Quantitation of Factor VII in the Plasma of Normal and Warfarin-Treated Individuals by Radioimmunoassay

By Daryl S. Fair

Highly purified single-chain factor VII was isolated from plasma and used to generate monospecific antibodies. A double-antibody equilibrium radioimmunoassay was constructed. The assay was tested for and met all the criteria required for a specific, sensitive, and accurate determination of factor VII in plasma. The range of sensitivity of the assay was between 1 and 500 ng factor VII/ml, and the coefficient of variation was 1 %-3% within assay and 12%-16% between assays. Pure factor VII and plasmic factor VII from normal, warfarin-treated, and hereditary deficient individuals inhibited competition assays with parallel slopes, indicating the expression of similar epitopes by these molecules and validating the measurement of this protein in plasma. The concentration of factor VII in normal plasma (n = 41) was 470 ± 112 ng/ml, and the measurement of factor VII antigen correlated with activity (r = 0.82). Factor VII concentration in the plasma of individuals on warfarin therapy (n = 24) was 238 ± 73 ng/ml. Factor VII activity was about 38% of normal and correlated less well with factor VII antigen (r = 0.53). The specific activity of these molecules was 78% of normal (p < 0.01), suggesting the presence of nonfunctional or partially functional molecules in the circulation of individuals undergoing drug therapy. Analysis of two hereditary deficient patients revealed that, while there were significant levels of factor VII protein, the procoagulant activity was <2%, indicating a discordant relationship of these parameters in individuals expressing the deficient factor VII phenotype.

Factor VII is a single-chain, vitamin K-dependent protein present in plasma in trace quantities. Factor VII or the activated form, VIIa, alone has little biologic activity. However, in the presence of calcium, phospholipid, and tissue factor, the extrinsic factor X activating complex is formed, increasing the rate of factor X activation 16,000-fold over that observed for the enzyme alone. Both factors VII and VIIa bind to tissue factor with similar affinities. Analogous findings have been reported for the binding of factors VII and VIIa to lipopolysaccharide-stimulated monocytes. The endogenous procoagulant activity associated with the zymogen has been suggested to be sufficient to initiate coagulation in the presence of its cofactor, phospholipid surface, and calcium. In addition to the cleavage of factor X, factor IX can also be activated by the factor VII (VIIa) containing complex.

Only recently has the human molecule been isolated to homogeneity and characterized. Factor VII has a molecular weight of 48,000-50,000 and contains about nine γ-carboxy glutamic acid residues. Although factor VII appears to be an active zymogen, its activity is increased 25-40-fold when converted to its active form by factors Xa, IXa, XIa, or thrombin. Factor VIIa is a two-chain serine protease whose 26,000 and 22,000 dalton chains are held together by disulfide bonds. This enzyme can be inhibited by antithrombin III, when sufficient concentrations of heparin are present.

The concentration of factor VII in plasma has been estimated from purification studies to be 150 ng/ml to 1 μg/ml. To date, no direct measurements of this protein in plasma have been reported. The present study used purified factor VII to generate polyclonal antibodies and construct a specific radioimmunoassay. The concentration of factor VII has been measured in the plasma of normal, warfarin-treated, and two factor VII-deficient individuals.

MATERIALS AND METHODS

Coagulation Assay

Factor VII activity was measured in a one-stage coagulation assay. To 50 μl of factor VII-deficient plasma (George King Bio-Medical, Inc., Overland Park, KA) was added 50 μl of test plasma (1/20 and 1/40) or column fractions diluted in 0.02 M Tris/HCl (pH 7.4) containing 0.14 M NaCl (TBS) and 1 mg/ml bovine serum albumin (TBS-BSA). After incubation at 37°C for 1 min, 100 μl of prewarmed rabbit brain thromboplastin containing 12.5 mM CaCl2 (General Diagnostics, Morris Plains, NJ) was added, and the time for clot formation measured. Standard curves of factor VII activity were constructed from dilutions of pooled normal human plasma and were linear on log-log plots from 0.0025 to 0.2 U/ml. One unit of factor VII activity was defined as the amount of factor VII present in 1 ml of pooled normal human plasma. The values of factor VII activity measured in individual plasmas represent the mean of four determinations.

