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SEPARATION OF HUMAN FACTOR X FROM FACTOR Xa BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Human factor X is the vitamin K-dependent proenzyme of a plasma serine protease that participates in the cascade of events leading to blood coagulation. It is converted to its active form, factor Xa, after specific cleavage by other plasma proteases or the protease from Russel's Viper venom. We have separated Factor X from factor Xa by reversed-phase high-performance liquid chromatography using an increasing gradient of acetonitrile in 0.1% trifluoroacetic acid. The factor X and factor Xa activities were well separated from each other on a wide-pore diphenyl column (Whatman Protesil 300) in less than 30 min. Both factor X and factor Xa activities were found to be essentially unaffected by the solvent system. This system was used to evaluate the purity of several factor X and factor Xa preparations. The kinetics of the Russel's Viper venom catalyzed conversion of factor X to factor Xa was also studied by using this chromatography system. A time-dependent decrease in the protein peak corresponding to factor X and a corresponding increase in the factor Xa protein peak was observed upon incubation with Russel's Viper venom.

INTRODUCTION

Factor X acts in a concerted manner with blood platelets and other plasma coagulation factors to clot blood at the site of vascular injury. Activated factor X (factor Xa), activated factor V, and Ca^{2+} ions are believed to assemble on the platelet surface to form a complex, which converts prothrombin to thrombin[1,2]. Thrombin catalyzes the conversion of fibrinogen to fibrin which then polymerizes to help form the clot.

The conversion of factor X to factor Xa is catalyzed by activated factor IX and activated factor VIII. Most patients with severe bleeding disorders have been found to lack either functional factor VIII (hemophilia A) or factor IX (hemophilia B). The treatment of these patients normally involves replacement therapy with concentrated preparations of the missing factor. In a relatively small number of cases, bleeding disorders have been found to be associated with the lack of functional factor X. In these situations, the availability of a safe, well defined preparation of factor X would be highly desirable.

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Several workers have described the isolation of human factor X from plasma and its subsequent conversion to factor Xa[3–5]. Factor Xa was found to be exceedingly more active than its zymogen form in promoting blood coagulation. Any therapeutic preparation of factor X should therefore be well characterized and contain no factor Xa, which could potentially cause severe thrombotic complications. We describe here a high-performance liquid chromatography (HPLC) method which is capable of detecting factor X and factor Xa and of rapidly separating them from each other in various factor preparations.

EXPERIMENTAL

Apparatus and materials

The liquid chromatograph consisted of either Model 6000 A pumps (Waters Assoc., Milford, MA, U.S.A.) or Model 100 A pumps (Beckman Instruments, Fullerton, CA, U.S.A.), a Beckman Model 210 injector and a Waters Model 450 Variable-wavelength detector. The Protesil 300 diphenyl column (250 × 4.6 mm I.D., 10 μm) was obtained from Whatman (Clifton, NJ, U.S.A.). Acetonitrile was a Burdick & Jackson (Muskegon, MI, U.S.A.) HPLC-grade solvent, and HPLC-grade water was obtained from J. T. Baker (Phillipsburg, NJ, U.S.A.). All other chemicals were of reagent or analytical grade.

Protein determination

The protein concentration was measured by the method of Bradford[6], using bovine serum albumin (fraction V, Sigma, St. Louis, MO, U.S.A.) as a standard.

Factor X and factor Xa assay

Human factor X was assayed as described by Denson[7]. In this procedure, samples were incubated with factor VII/factor X deficient plasma and Russel's Viper venom (RVV), purchased from Sigma, platelet substitute (Thrombofax) obtained from General Diagnostics (Morris Plains, NJ, U.S.A.) and calcium chloride. Clot formation was detected in either a Hyland Clotek or MLA Electra 750A clot detection system. The reference standards used for the factor X assays were frozen pools of 30 normal plasmas. Factor Xa assays were carried out in the absence of RVV.

Purification of factors X and Xa

Human heat-treated prothrombin complex concentrate (Profilnine®) was produced from plasma at Alpha Therapeutic Corporation by standard manufacturing techniques which included chromatography of cryoprecipitate supernatant plasma on a DEAE ion exchange resin. Prothrombin complex concentrate was precipitated by the addition of 1 *M* barium chloride, and the precipitate was collected by centrifugation, resuspended, and applied to a column containing dextran sulfate agarose[8]. The column was eluted with a linear salt gradient and those fractions containing the highest factor X activity were pooled, concentrated, and dialyzed by ultrafiltration. If necessary, the factor X pool was further purified by applying it to a second dextran sulfate agarose column and eluting as before. The factor X pool from this second dextran sulfate agarose chromatography step was also concentrated and dialyzed.

