor VIII procoagulant protein (VIII:C) is a plasma protein that participates in the cascade of events leading to blood coagulation. It is absent or defective in patients with hemophilia A. *In vivo* Factor VIII:C associates with Von Willibrand factor and its multimers to form a high-molecular-weight particle that can be dissociated into a lower-molecular-weight form in the presence of high concentrations of salt. We have been able to purify rapidly Factor VIII:C on a large scale by sequential high-performance size-exclusion chromatography (HPSEC) under conditions of first low salt and then high salt concentration. Reconstituted commercial Factor VIII:C concentrate was purified by chromatography on a preparative HPSEC column (Toyo Soda, 60 × 2.5 cm, 300 ml) in 0.05 *M* imidazole buffer, (pH 7.0), containing 0.15 *M* sodium chloride. Factor VIII:C activity was eluted in the void volume in less than 20 min as a high-molecular-weight particle, well separated from low-molecular-weight contaminants. Purification was 20-fold, with a yield of 80%. Up to 4 g of Factor VIII concentrate could be purified at one time in this manner. This material was then concentrated and made 0.35 *M* in calcium chloride prior to re-chromatography on the same column in a buffer containing 0.30 *M* calcium chloride. Under these conditions, Factor VIII:C activity was eluted in the inner volume of the column at a position corresponding to a molecular weight of several hundred thousand in less than 1 h. It was well separated from both larger proteins and smaller peptide fragments. Analysis of the preparation with radiolabelled antibody to human Factor VIII:C antigen indicated that at least two molecular weight forms of Factor VIII:C were present.

INTRODUCTION

For several decades, there has been considerable interest in the human plasma protein Factor VIII because of its commercial value in the treatment of hemophilia A. Circulating Factor VIII is known to be a large complex, composed of two different proteins[1], a carrier protein (Von Willibrand factor) and the procoagulant protein (Factor VIII:C), which are linked together by non-covalent bonds. Hemophilia A

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patients lack functional Factor VIII:C and require infusion of the normal protein in order to overcome bleeding episodes. Several companies currently manufacture a concentrated Factor VIII product, used in treatment of hemophilia A patients, which is produced by fractionation of plasma from healthy donors. These preparations contain some proteases, fibrinogen, and other contaminants, which severely limit product stability in solution. Also, the small percentage of Factor VIII in these crude preparations requires that large volumes of the product be infused into the patient for optimal results. Therefore, an efficient method for further commercial purification of this material would be desirable.

Several methods have been described previously[2,3] for the purification of small amounts of Factor VIII:C from commercial concentrate. We describe here a high-performance size-exclusion chromatographic (HPSEC) method for large-scale one day partial purification of Factor VIII:C from commercial Factor VIII concentrate, which is amenable to further scale-up. This method may have application as a final step in the preparation of a commercial Factor VIII product or as an initial step in the preparation of a homogenous Factor VIII:C, to be used for structural studies.

EXPERIMENTAL

Apparatus and materials

The liquid chromatograph consisted of a Model 6000A pump, a Type UK6 injector containing a 10-ml sample loop, and a Model 450 variable-wavelength detector, all products of Waters Assoc. (Milford, MA, U.S.A.). The semi-preparative TSK 5000PW column (60 × 2.5 cm, 300 ml, bead size 17 μm) and TSK 6000PW guard column (7.5 × 2.5 cm, 37 ml) were from Toyo Soda (Tokyo, Japan). Buffers were made with HPLC-grade water, obtained from J. T. Baker (Phillipsburg, NJ, U.S.A.). All other chemicals were of reagent or analytical grade. Human ^{125}I-labelled anti-VIII:C Fab (antigen binding fragment) was prepared as described[4] and was kindly donated by Dr. Mark Weinstein (Boston City Hospital, Boston, MA, U.S.A.).

Protein determination

Protein concentration was measured either by fluorometric assay[5] or by the method of Bradford[6] with bovine serum albumin (fraction V, Sigma) as a standard.

Coagulation assays

Factor VIII:C activity was measured either by a two stage thromboplastin generation test (TGT) time procedure[7] or by a one-stage activated thromboplastin time test procedure[7,8]. In both procedures, Factor VIII-deficient plasma, obtained either directly from hemophilia A patients or purchased from George King Biomedical (Overland Park, KS, U.S.A.) was used as substrate. All VIII:C assays were performed with a Factor VIII:C concentrate house standard, calibrated against the standard for blood coagulation Factor VIII Human (80/556), obtained from the National Institute for Biological Standards and Control (London, U.K.). Duplicate dilutions at 1/100, 1/200, 1/400, and 1/800 were tested for each sample (except for column fractions) and the standard. In some cases Factor VIII:C activity in column fractions was determined colorimetrically by using the Kabi Vitrum COATEST assay kit (Kabi Vitrum, Stockholm, Sweden). This test determines Factor VIII:C activity on the basis

of its ability to convert Factor X to Factor Xa. It was found to yield results identical to those obtained by the coagulation assays.

