A Radioreceptor Assay for Quantitating Plasma Factor VIII/von Willebrand's Protein

By Kuo-Jang Kao, Salvatore V. Pizzo, and Patrick A. McKee

Human factor VIII/von Willebrand's factor (FVIII/vWF) is a plasma glycoprotein(s) with two distinct biologic activities. One is procoagulant activity (FVIII activity), which corrects the blood coagulation defect of hemophilia A patients; the other is platelet aggregating activity (vWF activity), which corrects the prolonged bleeding time of von Willebrand's disease (vWD) patients. From measurements of plasma FVIII/vWF levels by immunoassays or by ristocetin-induced platelet aggregation tests, it is accepted that the plasma concentration of FVIII/vWF protein is normal or elevated in hemophilia A patients. In contrast, most vWD patients have a deficiency of both plasma FVIII/vWF protein and platelet-aggregating activity. Other studies suggest that elevated plasma concentrations of FVIII/vWF in different disease states may contribute to increased platelet aggregability and as a consequence, an increased risk of atherothrombotic disease. Therefore, an accurate and rapid quantitative assay of plasma FVIII/vWF may be useful in both clinical practice and research.

In the past few years, several different quantitative assays for plasma FVIII/vWF have been developed. However, all are either platelet aggregation assays or immunologic assays. Immunologic assays, a heterologous antifactor VIII/vWF antiserum is prepared and used for quantitative immunoelectrophoresis, radioimmunoassay, or immunoradiometric assay. Although the sensitivity and specificity of these assays are probably acceptable, most are time consuming and only give semiquantitative estimations of plasma FVIII/vWF concentration.

Recently, we have reported the existence of FVIII/vWF receptors on human platelets and aspects of this work have been confirmed. The stability, high affinity and specific binding properties of these receptors allowed us to develop a rapid and accurate radioreceptor assay for measuring human plasma FVIII/vWF concentrations.

MATERIALS AND METHODS

Materials

Intermediate purity human FVIII/vWF concentrate and alpha-acid glycoprotein were obtained from the American National Red Cross, Bethesda, Md. Ristocetin was purchased from H. Lundbeck & Co., Copenhagen, Denmark. Carrier-free Na125I was from New England Nuclear, Boston, Mass. Fibrinogen was a product of A. B. Kabi, Bethesda, Md. Ristocetin was purchased from H. Lundbeck & Co., Copenhagen, Denmark. Thrombin was obtained from the Division of Biological Standards, NIH, Bethesda Md. Bovine lactoperoxidase and RC-60 (a lectin from Ricinus communis) were from Boehringer Mannheim Co. and Calbiochem Co., respectively. Biogel A-15m or Sepharose 4B was obtained from Bio-Rad or Pharmacia, respectively. Alpha2-macroglobulin was provided by Dr. W. Hubbard, Department of Immunology, Duke Medical Center. All other reagents used in our experiments were the best grade available.

Purification of FVIII/vWF Protein

Human FVIII/vWF was purified from intermediate purity FVIII/vWF concentrate by polyethylene glycol precipitation and agarose gel chromatography (Biogel A-15m) as previously described. Following reduction with beta-mercaptoethanol, the final product was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver staining.

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purified FVIII/vWF protein showed a single protein band by sodium dodecyl sulfate-urea gel electrophoresis. Protein concentrations were determined by the Lowry method using crystalline bovine serum albumin as the standard.

Radioiodination of FVIII/vWF

Purified FVIII/vWF was labeled with carrier-free $^{125}$I by solid phase lactoperoxidase as described by David and Reisfeld. The procedures for iodination were as previously described. After radiolabeling, free $^{125}$I was removed from $^{125}$I-labeled FVIII/vWF ($^{125}$I-FVIII/vWF) by applying the reaction mixture to a Sephadex G-25 column (1 x 20 cm) and eluting with 0.15 M NaCl-0.025 M cacodylate buffer, pH 6.8. The $^{125}$I-FVIII/vWF eluted in the first radioactive peak which was pooled and the specific activity defined by the ratio of its radioactivity to protein concentration. $^{125}$I-FVIII/vWF retained its full ability to serve as a cofactor in the ristocetin-induced platelet aggregation assay.

Preparation of Paraformaldehyde-Fixed Platelets

Washed human platelets were prepared from 15 ml of freshly collected citrated venous blood as previously reported. The washed human platelets were suspended in 3 ml of 0.15 M NaCl-0.025 M Tris-HCl (Tris-saline), pH 7.4, before fixation. The platelet suspension was mixed with an equal volume of freshly prepared 4% paraformaldehyde in 0.15 M Na$_2$HPO$_4$, the pH adjusted to 7.4 with 0.2 M HCl and then left at 4°C for 12 hr. The fixed platelets were recovered by centrifuging at 4000 g for 10 min, washed twice with Tris-saline, and resuspended in Tris-saline for storage at 4°C. The paraformaldehyde-fixed platelets could be stored and used in the radioreceptor assay for at least 2 mo.

