Enzyme Immunoassay for Factor VIII-Related Antigen

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I describe a simple enzyme-linked immunosorbent assay (ELISA) for the quantitation of Factor VIII-related antigen in plasma with use of commercially available peroxidase-labeled antiserum and solid-phase support. Regression analysis of 85 plasma samples analyzed by this technique (y) and by a commonly used electroimmunoassay (Anal. Biochem. 15: 45–52, 1966) (x) gave the equation y = 0.223 + 0.77x (r = 0.973). The present method was also compared with enzyme immunoassay in which a phosphatase-labeled antiserum prepared in our laboratory was used; the correlation between the two assays was very good. The simplicity and specificity of the ELISA technique should make it a useful alternative to the more difficult and time-consuming Laurell method.

Additional Keyphrases: von Willebrand’s disease - hemophilia - enzyme-linked immunosorbent assay - peroxidase-labeled antibody and alkaline phosphatase-labeled antibody compared - reference interval

Factor VIII-related antigen (FVIII:RAg)1 represents the antigenic determinants associated with von Willebrand factor protein and can be detected by heterologous antisera to purified Factor VIII. Low or undetectable amounts of FVIII:RAg are found in sera of patients with von Willebrand’s disease (1), whereas normal or increased concentrations of the antigen occur in patients with classic hemophilia (2). The quantitation of FVIII:RAg can thus be used for the differentiation of these two disorders. The discovery (3) of lower than normal ratios of Factor VIII clotting activity to FVIII:RAg in carriers of hemophilia A led to a widespread use of FVIII:RAg quantitation for detecting female carriers of hemophilia A (4–6).

The most widely used method for the quantitation of FVIII:RAg is Laurell’s electroimmunoassay (7), used in the initial studies on hemophilia carrier detection (2, 3) and adopted or modified later by other investigators. Subsequently, radioimmunoassay (8), immunoradiometric assay (9, 10), and fluoroimmunoassay (11) were developed to provide higher sensitivity. In 1976, Bartlett et al. (12) introduced an enzyme immunoassay for FVIII:RAg involving alkaline phosphatase-labeled antibody to FVIII:RAg and microhemagglutination plates as solid-phase support; it correlated well with the Laurell method and satisfactorily separated obligate carriers of hemophilia A from normal women by combining Factor VIII clotting activity and FVIII:RAg data. The indirect enzyme immunoassay described recently by Neas and Perkins (13) is relatively complex and more laborious.

In this paper I describe a simple and reliable test for the quantitation of FVIII:RAg with commercially available peroxidase (EC 1.11.1.7)-labeled antibody. The method, based on enzyme-linked immunosorbent assay (ELISA), obviates the necessity of preparing the enzyme–antibody conjugate in the laboratory and can be adapted for automation. The correlation of the present method with the Laurell technique and with the alkaline phosphatase (EC 3.1.3.1) immunoassay is presented, together with FVIII:RAg concentrations in normal individuals.

Materials and Methods

Materials

Samples. Blood samples from volunteer normal individuals, suspected carriers of hemophilia, and relatives of hemophilic patients were collected with plastic syringes and immediately mixed with citrate buffer in plastic test tubes (nine volume of blood + one volume of sodium citrate buffer, 130 mmol/L, pH 5.0). The plasma was separated within 1 h of venipuncture and stored in polystyrene tubes at −70 °C. A standard pool of 12 aliquots of plasma from healthy men was prepared every six months and kept frozen at −70 °C in 0.3-mL portions; the FVIII:RAg concentration of this pool was defined as 1.0 antigenic unit/mL (Ag unit/mL). An assayed reference plasma (“ARP” reference material) obtained from Helena Laboratories, Beaumont, TX 77704, was used as an internal control in each run.

Reagents. Rabbit anti-FVIII:RAg (IgG fraction, lot 010B) and horseradish peroxidase-labeled rabbit IgG to human FVIII:RAg (lot 011) were Dako (Copenhagen, Denmark) products obtained from Accurate Chemical and Scientific Corp., Westbury, NY 11590. IgG fraction of goat antiserum to human FVIII:RAg (lot no. 1000) and horseradish peroxidase-labeled rabbit IgG to human FVIII:RAg (lot 011) were Dako (Copenhagen, Denmark) products obtained from Accurate Chemical and Scientific Corp., Westbury, NY 11590. IgG fraction of goat antiserum to human FVIII:RAg (lot no. FEB-IGG-009-1) was purchased from Atlantic Antibodies, Scarborough, ME 04074. To conjugate this fraction with alkaline phosphatase, I used the glutaraldehyde method described by Voller et al. (14) with 2 mg of immunoglobulin and 5 mg of enzyme. The conjugate was purified on a 0.9 × 60 cm Sephadex G-200 column equilibrated with a buffer containing, per liter, 50 mmol of Tris ∙ HCl and 500 mmol of NaCl, pH 8.0, then stored in the dark at 4 °C in the same buffer to which were added bovine serum albumin (10 g/L) and NaNO3 (0.2 g/L).

