A Colorimetric Assay for Releasable Plasminogen Activator

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We describe an equilibrium assay for measuring release of plasminogen activator from blood-vessel walls and report data from 125 individuals free of overt thromboembolic disease. Excess human plasminogen is added to the egulobulin fraction of plasma obtained before and after venous occlusion at mean systolic pressure. To measure plasminogen generation in these samples, we used the chromogenic plasmin substrate D-Val-Leu-Lys-p-nitroanilide, which liberates p-nitroaniline upon cleavage. Releasable plasminogen activator in 24 subjects was determined by this colorimetric assay and by the radioassay previously reported by this laboratory (Am. J. Clin. Pathol. 78, 403–409, 1981), and the results were compared. The correlation coefficient was 0.97. The colorimetric assay offers several advantages over the radioassay assay: shorter incubation (6 vs 16 h) and no preparation or quantification of a radioactive substrate and its cleavage products.

Additional Keyphrases: fibrinolysis • coagulation assays • reference interval • colorimetry • thromboembolic diseases • methods for the small laboratory • effects of oral contraceptives

Inadequate fibrinolysis involving either plasminogen or plasminogen activator has been established as one mechanism of hypercoagulability (1, 2). The coagulopathy primarily presents as venous thromboembolic disease and its consequences (1–5, 7–9), although defective fibrinolysis has also been associated with myocardial infarction and thrombotic strokes (6, 11). Defective plasminogen as a cause of thromboembolic diseases are rare (10) compared with defects in fibrinolysis resulting from deficient plasminogen activator (1–5, 7–9).

Plasminogen activation is a complex process, involving several potential plasminogen activators (1, 2). Kallikrein, and to a lesser extent Factor XIIa, both of which are generated during coagulation, are capable of activating plasminogen (12–15). Recently, plasma protein C has also been shown to stimulate fibrinolysis (16, 17). Protein C is itself activated only during coagulation, and it functions indirectly by stimulating release of vascular plasminogen activator (16, 17).

Vascular plasminogen activator appears to be the primary plasminogen activator functioning to activate the fibrinolytic system in vivo (1, 2); a defect in release of this activator has been demonstrated in all the studies of altered plasminogen activation and thrombotic diseases cited above (1–5, 7–9). Measurement of vascular plasminogen activator involves some procedure to stimulate release of the activator into the blood (1, 2). Venous occlusion at a pressure midway between systolic and diastolic pressure for 5 min has been developed as a standardized procedure to stimulate release of vascular plasminogen activator (18, 19). The activator is measured in the egulobulin fraction of blood, prepared by diluting and acidifying plasma, because this procedure removes inhibitors of the fibrinolytic system (19). The pre-occlusion blood samples so studied contain no vascular plasminogen activator (20, 21), and any plasminogen activator activity presumably results from kallikrein and Factor XIIa (12–15, 21). The post-occlusion blood sample contains vascular plasminogen activator, kallikrein, and Factor XIIa (20, 21). Subtraction of the post-occlusion value from the post-occlusion value thus provides a corrected measurement of releasable vascular plasminogen activator (8, 9, 18).

The recognition of a relationship between major thromboembolic events and extremely low concentrations of releasable plasminogen activator raises the possibility of identifying individuals at risk. Such individuals could be excluded from exposure to drugs or therapies known to induce hypercoagulability. Moreover, recent studies on the effects of moderate exercise on the fibrinolytic response (18, 20, 22) suggest that a planned exercise program might constitute a benign intervention for individuals with low concentrations of releasable plasminogen activator who are felt to be at risk for development of thromboembolic disease.

Draper et al. (23) recently reported a colorimetric assay for determining plasminogen activator secretion by mouse peritoneal macrophages (23). In the present report we present a similar method of quantification of releasable plasminogen activator, involving the chromogenic plasmin substrate D-Val-Leu-Lys-p-nitroanilide (S-2251) in the presence of excess exogenous plasminogen to measure the amount of plasminogen activator released from the vessel wall after a standardized venous occlusion. The assay is simple to perform, and the results are highly reproducible and compare well with those by a radiolabeled casein assay previously reported by this laboratory (9, 18, 24). Unlike the radioassay assay, no radiolabeled substrates are required and the incubation time is shorter. Measurements by this new technique are reported in standardized Committee on Thrombolytic Agents (CTA) units, to facilitate comparison of results from different laboratories.

Materials and Methods

Materials. The substrate, D-Val-Leu-Lys-p-nitroanilide (S-2251), was obtained either from Ortho Diagnostics, Raritan, NJ 08869 or the Kabi Group, Greenwich, CT 06830. Human plasminogen was prepared by affinity chromatography on lysine-Sepharose as previously described by Deutsch and Mertz (25). Urokinase (Calbiochem reference standard) was obtained from Calbiochem-Behring Corp, La Jolla, CA 92112. ³H]Casein was prepared as previously described (24). All other reagents were of the best available grade.

