Application of Enzyme Immunoassays to Coagulation Testing
Jean Amiral, Benedicte Adalbert, and Mariette Adam

Enzyme immunoassays are very useful for the detection of low concentrations of coagulation proteins and pathological markers in plasma. Analytes in the ng/mL range are measurable with good reproducibility with intra- and interassay CVs of less than 5% to 10%. "Sandwich" methods have been developed for von Willebrand factor (plasma concentration about 8 μg/mL), Factor IX (5 μg/mL), protein C (4 μg/mL), and Factor X (10 μg/mL). However, this technique is only suitable for macromolecules; for low-molecular-mass peptides such as fibrinopeptide A a competitive method is used. Normal concentrations of fibrinopeptide A are below 3 ng/mL, with greater values suggesting in vivo generation of thrombin; thus this test is quite useful in detecting thrombosis. Reagents for both the sandwich and competitive methods are commercially available and cost effective, and have a longer shelf-life than those for radioimmunoassays.

Additional Keyphrases: "sandwich" immunoassay · thrombosis · von Willebrand factor · Factor IX · Factor X · protein C · fibrinopeptide A · peptides · thrombin

Maintenance of blood in a fluid state and prevention of hemorrhage requires a balance of activators and inhibitors of coagulation and fibrinolysis. Although clotting methods are useful for investigating most hemorrhagic diathesis and for monitoring procoagulant or hypocoagulant therapy, they are ineffective in early diagnosis of prethrombotic states and are limited for studying the subclinical stages of hemostatic disorders. A dramatic expansion in coagulation testing is currently underway. Highly sophisticated, specific, and ultrasensitive methods have been introduced for determination of low concentrations of coagulation and related proteins in plasma that can be used as pathological markers. Purification and characterization of coagulation factors and related peptides have led to the development of immunoanalytical methods for these analytes. Besides their activity, the absolute concentrations of these analytes can be measured (1). Moreover, measurement of analytes that have no specific activity, such as degradation products or peptides resulting from activation processes, has become possible (2).

The relative amounts in which coagulation factors and related proteins or peptides are present in blood vary widely, ranging from nanograms to milligrams per milliliter—quantities that can be easily measured by using immunoassays with labels (radioimmunoassay, enzyme immunoassay, fluoroimmunoassay) (3). Immunoassays for coagulation factors can provide useful information in the study of functional deficiencies, inactive complexes, abnormal proteins induced in various disease states or subsequent to therapy, the presence of pathological inhibitors, an increased catabolism, or abnormal degradation. Other new applications include the measurement of activation peptides (4), which are of high predictive value in early diagnosis of hypercoagulability or fibrinolysis.

Enzyme immunoassays have been introduced for von Willebrand factor (5–8), Factor IX (9, 10), Factor X (11), protein C (12), and fibrinopeptide A (13), with new applications under development. Our laboratory has developed various enzyme immunoassays for plasma coagulation proteins and molecular markers of various pathologic states. Depending on the molecular mass of the analyte, we use two different analytical principles (14, 15): an ELISA (enzyme-linked immunosorbent "sandwich" assay) method or a competitive method. Here we explain these in detail as they have been adapted for use in the coagulation laboratory.

Materials and Methods

Reagents

All the reagents are analytical grade and purchased from Prolabo, Paris, France, except when specified otherwise. Preparation of antisera: Ultra-specific antibodies with the high affinity required for these analytes are obtained from hyperimmune animals, immunized with the purified antigen (16). Rabbit or goat sera are used. When necessary, trace amounts of nonspecific antibodies are removed by adsorption to solid phase. Specificity of antisera is tested by conventional methods (Ouchterlony, immunoelectrophoresis) and by two-dimensional electrophoresis with a high concentration of antiserum in the second migration step. Specificity is checked again in the more-sensitive enzyme immunoassay. Antibodies are purified either by ion-exchange chromatography on DEAE-Trisacryl (IBF, Ville-neuve la Garenne, France) or affinity chromatography on columns of agarose-linked specific antigens.

Preparation of enzyme immunoassay reagents: Figure 1 illustrates the preparation of reagents for the ELISA sandwich method. Peroxidase (type VI; Sigma Chemical Co., St. Louis, MO) was chosen to label antibodies because of its...
ready availability, low cost, high activity, and chromogenic characteristics (17). In our hands, the reaction of o-phenylendiamine with hydrogen peroxide produces high responses and good sensitivity. Labeling antibodies with alkaline phosphatase or β-galactosidase is also potentially useful. The respective substrates with these methods would be p-nitrophenyl phosphate and o-nitrophenyl β-galactoside (18). Antibody-peroxidase conjugates are prepared according to Nakane and Kawai (19). Unreacted proteins are removed by repeated salt fractionation with ammonium sulfate.