Purification of Factor VII

Factor VII was isolated from 12-20 liters of fresh frozen citrated plasma by a modification of the method of Broze and Majerus. To thawed plasma was added the final concentrations of the following inhibitors: Trasylol (10 U/ml), benzamidine (0.001 M), phenylmethylsulfonyl fluoride (PMSF; 0.001 M), heparin (1 U/ml), and...
soybean trypsin inhibitor (10 μg/ml). All steps were performed at 4°C. To stirring plasma was added 1/10 volume of 1 M BaCl₂ dropwise over a 60-min period. The precipitate was collected by centrifugation at 4,400 g for 20 min, washed 4 times with 5 liters of 0.02 M Tris/HCl (pH 8.0), 0.1 M NaCl, 0.02 M BaCl₂, and 0.005 M benzamidine, and the protein eluted in 4 liters of 0.1 M Tris/HCl (pH 8.0), 0.15 M sodium citrate, 0.01 M benzamidine, 0.02% NaN₃, and 10 μg/ml soybean trypsin inhibitor with continuous stirring overnight. The insoluble barium salt was removed by centrifugation at 3,000 g for 20 min, and the supernatant was fractionated by the addition of ammonium sulfate. The protein, precipitated with 28%-70% saturation, was redissolved in 100 ml of 0.025 M sodium citrate (pH 6.0), 0.001 M benzamidine, 0.05 M NaCl, and 0.02% NaN₃ (buffer A) containing 0.2 M EDTA and 10 μg/ml soybean trypsin inhibitor, dialyzed against 4 liters of buffer A with 5 changes, and chromatographed on a column of DEAE-Sephacel (5 x 64 cm) equilibrated in buffer A. Bound proteins were eluted with an 8-liter linear salt gradient to 0.4 M NaCl in buffer A at a flow rate of 220 ml/hr. Factor VII eluted ahead of the other vitamin-K-dependent proteins, and the active fractions were pooled, concentrated by ultrafiltration, and dialyzed against 0.05 M Tris/HCl (pH 7.5), 0.15 M NaCl, 0.002 M benzamidine, 0.02% NaN₃ (buffer B). Factor VII was applied to a QAE-Sephadex column (2.5 x 23 cm) equilibrated in buffer B. After extensive washing, factor VII was eluted from the column with 0.005 M CaCl₂ in buffer B at a flow rate of 20 ml/hr, and 5-ml fractions were collected into tubes containing 250 μl of 0.25 M EDTA (pH 5.9). Factor VII eluting from the column was immediately analyzed for activity, pooled, and concentrated by ultrafiltration. The protein was then applied to a Sephadex G-75 superfine column (2.3 x 145 cm) equilibrated in 0.02 M sodium phosphate (pH 6.0), 0.15 M NaCl, 0.001 M benzamidine, and 0.02% NaN₃. Factor VII eluted as a symmetrical peak and was pooled, concentrated to 0.4-0.6 mg/ml, and stored in aliquots at −70°C. The concentration of factor VII was determined by amino acid composition. The isolated factor VII was homogeneous and unactivated, as judged by sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis. It had an apparent molecular weight of 50,000 ± 2,000 and a specific activity 1,900 ± 250 U/mg.

Activation of purified factor VII by factor Xa was carried out according to the method of Bajaj et al.³

**Gel Electrophoresis**

Analytical SDS-polyacrylamide gel electrophoresis containing 7.5% acrylamide was carried out according to the method of Weber and Osborn.¹⁴ Molecular weight standards used were transferrin (78,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (29,000), and soybean trypsin inhibitor (20,000).