Purification of Factor VIII:C

Up to ten bottles of commercial Factor VIII:C concentrate (Profilate, Alpha Therapeutic Corp., Los Angeles, CA, U.S.A.) were each reconstituted in 3 ml of water and pooled. Buffer exchange of the pooled material was performed by application of the pooled sample to a Sephadex G-25 (Pharmacia) column and elution with buffer A (50 m*M* imidazole · HCl (pH 7.0), 150 m*M* sodium chloride, 0.02% (w/v) sodium azide). All detectable protein (as measured by absorbance at 280 nm) was eluted in the void volume fractions, which were subsequently pooled. Up to 10 ml of G-25-filtered Factor VIII concentrate was injected into a preparative Toyo Soda TSK 5000PW column connected to a TSK 6000PW guard column and eluted with buffer A at a flow-rate of 8.5 ml/min. Fractions of 0.5-min duration were collected and analyzed for absorbance at 254 nm in a Gilford (Oberlin, OH, U.S.A.) Model 2600 spectrophotometer. Aliquots from each fraction were assayed for Factor VIII:C activity. Fractions with procoagulant activity were pooled and concentrated by dialysis against solid Aquacide II (Boehringer-Calbiochem, San Diego, CA, U.S.A.). The concentrated protein sample was then made 0.35 *M* in calcium chloride by adding 1/9th volume of a solution containing 3.5 *M* calcium chloride in Buffer A. This material was then re-injected into the columns described above and eluted with buffer A containing 0.30 *M* calcium chloride, at a flow-rate of 3–5 ml/min. Protein was monitored continuously at 280 nm, and 1-min fractions were collected into silanized glassware. Aliquots from each fraction were diluted at least 8-fold in a buffer containing 0.05 *M* imidazole (pH 7), 0.02% (w/v) sodium azide prior to being assayed for Factor VIII:C activity. Active fractions were pooled and concentrated by vacuum dialysis against buffer A or buffer A containing 25% glycerol.

Gel electrophoretic analysis

Factor VIII:C was analyzed by gel electrophoresis, essentially as described by Moake *et al.*[4]. A 25-μl volume of partially purified Factor VIII:C was incubated with 27 μl of ^{125}I-labelled anti-Factor VIII:C Fab (13 000 total cpm), containing 5 m*M* diisopropyl fluorophosphate (Aldrich, Milwaukee, WI, U.S.A.), 95 NIH (National Institutes of Health) U/ml hirudin, 367 kallikrein inhibitor U/ml aprotinin (Trasylol, FBA Pharmaceuticals, New York, NY, U.S.A.), and 3.2% polyethylene glycol (average mol. wt. 4000). After incubation for 90 min at 37°C and then for 30 min at 0°C, the reaction mixture was warmed to room temperature, and 52 μl 0.18 *M* Tris–HCl (pH 6.8), containing 6% (w/v) sodium dodecyl sulfate (SDS), 15% (v/v) 2-mercaptoethanol and 30% (v/v) glycerol, was added, followed by incubation at 37°C with gentle agitation. After 30 min, the entire mixture was analyzed by electrophoresis on 5% polyacrylamide slab gels, containing 0.1% SDS, according to the procedure of Laemmli[9]. Protein molecular weight markers were electrophoresed in lanes of the slab gel adjacent to the lane containing the reaction mixture. After electrophoresis, the portion of the gel slab containing the molecular weight markers was removed and stained with a solution, 2-propanol–glacial acetic acid–water (25:10:65, v/v/v) containing 0.15% (w/v) Coomassie Blue, for 2 h and then destained in methanol–glacial acetic acid–water (5:10:85, v/v/v). The relative mobility of indiv-

idual markers in the gel was then determined. The portion of the gel slab containing the reaction mixture was fixed in 2-propanol–glacial acetic acid–water (25:10:65, v/v/v) for 2 h, then soaked in a solution, of ethanol–glacial acetic acid–water–glycerol (70:10:15:5, v/v), for 45 min, dried, and placed in contact with XAWR-5 X-Omat film (Kodak, Rochester, NY, U.S.A.) and Kodak X-Omatic Regular intensifying screens for 5–7 days at −70°C. The relative mobilities of bands visible in the autoradiograph were determined and compared to those of the protein molecular weight markers.