F(ab')2 fragments are obtained by digesting the specific immunoglobulin (20) with pepsin (Sigma). To check the specificity of the reagents [F(ab')2 fragments and antibody–enzyme conjugates] in the routine enzyme immunoassay procedure, we use plasma from patients homozygous for severe deficiencies in specific factors. Values for these samples must be similar to those obtained for buffer alone.

Dilution buffer: per liter, 50 mmol of phosphate, 150 mmol of sodium chloride, 1 mL of Tween 20, and 1 g of bovine serum albumin; final pH is 7.50.

Washing solution: per liter, 20 mmol of phosphate, 150 mmol of sodium chloride, and 1 mL of Tween 20, adjusted to pH 7.00.

Substrate: o-phenylenediamine, 0.4 g/L of phosphate/citrate buffer (50 mmol of phosphate and 25 mmol of citrate per liter, pH 6.00). Just before use, add 40 μL of a 30% hydrogen peroxide solution per 100 mL of substrate solution.

Solid-phase support: Microplates were purchased from several manufacturers: Nunc, Roskilde, Denmark; Costar, OSI, Paris, France; or Dynatech, South Windham, ME; Maxisorp tubes for ELISA were purchased from Nunc.

Procedures

ELISA "sandwich" method: This method is used for von Willebrand factor, Factors IX and X, and coagulation protein C. Coat a solid-phase support (microplate or tube) with excess specific F(ab')2 fragments; remove unbound material by repeated washes with washing buffer. Add standard or test sample (antigen binds to the surface-linked antibodies), wash once again, then add the immunocompoundate, which binds to free antigenic sites. Remove excess conjugate by washing again, then measure peroxidase activity by its oxidation of o-phenylenediamine. Stop the development of color by adding sulfuric acid (3 mol/L), then measure absorbance at 492 nm. F(ab')2 fragments, instead of whole immunoglobulin, are used to avoid possible interference from rheumatoid factor (21). The enzyme immunoassays we evaluated and report on here are from the Asserachrom® line (Diagnostica Stago, Asnières, France).

Competitive method for fibrinopeptide A: The competitive method for enzyme immunoassays, which has been reported in detail by Soria et al. (13), involves competition for a limited amount of rabbit antibodies to fibrinopeptide A (FPA) between the test FPA (free in the sample) and immobilized FPA (coated on a solid-phase support). Concentrations of bound antibodies, which are inversely related to the concentrations of test FPA, are measured with sheep immunoglobulins (anti-rabbit IgG) labeled with peroxidase. Subsequent steps are identical to those described for the ELISA "sandwich" method. Specificity of the determination is achieved by collecting samples into a special anticoagulant supplemented with inhibitors and by using bentonite to remove cross-reacting fibrinogen before analysis (22). We compared the results by a commercially available test (Asserachrom FPA, Diagnostica Stago) with those by a radioimmunoassay kit (Mallinckrodt, St. Louis, MO).

Plasma collection: Collect blood in sodium citrate (110 mmol/L; citrate/blood, 1/9 by vol). Centrifuge, then separate the plasma and freeze at −80 °C until used. Calibrate with small aliquots of plasma pooled from normal individuals (15, in our studies) that has been kept frozen (−80 °C).

To determine various proteins (Factor IX, von Willebrand's factor, and protein C) by electroimmunodiffusion, we used Assera*-Plates (Diagnostica Stago) and the method of Laurell.

Results

Table 1 summarizes the performance characteristics of the Asserachrom kits. Inter- and intra-assay reproducibilities are good, and results depend on the concentrations of the analytes. Detection limits are in the nanogram range and compare well with those obtained with radioimmunoassays for similar applications. Determinations take 3 to 5 h, depending on the incubation time (1 to 2 h) chosen for each step. These assay characteristics are well adapted to the concentrations and variations observed clinically with these analytes (Table 2).

Von Willebrand factor: Determination of this coagulation factor (also known as VIII R:Ag) is useful in the diagnosis of von Willebrand's disease. Although several types of immunoassays for it have been proposed (5, 8, 23, 24), the sandwich method appears to be the more practical because no pure antigen is needed and specific antibodies are available. This method has been fully applied in hemophilia A, in von Willebrand's disease (particularly in severe deficiencies, where very low quantities are present), in the identification and classification of carriers of hemophilia A, and in evaluating endothelial cell damage (25).

By the ELISA method for von Willebrand factor, the mean percentage of the factor in plasma from 30 normal individ-

<table>
<thead>
<tr>
<th>Table 1. Some Characteristics of Asserachrom Kits for Measuring Coagulation Proteins in Plasma</th>
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<tbody>
<tr>
<td><strong>Analyte</strong></td>
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<tr>
<td></td>
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<tr>
<td>Sandwich ELISA von Willebrand factor</td>
</tr>
<tr>
<td>Factor IX</td>
</tr>
<tr>
<td>Factor X</td>
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<tr>
<td>Protein C</td>
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<tr>
<td>Competitive enzyme immunoassay FPA</td>
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*: In terms of dilution of normal pooled plasma. b: ng/mL.