Alkaline phosphatase (bovine intestinal mucosa, type VII-S; spec. activity approx. 1000 kU/g of protein), D-nitrophenyl phosphate (“Sigma 104” phosphatase substrate tablets), o-phenylenediamine, and Tween 20 were purchased from Sigma Chemical Co., St. Louis, MO 63178. All other reagents were of the highest purity available.

Buffers. Coating buffer (carbonate buffer, 50 mmol/L, pH 9.6) is prepared by dissolving 1.59 g of Na2CO3, 2.93 g of NaHCO3, and 0.2 g of NaN3 in 1 L of distilled water. Store at 4 °C and prepare a fresh solution every two weeks.

Phosphate-buffered saline–Tween solution (PBS-Tween) is prepared by dissolving 8.0 g of NaCl, 0.2 g of KH2PO4, 1.16 g of Na2HPO4, 0.2 g of KCl, and 0.5 mL of Tween 20 in 1 L of distilled water.

Phosphate–citrate buffer, pH 5, is prepared by mixing 500 mL of citric acid (100 mmol/L) and 530 mL of Na2HPO4 (200 mmol/L). The substrate solution consists of 8 mg of o-phenylenediamine dissolved in 20 mL of phosphate–citrate buffer, pH 5, plus 10 μL of 300 mL/L HzO2 solution. Prepare immediately before use and protect from strong light.
Solid-phase support. Polystyrene cuvette strips (Cuvette-Pak; Gilford Instruments, Oberlin, OH 44074) were used as a solid phase for attaching the first antibody. Each cuvette strip has 10 individual wells that serve as reaction chambers and can be used as cuvettes for direct spectrophotometric reading in Gilford EIA manual or PR-50 automatic readers.

Procedure

Peroxidase ELISA. The principle of the technique is the same as that described by Voller et al. (14). Fill each well of the cuvette strip with 400 μL of rabbit anti-FVIIIRAg IgG (15 g/L) diluted 1500-fold in the coating buffer; this dilution, corresponding to an IgG concentration of 10 mg/L, was determined by “checkerboard” (14) titration with several standard plasma pools dilutions (two-, four-, and eightfold) and different anti-FVIIIRAg IgG concentrations (100, 10, 1.0, and 0.1 mg/L). After overnight incubation at 4 °C in a humid chamber, wash the antibody-coated wells three times with PBS-Tween. Then add 300 μL of 50-fold dilutions (in PBS-Tween) of freshly opened standard plasma pool, ARP reference plasma, and test samples to the individual wells; include additional dilutions of the standard plasma pool to produce 0.75, 0.50, and 0.25 Ag units/mL. Dilute samples with FVIIIRAg concentrations > 1 Ag unit/mL, to fit within the standard curve. Incubate the strips for 2 h at room temperature (humid chamber), wash three times with PBS-Tween, and add to each well 400 μL of the peroxidase-conjugated rabbit anti-FVIIIRAg, diluted 5000-fold with PBS-Tween. After incubation for 2 h at room temperature (humid chamber), wash the wells three times with PBS-Tween, and then dispense 300 μL of freshly prepared substrate solution into each well. After 30 min at room temperature, stop the reaction by adding 300 μL of H2SO4 (2 mol/L) and read the absorbance at 492 nm against a blank. Determine the FVIIIRAg concentration of test samples by comparison with the standard curve (on linear graph paper).

Alkaline phosphatase ELISA. The procedure is the same as that described above for peroxidase, except for the use of IgG fraction of goat anti-FVIIIRAg as the first antibody (10 mg/L in coating buffer) and alkaline phosphatase-labeled goat anti-FVIIIRAg IgG as the second antibody (diluted 1000-fold in PBS-Tween). p-Nitrophenyl phosphate (one 5-mg tablet in 5 mL of diethanolamine buffer, 1 mol/L, pH 9.8) is used as substrate, the enzyme reaction stopped by the addition of NaOH (3 mol/L), and the absorbance read at 405 nm.