RESULTS AND DISCUSSION

Factor VIII:C activity was separated from low-molecular-weight contaminants present in commercial Factor VIII preparations by HPSEC on a semipreparative Toyo Soda TSK 5000PW column in a low-ionic-strength buffer (Fig. 1). Under these conditions Factor VIII:C is known to be associated with Von Willibrand Factor and its multimers as a high-molecular-weight complex[2,10,11]. The Factor VIII:C activity was found to be eluted at the exclusion limit of the column at a molecular weight in excess of $2 \cdot 10^6$ (Fig. 1). The entire separation took place in less than 25

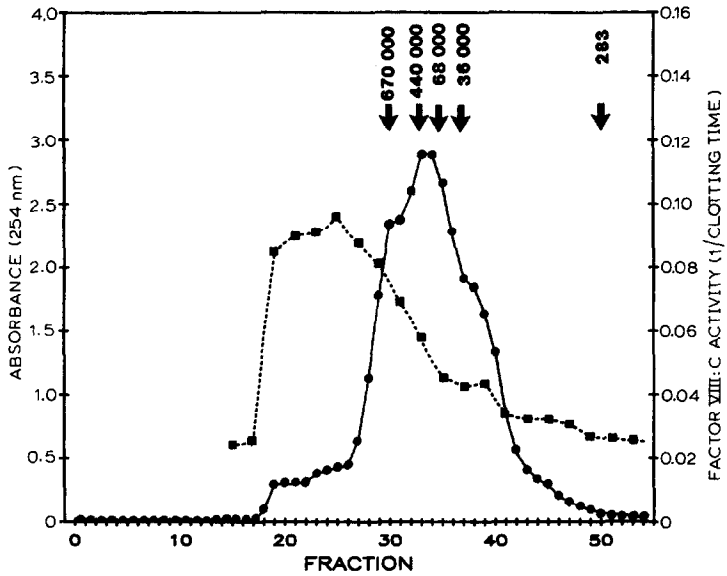

Fig. 1. Purification of Factor VIII:C concentrate by HPSEC. Approximately 1.5 g of commercial Factor VIII concentrate (2 vials Profilate), which had been passed through a Sephadex G-25 coarse column, was injected into a Toyo Soda TSK 5000PW column (60 × 2.5 cm), connected to a Toyo Soda TSK 6000PW guard column (7.5 × 2.5 cm) and eluted with buffer A at a flow-rate of 8.5 ml/min. Individual fractions were collected at 0.5-min intervals and the absorbance (●—●) of each fraction was determined spectrophotometrically at 254 nm. An aliquot from each fraction eluted after the void volume was diluted 400-fold and the length of time (seconds) required for clot formation in the thromboplastin generation test procedure at this dilution was determined. Activity (■---■) for each fraction is represented as the reciprocal of the clotting time, since clotting time for this assay is linearly related to Factor VIII:C activity in an inverse manner. Arrows indicate positions at which protein standards of the indicated molecular weight are eluted from the columns under identical conditions. Protein standards injected were thyroglobulin (mol.wt. 670 000), ferritin (440 000), bovine serum albumin (68 000), β-lactoglobulin (36 000) and guanosine (283).

TABLE I

PURIFICATION OF COMMERCIAL FACTOR VIII CONCENTRATE BY HPSEC

All values represent the mean of seven preparations.

	Volume (ml)	*Total protein (mg)*	*Total units*	*Specific activity (units/mg)*	*Degree of purification (fold)*	*Yield (%)*
Factor VIII concentrate (G-25 filtered)	26	965	726	0.75	1	100
TSK 5000 (no calcium chloride)	68*	37	571	15.0	20	76

* Volume at this step ranged from 6 ml after concentration to 275 ml before concentration.

min, and about 80% of the Factor VIII:C activity applied to the column could be recovered in the pool of the eluted active fractions (Table I). The purification by this step was 20-fold (Table I). This material was susceptible to thrombin activation. However, we have observed as much as a 45% decrease in activity upon subsequent concentration of the pool by either polyethylene glycol (average mol.wt. 4000) precipitation or dialysis against solid Aquacide II.