Table 2. Characteristics of Various Analytes Measured by Enzyme Immunoassay

<table>
<thead>
<tr>
<th><strong>Analyte</strong></th>
<th>Concentration, int. units/L (and μg/mL)</th>
<th>Normal range</th>
<th>Abnormal range</th>
</tr>
</thead>
<tbody>
<tr>
<td>von Willebrand's factor</td>
<td>&gt;1 × 10⁶</td>
<td>600–1500</td>
<td>&lt;600, &gt;2000</td>
</tr>
<tr>
<td>Factor IX</td>
<td>55 000</td>
<td>600–1400</td>
<td>&lt;600</td>
</tr>
<tr>
<td>Factor X</td>
<td>65 000</td>
<td>700–1300</td>
<td>&lt;600</td>
</tr>
<tr>
<td>Protein C</td>
<td>62 000</td>
<td>600–1400</td>
<td>&lt;600</td>
</tr>
<tr>
<td>FPA</td>
<td>1536</td>
<td>&lt;3</td>
<td>≥3</td>
</tr>
</tbody>
</table>

*: ng/mL.
uals and from 10 patients suffering from hemophilia A was 101.3 (SD 24.8)% and 103.2 (SD 22.8)% respectively (100% = the value in pooled normal human plasma). Results of single determinations on five patients with von Willebrand's disease were 0.1, 6.7, 18, and 30%. Correlation between these results and those by the Laurell electromunoimmunodiffusion method was good for normals, patients with hemophilia A, and patients with classical von Willebrand's disease (results not shown). Moreover, the presence of an abnormal protein (as in von Willebrand's disease type II) changes the results obtained with both the ELSA and the Laurell methods, indicating that a different electrophoretic mobility and a loss of immunoreactivity to antibodies is characteristic of the abnormal protein (6).

**Factors IX, X, and protein C**: These vitamin K-dependent factors, synthetized in the liver in low concentrations, are of diagnostic value in various pathological states. Deficiencies or abnormalities of Factor IX result in one of the many heterogeneous types of hemophilia B. Measurement of this factor allows classification of hemophilia B into subclasses (B⁺, B⁻, or B馕) (26), and may help to detect carriers of hemophilia B and make prenatal diagnoses of these deficiencies. Protein C has a major role in regulating in vivo activation of blood, by inactivation of thrombin-modified Factors VIII:C and V. Congenital or acquired deficiencies of protein C result in thrombotic tendencies (27), and its routine measurement in coagulation laboratories can aid in the diagnosis of thrombosis. Concentrations of Factor X, Factor IX, and protein C are decreased in liver cirrhosis and in patients treated with oral anticoagulant drugs, e.g., warfarin. Table 3 shows several applications of the measurement of these three factors in various groups of patients. In addition, patients with various subclasses of hemophilia B were tested for Factor IX, for which normal (B⁺), low (B⁻), and very low (B ينب) values were consistently obtained for the concentration of antigen. For these several groups of patients, there was a good correlation between the ELSA method and the Laurell electromunoimmunodiffusion method (results not shown). We observed no discrepancy between results by each, and mean values and standard deviations by both methods did not significantly differ.

In warfarin-treated patients, the concentrations of Factors IX and X and protein C, measured immunologically, are decreased, but usually less than as measured with clot-based functional assays. Although both normal and abnormal proteins are synthesized in the liver during therapy with anticoagulant, the total functional concentration of these in plasma is lowered. However, the antibodies used in these tests react with normal molecules as well as with the abnormal forms (hypo or acarboxylated) induced by anticoagulant therapy. Therefore the concentrations of antigenic and functional proteins do not always coincide. Protein C and Factor X are decreased to a comparable extent, with similar variations. Factor IX is less decreased in the warfarin-treated group. In patients suffering from cirrhosis of the liver, protein C is the factor decreased the most, reaching very low concentrations in some patients. Factor IX and Factor X present much greater variations than other factors, but their total decrease is less severe.

**Fibrinopeptide A** (FPA): This is a marker of high predictive value for the early diagnosis of hypercoagulability (28), and can be used to monitor procoagulant and hypocoagulant therapy, and to survey post-surgical states, heart diseases, progression of cancer, and risk of thrombosis. Above-normal concentrations, >3 mg/mL, indicate thrombin generation in vivo. Mean FPA values for 20 normal subjects and 10 patients with active thrombosis were 1.2 (SD 0.6) and 22.8 (15.4) ng/mL, respectively, with the Asserachrome FPA kit, and 1.1 (SD 0.8) and 23.6 (SD 14.8) with the competitive radioimmunoassay kit (Mallinckrodt).