Electroimmunoassay. For comparison, I used Laurell's electroimmunoassay (7, 15) to quantitate the FVIIIRAg. Cover 8 × 10 cm glass plates with 16 mL of agarose gel (10 g of agarose per liter of Tris-barbiturate buffer, 30 mmol/L, pH 8.8) containing 200-fold diluted rabbit antiserum to FVIIIRAg (Calbiochem-Behring, La Jolla, CA 92037). After 20 h at 15 mA/plate, wash the plates in isotonic saline, dry, and stain with Coomassie Blue R-250, 2.5 g/L. Plot the areas of the precipitin peaks of standard plasma pool dilutions vs concentration, and determine the FVIIIRAg of the unknowns by reading from the standard curve.

Results

The precision of the present method was evaluated by analyzing two plasma samples at different FVIIIRAg concentrations. Within-assay and between-assay results (CV) are given in Table 1. The minimum detectable FVIIIRAg concentration (the concentration significantly different from zero at the 95% confidence level) was 0.03 Ag unit/mL.

<table>
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<th>n</th>
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<td>0.030</td>
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Table 1. Precision of the Method

FVIIIRAg concentrations ranging from 0.2 to 4.55 Ag units/mL (Figure 1). Regression analysis of the data for ELISA (y) vs electroimmunoassay (x) gave the equation y = 0.223 + 0.77x (r = 0.973).

To compare the present method with the enzyme assay in which alkaline phosphatase serves as an antibody label, I prepared a conjugate of goat anti-FVIIIRAg IgG fraction with alkaline phosphatase. The agreement of the phosphatase-ELISA method (y) with the peroxidase-ELISA method (x) is given by the equation y = 0.017 + 1.02x (r = 0.967, n = 30).

The range of FVIIIRAg concentrations in healthy adult individuals, as determined by peroxidase-ELISA of 19 plasma samples from nine men and 10 women (ages 20 to 48), was 0.51 to 1.77 Ag units/mL (mean 1.01 Ag units/mL).

Discussion

The results of the precision studies (Table 1) compare favorably with those obtained by electroimmunoassay. CVs with the present method range from 3.9 to 4.4%; the data on FVIIIRAg obtained by the Laurell technique in different laboratories (16) showed CVs ranging from 6.2 to 22.9%.

Although the peroxidase-ELISA method showed a good correlation with electroimmunoassay, it gave somewhat higher results than the Laurell technique at low FVIIIRAg concen-

Fig. 1. Comparison of enzyme immunoassay (horseradish peroxidase-ELISA) and electroimmunoassay (Laurell method) for the quantitation of Factor VIII-related antigen

Regression analysis: y = 0.223 + 0.77x, r = 0.973, n = 85. Units are antigen units (see text). Only the 0.2–1.2 Ag units/mL concentration range is shown, although all the data, covering the range of 0.2–4.55 Ag units/mL, were used for the regression analysis.
trations. No standard preparation being available for the quantitation of this antigen, it is difficult to determine which of the two methods is biased. Given the variations in precipitin peak shapes between individual samples, the necessity of visual interpretation, and the difficulty in tracing often small and faint precipitin peaks in the Laurell patterns, the electroimmunoassay is more likely to give erroneous results, especially at low FVIIIIRAg concentrations. In any case, each laboratory involved in hemophilia carrier detection and evaluation of hemophilia and von Willebrand patients must establish its own range for normal FVIIIIRAg concentrations.

Very good correlation between the two enzyme assays suggests that both methods can substitute equally well for the Laurell technique. The use of commercially available reagents makes peroxidase-ELISA a very suitable method for any laboratory not equipped with instrumentation and expertise necessary for preparing and purifying the enzyme conjugate. The stability of both reagents is excellent; according to the manufacturer, both the peroxidase-conjugated and unconjugated rabbit anti-FVIIIIRAg IgG are stable for at least three years, stored refrigerated. I have not observed any change in the performance of either antisera during six months. However, each new lot of antiserum must be evaluated by checkerboard titration, to choose the proper dilutions for the antibody coating and color development.

A prerequisite for the effective use of the assay is to establish reference values for a normal healthy population. The range I obtained, 0.51–1.77 Ag units/mL, agrees well with the reference ranges of 0.50–1.60 Ag units/mL and 0.52–1.59 Ag units/mL obtained by Zimmerman et al. (2) and Peake et al. (6), respectively, by the Laurell method.2 The advantage of the present method over the Laurell technique is in its simplicity and convenience; it can be completed in one day, allows determination of many samples in a single run, and is economical of reagents. As with the microplate modification (12), the method is suitable for large-scale use; if an automated instrument such as Gilford PR-50 processor/reader is available, a fully automated process can be achieved. However, the assay can be performed without special equipment by transferring the final colored solution to a cuvette of a regular spectrophotometer; a standard aspirating cuvette is especially useful for this. Because of its convenience, specificity, and economy, this assay is a useful alternative to currently used techniques.

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References