A Novel Clotting Assay for Quantitation of Plasma Prothrombin (Factor II) Using Echis multisquamatus Venom

Ramona J. Petrovan, PhD,1 Samuel I. Rapaport, MD,2 and Dzung T. Le, MD, PhD1

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Abstract

Measuring plasma prothrombin activity seems useful for evaluating thrombotic risk and managing oral anticoagulant therapy as an adjunct to the international normalized ratio. Therefore, we designed a new plasma prothrombin assay based on the ability of Echis multisquamatus venom to activate prothrombin with only calcium as a cofactor. In this assay, 1 part of undiluted citrated plasma is added to 5 parts of a venom reagent and the clotting time is measured. The assay’s advantages are that dilution of the test plasma is required only when prothrombin activity exceeds 100%, a single standard curve can be used over months for a given batch of stock reagent, and barium-adsorbed plasma is used for dilution of test plasma and construction of the standard curve, thus eliminating the need for prothrombin-deficient plasma. However, one should be aware of the following: (1) test samples must contain at least 200 mg/dL fibrinogen; and (2) when prothrombin concentrations were below 50%, the venom-based assay often gave values up to 10% higher than the thromboplastin-based assay. Values obtained in 262 plasma samples tested with the venom-based assay and with a thromboplastin-based prothrombin assay correlated well ($r^2 = 0.93$).

Plasma functional prothrombin concentration can be determined by measuring its activity in clotting assays using a prothrombin-deficient substrate or by measuring native, fully carboxylated prothrombin antigen in an enzyme-linked immunosorbent assay.1-2 Since these assays require a dedicated coagulation laboratory with specialized instruments, they are not readily available in many clinical settings. Nevertheless, measuring prothrombin levels may be useful in addition to the international normalized ratio (INR) for monitoring oral anticoagulation, because the antithrombotic effectiveness of oral anticoagulants depends on reducing the plasma level of functionally active prothrombin.3-4 Moreover, a genetic variation in the 3’-untranslated region of the messenger RNA of the prothrombin gene (20210G to A), present in 2% to 3% of white populations, is a newly recognized risk factor for venous thrombosis5-9 that is associated with an increased plasma prothrombin level. Evidence has also been reported relating thrombotic risk to the level of plasma prothrombin activity.5 Hence, measuring plasma prothrombin activity may prove useful as part of an initial battery of coagulation tests to evaluate a person’s thrombotic risk.

The venom of the viper Echis multisquamatus contains an enzyme capable of activating prothrombin in the absence of cofactors other than Ca$^{2+}$10,11 Since the enzyme requires the posttranslational modification of the Glu domain in prothrombin, it can be used to measure functional prothrombin concentration in patients receiving an oral anticoagulant. We report the standardization of a specific functional prothrombin assay and an analysis of data from 262 individual samples in which the prothrombin concentration...
was measured with the venom-based assay and a standard thromboplastin-based prothrombin assay.

Materials and Methods

Materials

The crude venom from *E multisquamatus* was obtained from Latoxan (France) and Atroxin (Sigma, St Louis, MO). Innovin (recombinant human tissue factor thromboplastin) was purchased from Dade International (Miami, FL). Prothrombin-deficient plasma was Diagnostica Stago Deficient II, supplied by American Bioproducts (Persippany, NJ). Human fibrinogen (plasminogen-depleted) was purchased from Calbiochem (La Jolla, CA). Gradipore dRVVT- and LA-confirm reagents were supplied by Rainbow Scientific (Windsor, CT). Second International Standard for factors II, VII, IX, and X plasma (94/746) was obtained from NIBSC (South Mimms, Potters Bar, Hertz, UK).

Plasma Samples

Normal reference plasma was pooled plasma from 25 healthy donors. It was prepared from venous blood collected in one tenth of volume buffered citrate anticoagulant (0.065 mol/L sodium citrate, 0.045 mol/L citric acid, pH 7.4). Platelet-rich plasma (>150,000/µL) was prepared by centrifugation at 500g for 10 minutes at room temperature and stored in aliquots at −80°C. Platelet-poor plasma was separated by centrifugation 2 times at 1,800g for 15 minutes at room temperature, pooled, and stored in aliquots at −80°C. The prothrombin concentration of this reference plasma, which was 1 IU FII as determined against the International Standard Plasma, was assigned a value of 100%. Two control plasma samples were prepared similarly and stored in aliquots at −80°C: 1 from a healthy donor with a normal plasma prothrombin concentration and 1 from a person taking a stable dose of warfarin with a low plasma prothrombin concentration. Test samples were collected from healthy volunteers at the University of California San Diego (UCSD) and from patients at the UCSD Medical Center in accordance with the requirements of UCSD’s institutional review boards. Plasma samples were prepared by centrifugation for 1 or 2 times and assayed fresh or after storage at −80°C.