Preparation of Plasma Samples for the FVIII/vWF Radioreceptor Assay

Venous blood collected from each individual was mixed with one-tenth volume of 3.8% sodium citrate. Platelet-poor plasma was prepared by centrifuging the citrated venous blood at 6000 g for 10 min, after which it was separated and stored at –70°C. Essentially identical results were observed when fresh plasma samples were used prior to freezing. Just before assay, the plasma was thawed at room temperature and 2 ml were gel filtered on a Biogel A-15m column (1.9 x 7 cm) at 4°C, using Tris-saline as the eluting buffer at 10 ml/hr. One millilitre fractions were collected, and the absorbance at 280 nm was determined for each. As shown in Fig. 1, the first four fractions of the void volume protein peak were pooled for use in the radioreceptor assay; this pool could be stored at 4°C for at least 2 wk or at –70°C for months without affecting the results of the assay. Although there is FVIII/vWF activity beyond the fourth tube in the void volume, its concentration is very low and the contamination by other plasma proteins increases greatly. Hence, to avoid the dilution of FVIII/vWF concentration as well as potential interfering effects by contaminant proteins, fractions beyond the region indicated in Fig. 1 were not used. Each column was washed with 5 volumes of elution buffer before another plasma sample was applied. The percent recovery of FVIII/vWF protein was deter-

![Fig. 1. FVIII/vWF recovery after gel filtration. Two milliliters of a plasma sample were mixed with 2 μl of $^{125}$I-FVIII/vWF (53,400 cpm), applied to a 1.9 x 7 cm Biogel A-15m column and eluted at 10 ml/ph with 0.15 M NaCl-0.025 M Tris-HCl, pH 7.4. The absorbance at 280 nm and radioactivity of each 1-ml fraction were determined and the first 4 tubes (indicated by the arrows) of the void volume protein peak were pooled. The percent recovery of FVIII/vWF from the pooled eluent was calculated as described in Materials and Methods.](image-url)
determined separately by adding 2 μl of 125I-FVIII/vWF (54,000 cpm) to a 2-ml plasma sample that was then chromatographed on the column as described above. The absorbance at 280 nm and the radioactivity of each fraction were measured and the percent recovery of FVIII/vWF protein determined by dividing the total cpm of 125I-FVIII/vWF recovered in the pooled aliquot by the total cpm of 125I-FVIII/vWF applied to the column. The percent recovery was determined in quadruplicate for each column used in the assay. The average percent recovery of FVIII/vWF from a representative column was 66.3% ± 2.4% (mean ± SD, n = 4); similar recoveries were observed for hemophilic and vWF plasma samples. The mean percent recovery of FVIII/vWF for each column was used in the calculation of plasma FVIII/vWF concentration as described below.

**Radioreceptor Assay Method**

The assay was performed at 24°C in a 12 × 75 mm polystyrene tube. Each incubation mixture contained 0.1 μg 125I-FVIII/vWF in 100 μl 0.15 M NaCl-0.025 M Tris-HCl-0.1% bovine serum albumin, pH 7.4, (Tris-saline-BSA); 0.5 mg ristocetin in 100 μl Tris-saline; 200 μl FVIII/vWF standard in Tris-saline-BSA or 200 μl of a plasma eluent pool; and 100 μl of paraformaldehyde-fixed platelets (5 × 10⁶ cells as calculated from counts on a standard hemacytometer under a phase contrast microscope). The start of each incubation was defined by the addition of the 100 μl of platelet suspension. After standing for 100 min at 25°C, each incubation mixture was terminated by adding 1 ml of ice-cold Tris-saline and centrifuging immediately at 3500 g for 20 min at 4°C. After centrifugation, the supernate, which contained free 125I-FVIII/vWF, was aspirated and the pellet of fixed platelets with bound 125I-FVIII/vWF was counted in a Beckman Model 4000 gamma counter for 1 min. FVIII/vWF standards (0.025–50 μg) were prepared from a concentrated FVIII/vWF stock solution (2 mg/ml) by serial dilution with Tris-saline-BSA. All of the test plasma samples were assayed in duplicate or triplicate. Nonspecific binding was determined from incubation mixtures to which an excessive amount of unlabeled standard FVIII/vWF (50 μg) was added. The specific bound counts, (B₀), were determined by subtracting nonspecific binding from the bound counts observed when no unla-