**Amino Acid Compositions**

Samples were hydrolyzed for 24 hr in 6.0 N HCl in evacuated tubes at 110°C. A Beckman 121 M amino acid analyzer (Beckman Instruments, Inc., Fullerton, CA), using the two-column method, was employed for analysis. The concentration of factor VII was based on the mean of duplicate determinations, and the composition data represent the mean of four determinations.

**Antisera**

Two female hybrids of Checkered Giant and New Zealand red rabbits were each immunized with 30 μg of factor VII emulsified in Freund's complete adjuvant at multiple subcutaneous sites. Two weeks later, the animals were boosted with 30 μg of antigen in Freund's incomplete adjuvant (FIA), followed by a third injection of 20 μg of protein in FIA at week 4. The antiserum obtained at week 7 was used in this study.

**Antibody Neutralization**

Five milliliters of rabbit antiserum was adjusted to 45% saturation with ammonium sulfate. The precipitated protein was collected by centrifugation at 2,000 g for 15 min at 20°C, dissolved in 0.15 M NaCl to its original volume, and reprecipitated at 45% saturation of ammonium sulfate. The IgG fraction was obtained by dialyzing the redissolved protein against 0.02 M sodium phosphate (pH 8.0) and applying the solution to a DEAE-cellulose column (2.5 x 40 cm) equilibrated in the same buffer. The IgG was present in the unbound protein fraction and was concentrated by ultrafiltration (Amicon PM-10 membrane) to 2.3 mg/ml using E₅₀₀ = 13.4. This solution was heat inactivated at 56°C for 45 min. Neutralization of factor...
VII activity was performed by mixing 25 μl of normal human plasma (diluted 1/5 in TBS-BSA) with 25 μl of anti-factor VII IgG at varying dilutions. The reactions were carried out at 37°C for 30 min, and the remaining factor VII activity measured in a one-stage clotting assay.

Radioimmunoassay

Factor VII was radiolabeled using the method of Fraker and Speck.11 Iodogen (Pierce Chemical Co., Rockford, IL) was prepared according to the manufacturer's instructions, and 2 μg was dried in a 1.5-ml polypropylene tube. Factor VII (10 μg), 0.5 M sodium phosphate (15 μl), and 0.5 and mCi (1 μl) of carrier-free Na125I were added to the tube and permitted to react at 4°C for 2–3 min. The reactants were transferred to a second polypropylene tube containing 100 μl each of 1% KI and 1% ovalbumin in 0.02 M sodium phosphate (pH 7.2), 0.15 M NaCl, 0.02% NaN3 (PBS). The free iodine was separated from the protein by gel filtration on a 1.2-mI column of Sephadex G25 equilibrated in PBS containing 1% BSA and 0.001 M PMSF. The iodinated factor VII was 94%–97% precipitable in 10% trichloroacetic acid. The specific activity was 8.3–12.5 μCi/μg protein, assuming complete recovery of the protein.121I-factor VII retained about 50% of its clotting activity.

An equilibrium double-antibody radioimmunoassay for factor VII was constructed using a 4-compartment system: to (1) 250 μl of 125I-factor VII (0.1 nM) was added (2) 250 μl of buffer or dilution of competing protein or plasma and (3) 250 μl of rabbit anti-factor VII diluted to give 40% binding. After incubation for 16 hr at 4°C (4), 250 μl of goat anti-rabbit IgG was added. Following incubation for 6 hr at 4°C, the immunoprecipitates were collected by centrifugation at 2,000 g for 15 min at 4°C, and 500 μl of the supernatants were counted in a Iso-Data 20/20 series gamma counter (Iso-Data, Inc., Palatine, IL). All components were diluted in 0.042 M borate buffer (pH 8.3), 0.025 M NaCl, 2% heat inactivated normal rabbit serum, 0.02% sodium azide, 0.001 M PMSF, 0.01 M benzamidine, and 5 U/ml Trasylol. The goat antiserum was heat inactivated (56°C/45 min) and contained 0.01 M benzamidine.