Factor Xa was prepared from purified factor X by incubation with either partially purified factor X-activating protein (from RVV), bound to Sepharose, or whole RVV, bound to Sepharose[6]. Between 0.5 and 1.0 mg of purified factor X in 2 ml of tris(hydroxymethyl)aminomethane (Tris) buffered saline (TBS) was incubated with 3 ml of Sepharose-bound RVV in the presence of 5 m*M* calcium chloride. The solution was mixed at 37°C for 30–60 min. Following activation, the Sepharose-bound RVV was removed by centrifugation.

Electrophoresis

Samples were analyzed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) on 11% acrylamide gels according to the method of Laemmli[9]. Factor X samples and protein standards were heated in a solution containing 2% (w/v) SDS, 5% (w/v) 2-mercaptoethanol, 0.05 *M* Tris–HCl (pH 6.8) and 10% (v/v) glycerol for 5 min at 95°C before electrophoresis. Immediately after electrophoresis the acrylamide gel slab was stained with a solution containing 25% (v/v) 2-propanol, 10% (v/v) glacial acetic acid, and 0.15% (w/v) Coomassie Blue for 2 h and then destained in 5% (v/v) methanol, 10% (v/v) glacial acetic acid.

RESULTS

Highly purified human factor X was prepared from plasma by DEAE ion-exchange chromatography, barium citrate precipitation, and dextran sulfate agarose chromatography. These preparations had specific activities of between 40 and 70 factor X units per mg. Analysis of the factor X matrial by SDS-PAGE in the presence

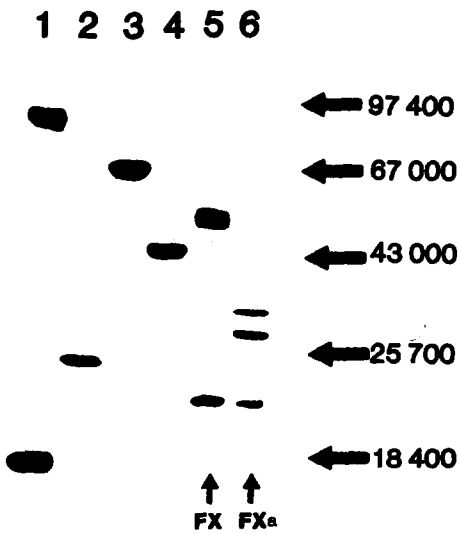

Fig. 1. Polyacrylamide gel electrophoresis of purified factor X and factor Xa. Approximately 10–20 μg of the following samples were loaded onto the gel: lane 1, phosphorylase *b* (97 kD) and β-lactoglobulin (18 kD); lane 2, chymotrypsinogen (25 kD); lane 3, bovine serum albumin (67 kD); lane 4, ovalbumin (43 kD); lane 5, factor X; lane 6, factor Xa.