**Assay of Ristocetin-Induced Platelet Aggregation**

Platelet-aggregating activity was assayed by the turbidimetric method of Born²⁶ in a dual sample aggregometer (Sienco Inc., Morrison Colo.). Fresh platelets were washed and resuspended as described previously.²² The platelet concentration was adjusted to 7.5 × 10⁵/μl for the assay. Platelet aggregation was monitored in a siliconized glass cuvette at 25°C by mixing the following at 1000 rpm: 0.4 ml of platelet suspension; 50 μl of purified FVIII/vWF standard (0.1–1.0 μg), plasma or plasma eluents; and 50 μl of 0.5

![Fig. 2. Platelet-aggregating activity of 125I-FVIII/vWF. The platelet-aggregating activities of nonlabeled purified FVIII/vWF and 125I-FVIII/vWF were assayed by ristocetin-induced platelet aggregation as described in Materials and Methods. The ordinate is the reciprocal of the initial velocity (v) of the platelet aggregation curve; the abscissa is the reciprocal of the FVIII/vWF concentration in each mixture.](https://www.bloodjournal.org/content/58/2/581)
The specificity of '25I-FVIII/vWF binding to fixed platelets was studied by using high concentrations of different human plasma proteins, a lectin of Ricinus communis, a plasma eluent from pooled normal plasma, or a plasma eluent from vWD plasma to compete with '25I-FVIII/vWF for binding. Figure 3 shows that only unlabeled FVIII/vWF and the plasma eluents from pooled normal plasma effectively competed with '25I-FVIII/vWF for binding. As shown in Fig. 4, Scatchard analysis of these data also indicated that FVIII/vWF has a very high affinity ($K_d = 0.84 \text{nM}$) for the fixed platelets. The number of FVIII/vWF receptor sites calculated from the intercept of the abscissa of the Scatchard plot is about 39,000 per fixed platelet if it is assumed that only one FVIII/vWF molecule of $1.1 \times 10^6$ daltons binds to each site. These data clearly show that the binding properties of FVIII/vWF to paraformaldehyde-fixed platelets are essentially the same as those previously reported by us for fresh washed human platelets.

**RESULTS**

'25I-FVIII/vWF Binding to Paraformaldehyde-Fixed Platelets

The specificity of '25I-FVIII/vWF binding to fixed platelets was studied by using high concentrations of different human plasma proteins, a lectin of Ricinus communis, a plasma eluent from pooled normal plasma, or a plasma eluent from vWD plasma to compete with '25I-FVIII/vWF for binding. Figure 3 shows that only unlabeled FVIII/vWF and the plasma eluents from pooled normal plasma effectively competed with '25I-FVIII/vWF for binding. As shown in Fig. 4, Scatchard analysis of these data also indicated that FVIII/vWF has a very high affinity ($K_d = 0.84 \text{nM}$) for the fixed platelets. The number of FVIII/vWF receptor sites calculated from the intercept of the abscissa of the Scatchard plot is about 39,000 per fixed platelet if it is assumed that only one FVIII/vWF molecule of $1.1 \times 10^6$ daltons binds to each site. These data clearly show that the binding properties of FVIII/vWF to paraformaldehyde-fixed platelets are essentially the same as those previously reported by us for fresh washed human platelets.

**Binding Assay**

To determine if whole plasma samples could be used directly for measuring FVIII/vWF content, different volumes (20–100 µl) of normal plasma were assayed by competitive receptor binding. As shown in Fig. 5, the lack of parallel relationships between the FVIII/vWF standard curve and the plasma samples suggests that an interfering factor(s) in the plasma samples inhibited '25I-FVIII/vWF binding disproportionately at larger plasma volumes. However, the interfering substance(s) could be removed by filtering each plasma sample through a 4% agarose gel column. As is evident in Fig. 5, the FVIII/vWF in the pool of the first four void volume fractions (Fig. 1) gave parallel competition curves when analyzed in mixtures of unlabeled purified FVIII/vWF and fixed platelets.
The standard curve for the assay was drawn by linear regression of the logit-transformed binding competition curve. Figure 6 shows the mean curve obtained by averaging four different standard binding curves. Each of four different samples of plasma eluent were also assayed in duplicate. The coefficients of intra- and interassay variation were 6% and 10%, respectively, thereby indicating that the radioreceptor assay for plasma FVIII/vWF is both precise and reproducible.