Barium-adsorbed plasma was prepared from donors with a fibrinogen concentration exceeding 300 mg/dL. Platelet-poor plasma was stirred with 20 mg/mL of barium chloride for 30 minutes at 4°C, followed by centrifugation at 3,600g for 30 minutes at 4°C. The procedure was repeated using 15 mg/mL of BaCl₂, and the supernatant was dialyzed against HEPES buffer (25 mmol/L HEPES (pH 7.5), 150 mmol/L sodium chloride) overnight at 4°C. Adsorption did not alter the ability of the fibrinogen to clot in the plasma; when decreasing concentrations of thrombin were added to plasma before and after adsorption, similarly increasing clotting times of up to 80 seconds were obtained.

Clotting Assays

All clotting times were determined in duplicate with a Diagnostica Stago ST4 semiautomatic coagulometer (American Bioproducts).

Thromboplastin-Based Prothrombin Assay

Fifty microliters of test plasma, diluted 1:5 and 1:10 in saline, was incubated for 3 minutes at 37°C with 50 µL of a prothrombin-depleted plasma reagent (Diagnostica Stago) and clotted by the addition of 100 µL of Innovin. Clotting times were converted to percentage of normal plasma prothrombin (% NP-FII) from a log-log curve prepared with dilutions between 1:5 and 1:40 (corresponding to 100% to 12.5% NP-FII) in saline of normal reference plasma. Standard curves were prepared on each day of testing.

Venom-Based Prothrombin Assay

A stock venom reagent of 0.2 mg/mL of lyophilized *Echis multisquamatus* crude venom in 25-mmol/L concentration of HEPES (pH 7.5), 150-mmol/L concentration of sodium chloride, and 1-mmol/L concentration of calcium chloride was prepared. Aliquots of 0.5 to 1 mL of this stock reagent remained stable for at least 6 months when stored at −80°C, and could be frozen and thawed at least 2 times without loss of activity. (Although not recommended, aliquots were stable for several weeks when stored at −20°C.)

A working venom reagent containing 16.7 µg/mL of venom in 25 mmol/L HEPES (pH 7.5), 150 mmol/L sodium chloride, and 5 mmol/L calcium chloride was prepared by adding 1 part of stock reagent to 11 parts of 25-mmol/L HEPES (pH 7.5), 150 mmol/L sodium chloride, and 5.4 mmol/L calcium chloride buffer. This working reagent usually was prepared daily but was found to be stable when frozen at −20°C and used on a following day.

In the routine assay, 25 µL of undiluted citrated test plasma was incubated at 37°C for 3 minutes and clotted by the addition of 125 µL of venom reagent that had been warmed to 37°C. For some test plasma samples (see the “Results” section), the assay was repeated after diluting the test sample 1:1 in barium-adsorbed plasma.

Clotting times were converted to prothrombin concentration from a log-log standard curve in which undiluted normal reference plasma was used for the 100% value. Serial
dilutions of the normal reference plasma up to 1:8 (12.5% NP-FII) were made in barium-adsorbed plasma.

Results

Standardization of the Assay

In preliminary experiments, the concentration of the *E. multissquamatus* and Ca\(^{2+}\) ions in the venom reagent and the final dilution of the test plasma in the reaction mixture were varied. Adding 25 \(\mu\)L of undiluted test plasma to 125 \(\mu\)L of a venom reagent containing 16.7 \(\mu\)g/mL of venom and 5 mmol/L calcium chloride were found to be the optimal conditions for the assay (data not shown). Clotting times of the normal prothrombin and low prothrombin control plasma samples were essentially constant when samples were tested immediately, after standing for up to 6 hours at room temperature or at 4°C, or after storage at -80°C for several months.

Two batches of stock venom reagent were used throughout the year of the study. Over a period of several months, standard curves made with working venom reagent prepared from either stock reagent were virtually identical. In contrast, a higher degree of variability was found for standard curves of the thromboplastin-based prothrombin assay. However, the slope of a composite standard curve of the venom-based assay, constructed from 6 individual curves, was similar to the slope of a composite standard curve of the thromboplastin-based prothrombin assay constructed from 9 individual curves (Figure 1).