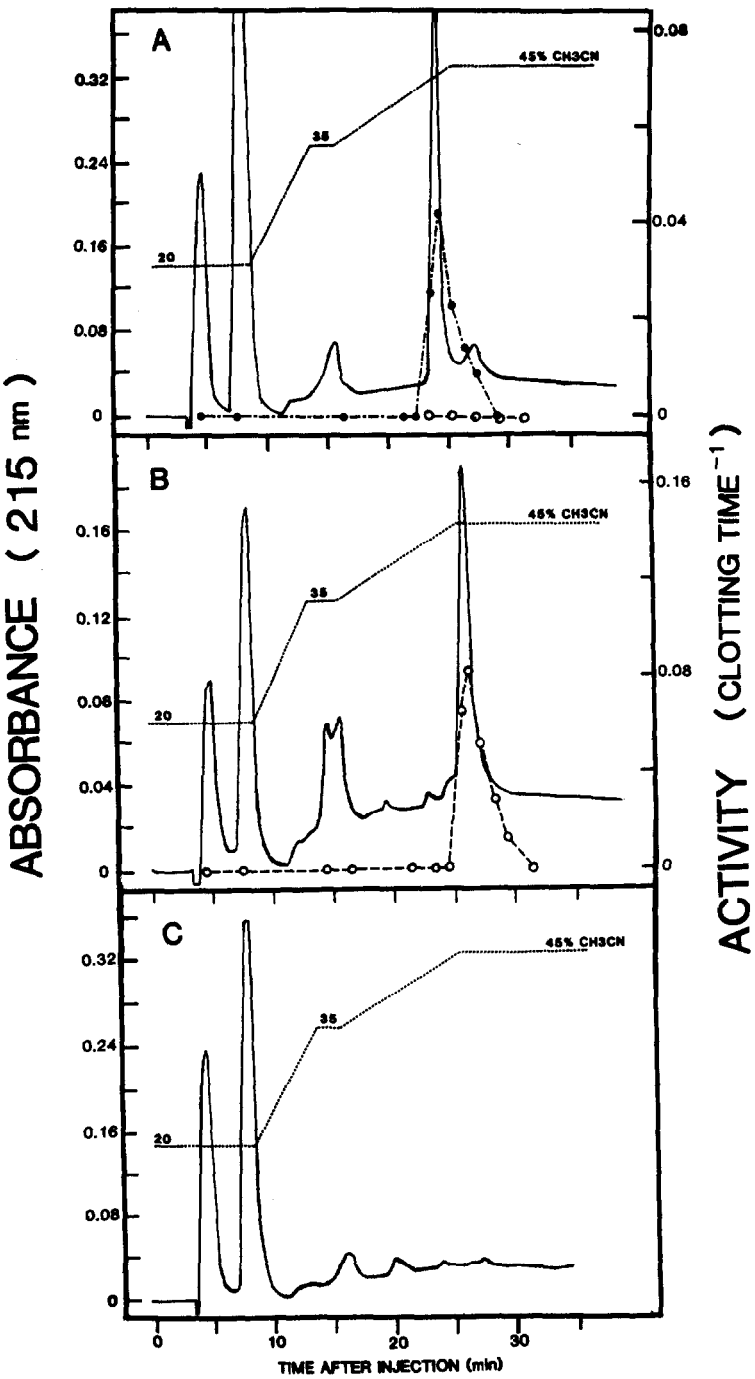

Fig. 2. HPLC separation of purified factor X and factor Xa on a wide-pore diphenyl column. A 1-ml volume of a eluent A [0.1% TFA in acetonitrile–water (1:4)] containing (A) *ca.* 50 μg of factor X, (B) 25 μg of factor Xa, or (C) solvent alone was applied to a Whatman Protesil 300 diphenyl column that had previously been equilibrated with eluent A. The flow-rate was 1 ml/min. The column was washed for 5 min with the same mobile phase, followed by a linear gradient (5 min) from 0 to 30% eluent B [0.1% TFA in acetonitrile–water (7:3)] and continued elution (2 min) with 30% eluent B. The column was then eluted with a linear gradient (10 min) from 30 to 50% eluent B. Absorbance was monitored continuously at 215 nm, using a Waters Model 450 spectrophotometer, and 1-ml fractions were collected, diluted 1:4 with TBS, and assayed for factor X (●----●) or factor Xa (○---○) activity.

of 2-mercaptoethanol (Fig. 1, lane 5) revealed two major polypeptides with apparent molecular weights of approximately 50 kilodalton (kD) and 20 kD.

After incubation of factor X with immobilized RVV, electrophoretic analysis revealed the complete absence of the 50 kD polypeptide, the presence of the 20 kD polypeptide, and the appearance of two new polypeptides with apparent molecular weights of 30 kD and 32 kD (Fig. 1, lane 6). This is consistant with the known activation scheme for factor X in which only the heavy chain of factor X, is cleaved to produce factor Xa. The 30 kD and 32 kD polypeptides most likely represent the α and β forms of the heavy chain of factor Xa[2]. The small activation peptides resulting from the cleavage of the heavy chain of factor X were not visible in this gel system.

The gel electrophoresis results suggest that purified factor X, prior to activation, contained no factor Xa. The RVV activation procedure was sufficient to convert factor X to factor Xa completely. Following activation, no factor X was present in the factor Xa preparations (as demonstrated by the lack of a 50 kD polypeptide in these preparations).

The purified preparations of human factor X and factor Xa were analyzed by reversed-phase HPLC on a Whatman Protesil 300 diphenyl column, as shown in Fig. 2. Analysis of both factor X (Fig. 2A) and factor Xa (Fig. 2B) revealed several UV-absorbing peaks. Two peaks, appearing immediately after sample injection, were observed in every analysis, including buffer alone (Fig. 2C), and are injection artifacts. A major UV-absorbing peak which eluted at 42% acetonitrile, *ca.* 24 min after injection of the sample, was observed in the factor X preparation (Fig. 2A). This peak coincided with a peak of factor X activity and contained no factor Xa activity. This peak was hardly visible when the factor Xa preparation was analyzed (Fig. 2B). Instead a major UV-absorbing peak which eluted at 45% acetonitrile, about 26 min after injection, was observed. This peak coincided with the peak of factor Xa activity.