The FVIII/vWF concentrations determined by this assay for four different plasma samples are shown in Table 1. As can be seen, the plasma levels of FVIII/vWF for two vWD patients were 41.7% and 19.6% of pooled normal plasma. The plasma FVIII/vWF concentration was three times higher than that of pooled normal plasma in one hemophilia A patient. Plasma samples from 10 normal adults, which were

<table>
<thead>
<tr>
<th>Source of Plasma</th>
<th>Assay No.</th>
<th>Mean ± SEM (µg/ml, n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal pooled</td>
<td>1</td>
<td>15.0 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>16.8 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>17.1 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>16.3 ± 0.5</td>
</tr>
<tr>
<td>Hemophilia A</td>
<td>1</td>
<td>51.1 ± 2.5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>57.1 ± 3.8</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>51.1 ± 2.6</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>51.1 ± 2.6</td>
</tr>
<tr>
<td>vWD (J.D.)</td>
<td>1</td>
<td>5.7 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>6.5 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>9.0 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>6.0 ± 0.4</td>
</tr>
<tr>
<td>vWD (B.C.)</td>
<td>1</td>
<td>2.9 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3.2 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>3.0 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>3.6 ± 0.2</td>
</tr>
</tbody>
</table>

The plasma eluent of each plasma sample was assayed at least in triplicate on four different dates. Only the means of the triplicate or quadruplicate assays are given for each of the 4 days; intra- and interassay variations were 6% and 10%, respectively.

Plasma FVIII/vWF concentration (µg/ml) =

\[
\text{Plasma FVIII/vWF concentration (µg/ml)} = \frac{\text{PLASMA (µL; O, a)}}{2 \times \text{Percent recovery of FVIII/vWF from the column}}
\]
assayed individually to determine the range of normal plasma FVIII/vWF concentration, varied within a range of 8.3–24.9 µg/ml, with the average normal plasma FVIII/vWF concentration being 15.4 ± 4.6 µg/ml (mean ± 1 SD). This latter value is in good agreement with that determined for pooled normal plasma (Table 1) from 6 other adults.

**Correlation of the FVIII/vWF Radioreceptor Assay With Ristocetin-Induced Platelet Aggregation**

The FVIII/vWF concentrations in the four plasma samples in Table 1 were also assayed by ristocetin-induced platelet aggregation. The FVIII/vWF concentration of each plasma sample was determined using a standard curve of values for serial dilutions of pooled normal plasma. The FVIII/vWF concentration of each plasma eluent was assayed against different concentrations of purified standard FVIII/vWF (0.1–1.0 µg). As shown in Fig. 7, the plasma FVIII/vWF concentrations determined by ristocetin-induced platelet aggregation correlated very well with those determined by the radioreceptor assay. These results establish the validity of preparing assay samples by gel filtration and also support the functional relevancy of the radioreceptor assay.

**DISCUSSION**

In this report, we have described how human platelet FVIII/vWF receptors can be used for quantifying plasma FVIII/vWF concentration. Our results demonstrate that the assay is highly specific, sensitive, precise, and reproducible for measuring plasma FVIII/vWF levels. Moreover, our finding that FVIII/vWF receptors are preserved on paraformaldehyde-fixed platelets explains the previous finding that fixed platelets can be agglutinated by FVIII/vWF in the presence of ristocetin.

When assayed directly, it was apparent that whole plasma contains a substance that interferes with the binding of FVIII/vWF to platelets. Although the biochemical nature of this interfering factor(s) has not been identified, it can be removed by gel filtration of plasma on a 4% agarose column. The validity of using this procedure to prepare plasma samples for the radioreceptor assay is evident since the plasma FVIII/vWF concentrations measured by the radioreceptor assay correlate very well with those determined for whole plasma samples by ristocetin-induced platelet aggregation (Fig. 7).

The concentration of FVIII/vWF was measured in one hemophilia A patient and two vWD patients by the radioreceptor assay. The plasma FVIII/vWF concentration in hemophilia A plasma was three times higher than in pooled normal plasma; however, FVIII/vWF levels were much lower in the two vWD plasmas. Our data are consistent with results reported by others who determined concentrations of plasma FVIII/vWF by immunologic methods or ristocetin-induced platelet aggregation.

Previously, we demonstrated that FVIII/vWF receptor binding is a prerequisite for platelet aggregation induced by ristocetin. Similarly, we show in the present study that plasma FVIII/vWF concentrations from the radioreceptor assay correlate closely with levels of FVIII/vWF ristocetin cofactor activity. Therefore, it is foreseeable that a combination of the radioreceptor assay, a quantitative immunoassay and ristocetin-induced platelet aggregation will provide a useful approach for categorizing the molecular defects of von Willebrand’s disease. While quantitative immunologic assays depend on the antigenicity of FVIII/vWF, the preservation of FVIII/vWF antigenicity is not necessarily equivalent to the retention of vWF activity. More specifically, since FVIII/vWF binding
essentially equates with function, it is to be expected that the radioreceptor assay will give low binding despite high antigen levels by immunoassay in those patients having the “high antigen, low vWF activity” form of vWD.30,31 Along these lines it is of interest that we have been able to modify the carbohydrate side-chains of purified normal FVIII/vWF in vitro to generate a species having low receptor binding affinity, low platelet-aggregating activity, but normal antigenicity.21,32,33

REFERENCES

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A radioreceptor assay for quantitating plasma factor VIII/von Willebrand's protein

KJ Kao, SV Pizzo and PA McKee