Intra-Assay and Interassay Variations

A normal prothrombin control plasma sample and a low prothrombin control plasma sample were tested 10 times in a single run of the venom-based assay. Means ± SD for the clotting times were as follows: for the normal prothrombin plasma, 22.9 ± 0.3 seconds, coefficient of variation (CV) = 1.6%; for the low prothrombin plasma sample, 40.4 ± 0.8 seconds, CV = 1.9%. The corresponding values for percentage prothrombin concentration were as follows: for the normal prothrombin plasma sample, 112% ± 4%, CV = 3.1%; for the low prothrombin plasma sample, 30% ± 1%, CV = 3.9%.

Aliquots of the 2 control plasma samples were thawed on 10 different days and tested with working venom reagent prepared each day from the same batch of stock venom reagent. Means ± SD for the clotting times were as follows: for the normal prothrombin plasma sample, 22.0 ± 0.4 seconds, CV = 1.8%; for the low prothrombin plasma sample, 40.9 ± 0.7 seconds, CV = 1.6%. The corresponding percentage prothrombin values were as follows: for the normal prothrombin plasma sample, 114% ± 4%, CV = 3.6%; for the low prothrombin plasma sample, 30% ± 1%, CV = 3.2%.

We also determined the intra-assay variation for the thromboplastin-based assay using the same normal prothrombin control plasma sample but a different low prothrombin plasma sample. Means ± SD for the clotting times (n = 10) were as follows: for the normal prothrombin plasma, 15.6 ± 0.4 seconds, CV = 2.7%; for the low prothrombin plasma sample, 26.7 ± 0.8 seconds, CV = 2.9%. The corresponding percentage prothrombin values were as follows: for the normal prothrombin plasma, 115% ± 7%, CV = 5.8%; for the low prothrombin plasma sample, 18% ± 1%, CV = 6.0%.

Use of a Composite Standard Curve for the Venom-Based Assay

Because it was found that the standard curves for the venom-based assay were virtually identical on different days and the interassay variations of the normal prothrombin and low prothrombin control samples were minimal, it was not necessary to prepare a new standard curve each day for the venom-based assay. Rather, a composite standard curve was prepared from several standard curves (Figure 1) and used thereafter for a given
batch of stock reagent. On each test day, the working venom reagent was validated by assaying a thawed aliquot of the normal prothrombin control plasma sample and of the low prothrombin control plasma sample. After verifying that the working venom reagent yielded the expected clotting times for each control plasma sample, clotting times from test plasma samples were converted to percentage prothrombin concentration from the composite standard curve.

Lack of Effect of Platelet or Citrate Concentration of a Test Sample

Platelet-rich and platelet-poor plasma samples were prepared from a healthy person and from a person receiving warfarin. Aliquots of each were tested fresh and after repeated freezing and thawing to liberate phospholipid from lysed platelets. Clotting times were essentially identical for all aliquots: ± 0.3 seconds for fresh platelet-rich vs fresh platelet-poor plasma; ± 0.4 seconds for the samples after 5 freeze-thaw cycles. In a second experiment, plasma samples were prepared from whole blood collected from these persons in 1/10 vol/vol of 50-, 100-, or 150-mmol/L concentrations of buffered citrate. Clotting times varied by only ± 0.2 seconds for the plasma sample with a normal prothrombin level and by only ± 0.5 seconds for the plasma sample with a low prothrombin concentration.

Effect of Heparin on the Venom-Based Assay

Heparin was added to the normal prothrombin and the low prothrombin control plasma samples to yield a final heparin concentration of up to 2 U/mL. The resulting clotting times were as follows: for the normal prothrombin plasma sample, without heparin 22.0 seconds, with 1 U/mL heparin 22.4 seconds, and with 2 U/mL heparin 22.9 seconds; for the low prothrombin plasma sample, without heparin 45.2 seconds, with 1 U/mL heparin 47.8 seconds, and with 2 U/mL heparin 48.3 seconds.