**Discussion**

Amounts in which coagulation factors and related proteins or peptides are present in blood vary widely, ranging from nanograms to milligrams per milliliter. Thus a wide variety of immunological methods, with various detection sensitivities, can be used. Conventional methods (radioimmunoassay, electromunoimmunofiltration, immunonephelometry, or agglutination) can be used for proteins present in medium or high concentrations (above 10 μg/mL). Small quantities such as micrograms or nanograms per milliliter can be easily measured by using labeled immunoassay or fluorimmunoassay techniques. For protein concentrations in the range 1–20 μg/mL, both types of methods may be considered, depending on the accuracy and sensitivity needed. Figure 2 summarizes plasma concentrations of the main coagulation proteins and related peptides, and shows the working range of the main immunological methods used for their assay.

For some coagulation factors, immunoassays provide useful information. In physiological states, there is usually a strong correlation between the quantity of a specific protein and its associated activities, although there are discrepancies in various circumstances. Immunoassays based on polyclonal antibodies measure the entire protein. If the antiserum has been obtained from hyperimmune animals, there is a complete recognition of the structure of the molecule, and normal and abnormal proteins are determined to the same extent. The development of monoclonal antibodies, however, introduces quite a different kind of analysis. Monoclonal antibodies can be selected to react specifically with a single epitope and can provide a better knowledge of protein structure and intermolecular interactions.

Obtaining a good surface coating is of crucial importance to the performance of these assays. The solid phase to be coated must be carefully chosen. It must offer high binding capacities and stable biological and immunological reactivity of linked material, as well as good reproducibility within batch and from batch to batch. The amount of coated material determines the performance of the assay; use of too little coating severely reduces the rate of color development. We successfully used the three microplates (Nunc Type 1, Dynatech M129B, or Costar 3590) and the Nunc Maxisorp tubes for the enzyme immunoassays reported here. In these assays the reaction takes place on a solid surface, so that immunological equilibrium is reached slowly, taking at least 2 h at room temperature. However, one can reduce incubation times to 1 h (or even 30 min) without affecting

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Factor IX</th>
<th>Factor X</th>
<th>Protein C</th>
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<tbody>
<tr>
<td>Normal (n = 40)</td>
<td>102.3</td>
<td>19.5</td>
<td>98.6</td>
</tr>
<tr>
<td>Dicoumarol-treated (n = 25)</td>
<td>54.6</td>
<td>16.2</td>
<td>44.1</td>
</tr>
<tr>
<td>Liver cirrhosis (n = 12)</td>
<td>54.3</td>
<td>19.2</td>
<td>37.5</td>
</tr>
<tr>
<td>Hemophilia (n = 5 each)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B⁺</td>
<td>92</td>
<td>23.1</td>
<td></td>
</tr>
<tr>
<td>B⁻</td>
<td>22</td>
<td>8.1</td>
<td></td>
</tr>
<tr>
<td>B ينب</td>
<td>0.48</td>
<td>0.27</td>
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Table 3. Factors IX, X, and Protein C Measured by ELSA Sandwich for Different Groups of Subjects (% normal human pooled plasma = 100%)

1514 CLINICAL CHEMISTRY, Vol. 30, No. 9, 1984
the results, provided the incubation times are exactly the same for the standard and test dilutions. With standard working conditions, color develops in 2 to 3 min; shortening the incubation times increases the time for this development, usually to 5 min. Absorbance at 492 nm is between 1 and 1.5 for the highest point of the calibration curve (Figure 3).

Enzyme immunoassays are convenient methods for routine use in clinical laboratories. Numerous possibilities for automation exist (29). A multi-channel pipette for distribution, a washing machine, and a reader (allowing good linearity up to an absorbance of 2.0) are useful for obtaining reproducible results. Washing is critical because nonbound material must be removed completely to minimize nonspecific results.

Introduction of enzyme immunoassays in the field of hemostasis allows determination of low-concentration coagulation proteins in plasma and trace amounts of molecular markers of pathologic states. Use of commercial kits for these determinations standardizes performance characteristics and makes it possible to include these tests in the routine coagulation laboratories. Simplified procedures and specific and automatic equipment allow for analysis of large series of samples. This breakthrough in the practice of coagulation testing is due in part to the introduction of advanced immunotechnology.

Sensitivities in the nanogram range are available in most specialized laboratories. Important developments to be expected in the future will include the evaluation of molecular markers, which should improve the detection of prethrom-
botic states and prevention of thrombosis (4). Determination of FPA is, in this way, a very important test for this sensitive marker of thrombin generation in vivo. In coming years development of hybridoma technology will introduce more standardized tests with well-characterized monoclonal antibodies. In addition, studies of molecular interactions that result from minor but pathological enzymic activities will become available.

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References