Up to 4 g of commercial Factor VIII concentrate could be chromatographed on this column at one time. However, a maximum volume of only 10 ml was injected at any one time, so that the actual amount of protein sample applied to the column was limited by its concentration. In some cases, several consecutive HPSEC separations were required to purify the desired amount of material. Filtration of the Factor VIII concentrate prior to high-performance liquid chromatography (HPLC) is recommended. We found less than 10% loss in the activity of Factor VIII concentrate after chromatography on a Sephadex G-25 coarse column.

To determine the molecular weight forms of procoagulant VIII:C present in the HPSEC preparation, this material was incubated with human ^{125}I-labelled anti-VIII:C Fab and analyzed by SDS-polyacrylamide gel electrophoresis in slab gels containing 5% polyacrylamide and autoradiography (Fig. 2). The largest and predominant VIII:C Ag + ^{125}I-labelled anti-VIII:C Fab complex was a broad band with upper and lower molecular weight limits of $3.4 \cdot 10^5$ and $2.7 \cdot 10^5$, respectively (Fig. 2A). These amounted to upper and lower molecular weight limits of $2.9 \cdot 10^5$ and $2.2 \cdot 10^5$ after subtracting the $0.5 \cdot 10^5$ molecular weight of the ^{125}I-labelled anti-VIII:C Fab. This molecular weight (approximately $2.6 \cdot 10^5$) is similar to that reported by others[2,3] as the molecular weight of intact procoagulant VIII:C. It is the molecular weight form of VIII:C that predominates in fresh plasma but which is diminished after activation of the molecule by poroteolytic cleavage. A lower-molecular-weight form of VIII:C is also evident in the autoradiogram. It has a calculated molecular weight of $1.1 \cdot 10^5$. A form of VIII:C with this molecular weight has been reported[3] and is believed to be derived from the $2.6 \cdot 10^5$ molecular weight form by proteolytic cleavage. Additional forms of VIII:C with molecular weights of less than $2.6 \cdot 10^5$ are known to exist[2,3,12] but were not observed in the autoradiogram. This suggests that they were not present in the high-molecular-weight-complex isolated by HPSEC or were not recognized by the ^{125}I-labelled anti-VIII:C Fab.

Complexed Factor VIII:C can be dissociated from Von Willibrand Factor in

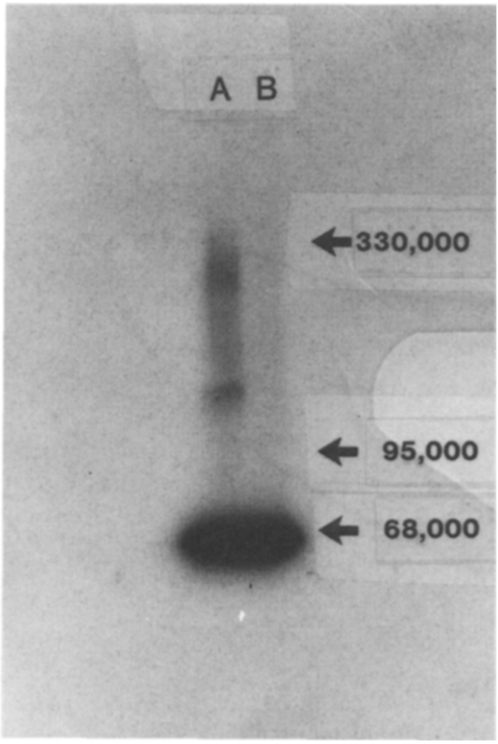

Fig. 2. SDS-polyacrylamide gel electrophoresis autoradiogram of ^{125}I-labelled Fab-VIII:C Ag in partially purified Factor VIII:C. Either (A) 25 μl of Factor VIII:C, purified by HPSEC, as described in Fig. 1, or 25 μl of (B) buffer A were incubated with ^{125}I-labelled anti-VIII:C Fab, and analyzed by SDS-polyacrylamide gel electrophoresis in 1.5-mm slab gels containing 5% polyacrylamide as described in Experimental. The corresponding positions in the gel of molecular weight markers thyroglobulin (subunit mol.wt. 330 000), phosphorylase *a* (95 000), and bovine serum albumin (68 000) are indicated by arrows.