The results of Fig. 2 demonstrate that factor X and factor Xa can easily be separated and identified by reversed-phase HPLC. The HPLC results were similar to those obtained by gel electrophoresis. Purified factor X, prior to activation, appeared to contain no factor Xa. Following activation, essentially no factor X was present in the factor Xa preparations (as demonstrated by the disappearance of the factor X

TABLE I

EFFECT OF HPLC SOLVENT SYSTEM ON THE ACTIVITY OF FACTOR X AND FACTOR Xa

Samples of purified factor X or factor Xa were diluted 80-fold with either a 0.1% TFA solution containing 45% acetonitrile (indicated by a "+" in the table) or TBS indicated by a "−" in the table) and incubated at room temperature for 5 min. Each sample was then diluted 1:3.5 with TBS and assayed for activity as described in Experimental.

Factor	*0.1% TFA 45% acetonitrile*	*Factor X (U/ml)*
1 X	−	42.3
2 X	+	49.5
3 Xa	−	11.1
4 Xa	+	7.2

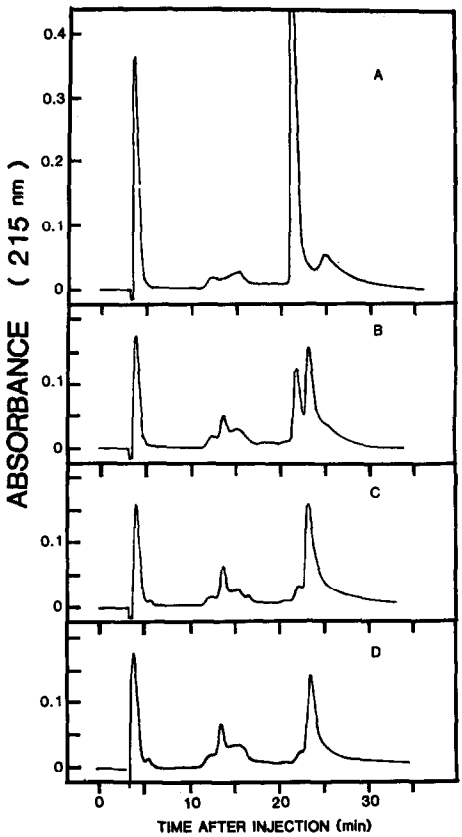

Fig. 3. RVV-catalyzed conversion of factor X to factor Xa. Purified factor X was mixed with Sepharose-bound RVV prior to analysis by reversed-phase HPLC. At various times, the mixture was centrifuged briefly (10 s), and 50 μl of supernatant, containing *ca.* 12.5 μg of partially activated factor X was removed. Any residual RVV activity in the samples was removed by the addition of 0.95 ml of eluent A [0.1% TFA in acetonitrile–water (1:4)]. The samples were then analyzed as described in Fig. 2. The incubation conditions were as follows: (A) factor X prior to incubation; (B) 3-min incubation with Sepharose bound-RVV; (C) 20-min incubation; (D) 60-min incubation.

peak in these preparations). The α and β forms of factor Xa could not be distinguished in this system.

The effect of the chromatographic solvent system on factor X and factor Xa activity was determined, and the results are presented in Table I. When factor X was incubated for 5 min with a solution containing 0.1% trifluoroacetic acid (TFA) and 45% acetonitrile and then assayed, no loss in activity was observed (Table I, lines 1 and 2). When factor Xa was incubated with a solution containing 0.1% TFA and 45% acetonitrile under the same conditions, a 35% loss in activity was observed (Table I, lines 3 and 4).

This would suggest that all of the factor X and most of the factor Xa activity could be recovered after reversed-phase HPLC.

The conversion of factor X to factor Xa by RVV was monitored over time by

reversed-phase HPLC, as shown in Fig. 3. Prior to incubation with RVV, almost no factor Xa was present in the factor X preparation (Fig. 3A). When factor X was incubated with RVV (in excess) for 3 min and the reaction was stopped, HPLC analysis indicated that 53% of the factor X present at the start of the incubation was converted to factor Xa (Fig. 3B). After a 20-min incubation with RVV, 85% of factor X was converted to factor Xa (Fig. 3C). No further change was observed when factor X was incubated for a total of 60 min with RVV (Fig. 3D), suggesting that a small portion of the factor X preparation was not capable of being activated.

The retention times described above for factors X and Xa were found to be consistent for the many different factor X and Xa preparations which were analyzed. We therefore consider this reversed-phase HPLC method to be a convenient and reliable means for analyzing the purity of factor X and Xa preparations. The method may also prove to be a useful purification step in the production of these factors. While the method may not be applicable as an early plasma purification step, it could be used as a final purification step to remove trace contaminants and factor X or Xa from factor Xa or factor X preparations, respectively.

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