Statistical analyses of the data were handled according to Rodbard.14 Values reported for the plasma samples represent the mean of 4 determinations. The coefficient of variation within assays was 1%–3% and between assays 12%–16%.

Plasma Samples

Most of the plasma from normal and warfarin-treated individuals were obtained from the coagulation laboratory of Scripps Clinic and were anticoagulated with acid-citrate-dextrose or 0.5% sodium citrate. Factor VII-deficient plasmas were purchased from George King Biomedical, Inc.

RESULTS

Factor VII purified from human plasma was homogeneous by SDS-polyacrylamide gel electrophoresis in the presence and absence of 2-mercaptoethanol (Fig. 1). Radiolabeled protein analyzed in a similar manner yielded a single component by each analysis with a migration coincident with that observed on stained gels, indicating that the molecule remained structurally intact after labeling (Fig. 1). The apparent molecular weight of factor VII determined by these methods was 50,000 ± 2,000. Amino acid composition of the isolated protein agreed, within experimental error, with the values reported by others.6,7

The IgG fraction of rabbit antisera to factor VII was tested for its ability to neutralize factor VII activity present in normal human plasma. Dilutions of antibody were added to a constant volume of normal human plasma and incubated at 37°C for 30 min. The remaining activity was measured in a one-stage factor VII coagulation assay. While 1 ml of the early antiserum (used for radioimmunoassay) had the capacity to neutralize factor VII present in 6 ml of normal plasma, antisera obtained from later bleeds could neutralize factor VII activity equivalent to that present in 15 ml of normal plasma. Normal rabbit serum IgG had no effect on factor VII activity.

To initially characterize the factor VII radioimmu-
noassay, the reaction between the labeled ligand and antibody was examined. Figure 2 depicts an antibody-binding experiment with the radiolabeled factor VII and serial two-fold dilutions of the antiserum. At high concentrations of antibody >90% of the ligand was bound, and with antibody dilution, ligand binding decreased. Nonspecific binding was insignificant (<1%). The antibody dilution that bound 40% of the iodinated factor VII was used in this study, although 25% binding was occasionally used for increased sensitivity. When the immunoprecipitate containing the labeled ligand was analyzed by SDS-polyacrylamide gel electrophoresis under reduced conditions, a single iodinated protein species was observed to migrate as native factor VII, indicating that activation or other protein modification had not occurred during the analysis.

Epitopes expressed by $^{125}$I-factor VII and the native protein were identical, as indicated by experiments in which the total concentration of factor VII was held constant, but the proportion of labeled ligand varied from 20%-100%. High concentrations of antiserum bound between 82% and 94% of the ligand (Fig. 3A). With dilution of the antibody, all the binding profiles superimposed, indicating that the affinity of the antibody for factor VII was not perturbed by labeling. In addition, the iodinated (nonradioactive) ligand and the unmodified protein were identical in their ability to inhibit the binding of $^{125}$I-factor VII to its antibody (Fig. 3B). Thus, the antiserum used in the assay reacted equally with both the labeled and native factor VII molecule.

To examine the specificity of the radioimmunoassay, purified antithrombin III, prothrombin, factor IX, factor X, factor VII, and protein C were used as competitors (Fig. 4). The first four proteins did not inhibit the binding of $^{125}$I-factor VII to its antibody, even at a 1,000-fold weight excess. Factor VII completely inhibited the binding. A slight inhibition was noted with protein C, and the level of competition was consistent with a 0.3% factor VII contamination of this protein preparation. Normal human plasma preabsorbed with barium citrate to remove vitamin K-dependent proteins was unable to inhibit, indicating the specificity of the assay only for factor VII and not other molecules in plasma. The linear range of sensitivity for this assay varied from 7–500 ng factor VII/ml at 40% binding to 1–250 ng factor VII/ml at 25% binding of the labeled ligand.