Effect of Plasma Fibrinogen Concentration on the Clotting Time

The test plasma, which is diluted 6-fold in the reaction mixture, is the sole source of fibrinogen for the assay. Therefore, we determined the effect of the fibrinogen concentration of a test plasma sample on its clotting time. A plasma sample with a normal prothrombin concentration and a plasma sample with a low prothrombin concentration from a patient receiving stable, long-term, prophylactic warfarin therapy were defibrinated with Atroxin.12 These defibrinated plasma samples were mixed in different proportions with their respective untreated plasma samples to vary the fibrinogen concentration of the mixtures while keeping their prothrombin concentrations constant. Variation of fibrinogen concentration varied by only ± 0.2 seconds for the plasma sample with a normal prothrombin level and by only ± 0.5 seconds for the plasma sample with a low prothrombin concentration.

Effect of Heparin on the Venom-Based Assay

Heparin was added to the normal prothrombin and the low prothrombin control plasma samples to yield a final heparin concentration of up to 2 U/mL. The resulting clotting times were as follows: for the normal prothrombin plasma sample, without heparin 22.0 seconds, with 1 U/mL heparin 22.4 seconds, and with 2 U/mL heparin 22.9 seconds; for the low prothrombin plasma sample, without heparin 45.2 seconds, with 1 U/mL heparin 47.8 seconds, and with 2 U/mL heparin 48.3 seconds.
concentration had a minimal but noticeable effect on the clotting time at concentrations above 200 mg/dL. Figure 2. Thus, as the fibrinogen concentration of the low prothrombin control decreased from 300 mg/dL to 200 mg/dL, its clotting time increased from 45.0 seconds to 48.7 seconds (24.8% and 29.5% prothrombin concentrations, respectively). As the fibrinogen concentration of the normal control sample decreased from 300 mg/dL to 200 mg/dL, its clotting time increased from 23.5 seconds to 25.0 seconds (101.1% and 82.3% prothrombin concentrations, respectively). On reducing the fibrinogen concentrations below 200 mg/dL, clotting times progressively increased to yield substantially lower values for prothrombin in the test sample. In an additional experiment, purified fibrinogen was added to a normal plasma sample to increase its initial fibrinogen concentration of 380 mg/dL to 530 mg/dL. The clotting times were as follows: 22.8 seconds at 380 mg/dL and 21.9 seconds at 530 mg/dL.

Results In Patients Receiving Warfarin and Patients Receiving a Lupus Anticoagulant

Prothrombin concentration was determined by the venom-based assay and the thromboplastin-based assay in plasma samples from 25 patients receiving long-term warfarin therapy for chronic thrombotic disease. The data and the linear regression equation are shown in Figure 3. The assays also were performed on samples from 25 additional patients with a lupus anticoagulant as demonstrated by a prolonged dilute Russell viper venom time (dRVVT) and a positive dilute viper venom-confirm test.13 The low prothrombin concentration (<65%) of many of the lupus anticoagulant samples also reflects the effect of warfarin therapy. The data and linear regression equation are shown in Figure 4.

Results With Samples Containing a High Concentration of Prothrombin

Samples with a prothrombin concentration of 90% or more as initially determined at a 1:5 dilution in the thromboplastin-based method were tested again at a 1:10 dilution in the thromboplastin-based assay and at a 1:1 dilution in barium-adsorbed plasma in the venom-based assay. The 1:1 dilution in the venom-based assay was necessary to bring all clotting times within the range of the standard curve in which 100% corresponds to the clotting time obtained with undiluted reference plasma. The data are included in Figure 5.

Results Obtained From All Plasma Samples

Prothrombin levels as determined by both methods in 57 healthy subjects and 205 patients (total, 262 plasma samples)
are plotted in Figure 5. The calculated linear regression line for the total data is $y = 0.84x + 9.8$, $r^2 = 0.93$.

Discussion

We describe a single-step clotting assay for plasma prothrombin activity based on an earlier observation that the venom from *E. multiscuamatus* efficiently activates $\gamma$-carboxylated prothrombin in the absence of cofactors other than Ca$^{2+}$.10-11 The major differences and similarities between the venom-based assay and the traditional thromboplastin-based assay are summarized in Table II and discussed in the following paragraphs.

The assay is easier to perform than thromboplastin-based assays for the following reasons:

- Test plasma samples usually do not require dilution before assay. Only if the test plasma sample has a shorter clotting time than the 100% point on the standard curve is a repeated assay on a 1:1 dilution of the test plasma sample needed.
- A new standard reference curve is not required on each test day. A single standard curve prepared as described in the "Materials and Methods" section can be used over many months because of the stability of the stock venom reagent and the very low intra-assay and interassay CVs. One needs only to check the working venom reagent on a given test day by measuring the clotting time of 2 control plasma samples, one with a normal and the other with a low prothrombin concentration.