the presence of high concentrations of calcium chloride[2,10,12]. We examined the effect of calcium chloride on Factor VIII:C, purified as described above. When this material was incubated with 0.35 *M* calcium chloride and rechromatographed on the semi-preparative TSK 5000PW column in the presence of 0.3 *M* calcium chloride, VIII:C activity was no longer eluted in the void volume, but instead it was eluted as a broad peak with a much lower apparent molecular weight, indicating that the factor had been dissociated from the high-molecular-weight compolex (Fig. 3). The peak of dissociated Factor VIII:C activity corresponded to an approximate molecular weight of between 100 000 and 300 000 and is probably composed of the $2.6 \cdot 10^5$ molecular weight form of the molecule (which was identified by ^{125}I-labelled Fab–SDS-polyacrylamide gel electrophoresis) and some of its cleavage products. When HPSEC in 0.3 *M* calcium chloride was used to separate VIII:C from the high-molecular-weight material isolated by HPSEC under low-ionic-strength conditions, a 2- to 3-fold increase in purity was generally observed. The highest specific activity we have observed after this step was 37 U/mg protein. However, only a 30% recovery of the applied activity was observed for this step (data not shown). While the recoveries for this

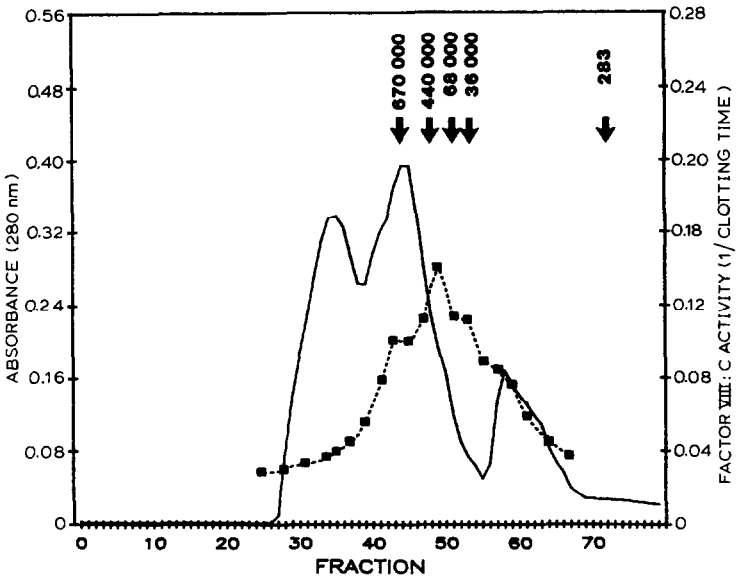

Fig. 3. HPSEC of Factor VIII:C in 0.3 *M* calcium chloride. Aproximately 20 mg of Factor VIII:C, which had been purified as shown in Fig. 1 and then concentrated and conditioned in 0.35 *M* calcium chloride, was chromatographed on the HPSEC columns described in Fig. 1 by elution was with buffer A, containing 0.3 *M* calcium chloride, at a flow-rate of 3 ml/min. The absorbance was monitored continually at 280 nm with a Waters Model 450 spectrophotometer. Each fraction was collected for 1 min and all fractions eluted after the void volume were diluted 20-fold in a buffer, containing 0.05 *M* imidazole, (pH 7) and 0.02% (w/v) sodium azide, and then assayed for Factor VIII:C activity, as described in the legend to Fig. 1. Arrows indicate positions at which protein standards of the indicated molecular weight are eluted from the columns under identical conditions. Protein standards as in Fig. 1. No dissociation of oligomeric protein molecular weight markers under these conditions was observed.

step are poor, we have found that isolation of dissociated VIII:C is generally required if further HPLC analysis is desired. The high-molecular-weight complex containing Factor VIII:C will not pass through analytical HPLC columns packed with particles of diameters of 10 μm or less, probably because the interstitial spacing between these particles is too small.

Fay *et al.*[2] have described a purification procedure for Factor VIII:C from commercial concentrate in which the first step is conventional size-exclusion chromatography on Bio-Gel A-15 in a low-ionic-strength buffer. The yield and extent of purification for this step is similar to what is reported here for HPLC of Factor VIII concentrate on a TSK 5000PW column. However, the HPLC step resolves similar amounts of concentrate in a much shorter time. One can easily fractionate 40 000 units of commercial Factor VIII concentrate (*ca.* 40 g) per day on the semi-preparative HPLC column described here. Larger TSK columns (preparative and pilot-plant-scale) and pumping systems are capable of separating considerably more Factor VIII concentrate in even less time. Conceivably, the dissociated form of Factor VIII could also be isolated on the same day using the above procedure. This should be of great value to the plasma fractionation industry and to researchers interested in the ultimate purification of large quantities of Factor VIII:C to homogeneity for use in structure–function studies.

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