Accuracy of factor VII measurements in plasma was established by adding varying amounts of purified factor VII to plasma and then determining the concentration of factor VII by radioimmunoassay (Fig. 5). A linear correlation was observed between the concentration of factor VII measured in plasma and the quantity of exogeneous protein supplemented. Extrapolation of these values by regression analysis resulted in a line that intersected the ordinate at 508 ng factor VII/ml, nearly the measured concentration of 428 ng factor VII/ml present in plasma without added protein. Hence, the measurement of factor VII could be accurately determined by this assay.

To validate the measurement of factor VII in plasma by radioimmunoassay, the competitive inhibition of the binding of $^{125}$I-factor VII to its antibody was compared between purified factor VII and several normal and abnormal factor VII plasmas (Fig. 6). The results observed for the normal human plasma pool and the plasma from a warfarin-treated patient were representative of all individuals within their respective groups, while the data reported for GK702 and GK704
were individual factor VII-deficient plasmas. For all plasmas, competition was dose-dependent, yielding regression lines whose slopes were parallel to that observed for the purified molecule. Displacement of the competition lines to the right indicated decreasing concentrations of factor VII antigen present in the individual plasmas. These results indicated that the epitopes expressed by factor VII in plasma were immunochemically identical to those present on the purified protein, and could be used to accurately measure the factor VII plasma concentration in normal, warfarin-treated, and factor VII-deficient individuals.

The possibility that significant levels of factor VIIa in plasma may influence the measurement of factor VII protein was examined. In competition assays, both factors VII and VIIa had the capacity to completely inhibit the reaction, with identical slopes of inhibition. Further, the inhibition curves of both molecules superimposed, indicating that the major epitopes present on factor VII were equally expressed on the activated two-chain molecule (data not shown).

The distribution of factor VII in the plasma of normal and warfarin-treated individuals was determined by radioimmunoassay. The mean concentration of factor VII measured in 41 normal plasmas was 470 ± 112 ng/ml, and the majority of individuals were between 300 and 500 ng/ml. Some samples (3/41) contained high concentrations of this protein. Plasmas from patients undergoing warfarin therapy (n = 24) had lower concentrations of factor VII (238 ± 73
ng/ml). Thus, warfarin therapy decreased the amount of factor VII antigen to about 50% of the level measured in normal plasma samples.

To correlate the measurements of factor VII protein with factor VII activity, plasmas from normal \((n = 26)\) and warfarin-treated \((n = 24)\) individuals were analyzed. Figure 7 depicts the individual datum points. Linear regression analysis of the data from normal plasmas yielded a line with an equation of \(y = 0.0021x + 0.019\) and a correlation coefficient \((r)\) of 0.82 \((p < 0.001)\). A reduction of both factor VII antigen and activity was observed in individuals on warfarin. Analysis of these plasmas generated a linear regression line described by the equation \(y = 0.0015x + 0.048\), with a \(r = 0.53\) \((p < 0.001)\). The slopes of the regression lines indicated that more protein than measured activity was present in the plasma of individuals on warfarin therapy relative to normal plasma, consistent with the presence of a less active vitamin K-dependent protease as a result of drug treatment. Similar findings were seen when normal and warfarin-treated individuals were analyzed as groups (Table 1). Whereas treatment with the vitamin K antagonists reduced the activity of factor VII to about 38% of that observed for the untreated group, factor VII protein concentration was about 51% of normal. Further, the specific activity of factor VII measured for the warfarin-treated group was about 78% of that determined for normal plasma factor VII. The difference between the specific activity was statistically significant \((p < 0.01)\).

Two individuals known to be deficient in factor VII activity were analyzed for factor VII activity and protein (Table 2). The competitive radioimmunoassay of each individual are presented in Fig. 6. While the plasma from patient GK702 contained about 12% of the normal factor VII concentration and about 2% of the procoagulant activity, patient GK704 contained 56% of factor VII protein and only 1%-2% procoagulant activity. These data indicate that factor VII antigen and activity may be discordant in individuals phenotypically expressing decreased levels of factor VII.