The venom-based assay has an additional advantage over thromboplastin-based assays because it eliminates the need for a costly prothrombin-deficient substrate plasma sample. A barium-adsorbed plasma sample, which can be stored in small aliquots for months at −80°C, is used for the construction of the standard curves and to dilute a test plasma sample as needed. Nevertheless, it should be emphasized that in preliminary experiments, the use of lyophilized normal or prothrombin-deficient plasma samples yielded unreliable results. Therefore, we recommend that the assay be used only with fresh plasma or stored frozen plasma samples.

The venom-based assay can be performed on plasma samples containing heparin, on plasma samples from patients receiving oral anticoagulant therapy (Figure 3), and on plasma samples from patients receiving a lupus anticoagulant (Figure 4). With more than 250 plasma samples tested to date, plasma prothrombin concentrations determined in the venom-based assay correlated well with plasma prothrombin concentrations obtained with a traditional thromboplastin-based assay ($r^2 = 0.93$).

However, as can be seen from the ideal correlation line of Figure 5 (dotted line), when prothrombin concentrations were below 50%, values in the venom-based assay were often up to 10% higher than values in the thromboplastin-based assay. Since virtually all values below 50% were from patients receiving oral anticoagulant therapy, their circulating prothrombin consisted of molecules with different degrees of $\gamma$-carboxylation. Although posttranslational modification of the Gla domain of prothrombin is required for the venom enzyme's activity,10 we suspect that the venom enzyme can activate partially $\gamma$-carboxylated prothrombin more efficiently than can the prothrombinase complex formed in the thromboplastin-based assay we used.

One limitation of the method deserves emphasis. The test plasma sample, which is diluted 1:6 in the reaction mixture, provides not only the prothrombin but also the fibrinogen needed for clotting. As seen in Figure 2, a test sample should contain not less than 200 mg/dL of fibrinogen, as measured by the Clauss method,14 to obtain an accurate test result. Hence, the assay is not recommended for

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<tr>
<td>Test plasma</td>
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<tr>
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<td>Minimal to 2 U/mL</td>
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<tr>
<td>Effect of fibrinogen</td>
<td>Yes, &lt;200 mg/dL</td>
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</tbody>
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FII = prothrombin; CV = coefficient of variation; ND = not done.

* According to the manufacturer.
† Except for very high-titer anticoagulant.
measuring prothrombin concentration in patients with a low fibrinogen level, as may be encountered in severe liver disease or disseminated intravascular coagulation. However, since the normal range for plasma fibrinogen concentration is 200 to 400 mg/dL and fibrinogen, an acute phase protein, is increased in most clinical disorders, the assay should prove reliable when it may be clinically useful to measure the plasma prothrombin concentration.

We believe that a simple rapid assay for plasma functional prothrombin can complement the use of the INR for managing patients receiving an oral anticoagulant. Substantial evidence exists that decreasing functionally active plasma prothrombin concentration is essential for the antithrombotic effectiveness of oral anticoagulation.\(^{3,4,15}\)

However, for several days after oral anticoagulant therapy is begun or changed, the INR predominantly reflects changes in the factor VII concentration rather than changes in the prothrombin concentration.\(^{15-18}\) Thus, for patients beginning anticoagulant therapy with both heparin and warfarin, supplementing the INR with determinations of functional prothrombin concentration can help decide when to discontinue heparin. Measuring the prothrombin concentration in addition to the INR also may have value when the oral anticoagulant dosage is varied abruptly, eg, before and after emergency surgery.

In a large study, all heterozygotes for the prothrombin gene mutation G20210A were found to have a plasma prothrombin concentration of 95% or more.\(^5\) Furthermore, an increased plasma prothrombin level was found to be an independent risk factor for thrombosis.\(^5\) Because of its simplicity, the venom-based assay should facilitate additional studies of the relation between the prothrombin gene mutation G20210A, plasma prothrombin activity, and an increased risk for thrombotic events. Such further data should enhance understanding of the role of prothrombin in the pathogenesis of thrombotic disorders.

From the Departments of 1Pathology and 2Medicine, University of California, San Diego, CA.

Address reprint requests to Dr Le: Department of Pathology, UCSD School of Medicine, 9500 Gilman Dr, La Jolla, CA 92093-0612.

References