Table 1. Comparison of Plasma Factor VII Activity and Antigen in Normal and Warfarin-Treated Individuals*

<table>
<thead>
<tr>
<th>Group</th>
<th>Normal</th>
<th>Warfarin</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>26</td>
<td>24</td>
</tr>
<tr>
<td>Factor VII activity ((U/ml))</td>
<td>(1.000 \pm 0.326)</td>
<td>(0.380 \pm 0.167)</td>
</tr>
<tr>
<td>Factor VII antigen ((ng/ml))</td>
<td>(467 \pm 127)</td>
<td>(238 \pm 72.7)</td>
</tr>
<tr>
<td>Factor VII specific activity ((U/\mu g))</td>
<td>(2.157 \pm 0.451)</td>
<td>(1.682 \pm 0.787)</td>
</tr>
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*Values given as mean ± standard deviation.

**DISCUSSION**

Factor VII is present as a trace protein in plasma, and its detection requires a sensitive and specific assay method. Highly purified human factor VII and monospecific polyclonal rabbit antibodies were characterized and used to construct a radioimmunoassay for the measurement of factor VII concentrations in plasma. This assay was tested for and met all the criteria required for a specific, sensitive, and accurate determination of factor VII in plasma. Iodination of the molecule did not affect the single-chain polypeptide structure, as evidenced by SDS-polyacrylamide gel electrophoresis. The antiserum had the capacity to bind >90% of the labeled ligand and to neutralize factor VII activity. Expression of antigenic determinants was not altered by labeling, as determined by equal binding of factor VII mixtures in which the proportion of iodinated factor VII varied and by the identical competitive inhibition results of iodinated and unmodified factor VII. The assay was specific for factor VII and did not react significantly with purified antithrombin III, factor IX, factor X, prothrombin, protein C or plasma depleted of vitamin K-dependent proteins. Depending on the amount of ligand bound, the range of sensitivity was between 1 and 500 ng factor VII/ml. Factor VII was quantitatively recov-

**Table 2. Characterization of Factor VII in the Plasma of Two Patients Deficient in Factor VII Procoagulant Activity**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Factor VII Activity ((U/ml))</th>
<th>Factor VII Antigen ((ng/ml))</th>
<th>Specific Activity ((U/\mu g))</th>
</tr>
</thead>
<tbody>
<tr>
<td>GK702</td>
<td>0.022</td>
<td>56.0</td>
<td>0.393</td>
</tr>
<tr>
<td>GK704</td>
<td>0.014</td>
<td>262</td>
<td>0.053</td>
</tr>
</tbody>
</table>
er from plasma supplemented with purified protein. The precision of the assay was indicated by a coefficient of variation of 1%–3% within assay and of 12%–16% between assays.

In competitive inhibition assays, plasma factor VII and pure factor VII inhibited the binding of $^{125}$I-factor VII to anti-factor VII completely, and the slopes of inhibition were parallel, validating the method for the measurement of factor VII in plasma. This parallelism of slopes was true for 25 individual samples analyzed in detail. The concentration of factor VII determined from 41 individuals was 470 ± 112 ng/ml, based on protein standards determined by amino acid composition. This value is within the estimated concentration of 430–580 ng/ml predicted from the purification of factor VII within the “normal” range of this assay. The decreased concentration of factor VII could result from an increase in the catabolic rate or a decrease in the synthesis and secretion of this abnormal protein.

Analysis of two individuals with hereditary factor VII deficiency revealed that there was a discordant relationship between factor VII protein and activity. Both plasmas studied had a significant concentration of antigen and little procoagulant activity. Similar findings have been observed by others.20,22,29–31

Of interest is the recent discovery that factor VII variants may be differentiated based on their activation patterns by various thromboplastins.29,30 This suggests that a genetic heterogeneity may exist for the variant forms of factor VII. The use of a specific, accurate, and independent measurement of plasma factor VII by radioimmunoassay will provide the quantitative basis for further examination of factor VII procoagulant activities in individuals expressing the aberrant factor VII phenotype.

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