Plasma collection. The protocol has been previously described (8, 9, 18). In brief, volunteers reported for venipuncture after at least 3 h of refraining from cigarette use and overnight abstinence from alcohol. Subjects were also asked to refrain from vigorous physical activity on the day of testing. They remained seated for about 10 min before venipuncture. Two blood samples were obtained from each subject, by a
two-syringe technique. A baseline sample was drawn from an antecubital vein after inflation of a sphygmomanometer to 30 mmHg (4 kPa) for no longer than 1 min. A second sample was drawn from the opposite arm after 5 min of venous occlusion at a pressure midway between the systolic and diastolic pressures.

The blood was anticoagulated with sodium citrate (final concentration, 3.8 g/L) and centrifuged at 6000 x g for 10 min at 4 °C. The euglobulin fractions were prepared from 1 mL of both pre- and post-occlusion samples by the method of Walker et al. (18). The precipitates were redissolved in 1 mL of a pH 8.0 solution containing 1 g of sodium borate, 9 g of sodium chloride, and 1 g of EDTA per liter, and stored on ice until used.

Fibrinolysis assays. We measured plasminogen activator in the euglobulin fractions by the radioassay we previously reported (18, 24) and by the new colorimetric assay. The substrate S-2251 is specific for plasmin under the conditions used (23, 26). We added 50 μL of each reconstituted euglobulin fraction to 100 μL of a human plasminogen solution (5.07 μg of plasminogen in a buffer containing, per liter, 150 mmol of NaCl and 50 mmol of sodium phosphate at pH 7.4). We also examined the activity of the euglobulin fraction to hydrolyze S-2251 in incubations with buffer replacing plasminogen solution.

After diluting a 3 mmol/L solution of S-2251 in distilled water 9.5-fold with pH 7.4 buffer containing 12 mmol of NaCl and 50 mmol of Tris HCl per liter, we added 350 μL of the solution to the euglobulin-plasminogen mixture, then incubated at 37 °C for 6 h.

To prepare a standard curve for quantifying plasminogen activator activity, we used various dilutions of the purified kidney plasminogen activator, urokinase (EC 3.4.21.31; Calbiochem reference standard, cat. no. 672123) in place of the euglobulin sample, to generate plasmin from plasminogen. Urokinase was dissolved at a concentration of 380 CTA units/mL in a buffer (150 mmol of NaCl and 50 mmol of sodium phosphate per liter, pH 7.4). Aliquots of the standard were stored at −80 °C until required for assay. Aliquots were used only once. The concentrated urokinase was diluted appropriately immediately before use and kept on ice until added to the assay mixture. The ability of urokinase to cleave S-2251 was determined by incubating urokinase with substrate in the absence of plasminogen. Background plasmin generation was also measured by adding buffer without urokinase to the incubation mixture containing plasminogen. After subtracting background values from the color yield of urokinase plus plasminogen, we plotted on semilogarithmic paper a standard curve of CTA units of urokinase vs absorbance and determined the units of plasminogen activator from this curve. Releasable plasminogen activator was determined by subtracting the value for pre-occlusion plasminogen activator from the post-occlusion value as previously described (8, 9, 18). Releasable plasminogen activator (RPA) was expressed as CTA units/mL of plasma as follows:

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\text{RPA} = \frac{[\text{post-occlusion activity (CTA units/50 μL)} - \text{pre-occlusion activity (CTA units/50 μL)}]}{20}
\]

The concentration of plasminogen we use in the colorimetric assay has been demonstrated (24) to be adequate to ensure that the rate of plasminogen activation by the subject's plasma is neither substrate limited nor affected by the amount of plasminogen present in the individual euglobulin precipitates (24).

Results

The color yields obtained when S-2251 was exposed to euglobulin fraction or urokinase alone were negligible. When plasminogen was added to the substrate in the absence of either activator, this was also the case. (Color yields at 405 nm were generally <0.035 vs a buffer blank.)

The data from 11 standard curves generated over a two-month period demonstrate reproducibility among urokinase samples prepared and analyzed in separate experiments (Figure 1). The standard curve is linear between the values of approximately 5 x 10^{-5} and 10^{-1} CTA units/0.5 mL of complete reaction mixture.

Multiple aliquots of plasma from five subjects were processed to obtain euglobulin precipitates, which were suspended and analyzed as described above. The plasminogen activator values were highly reproducible, even when these samples were analyzed on different days (Table 1).

Epsilon-aminocaproic acid (EACA), 10 mmol/L, and lysine, 0.1 mmol/L, reportedly increase the initial velocity of plasminogen activation in a rate assay in which urokinase is used, with EACA more effective than lysine at stimulating concentrations (27). Both lysine and EACA bind to plasminogen and alter its conformation, greatly enhancing the rate of activation (27). Lysine, 0.1 mol/L, inhibited the reaction mildly, but EACA, 10 mmol/L, completely inhibited color production (Table 2). We varied exogenous plasminogen concentrations in an attempt to enhance color yield and hasten the assay. Increasing the plasminogen concentration up to 25-fold increased the total color yield but did not increase the net color yield after background subtraction (data not shown).

![Fig. 1. Colorimetric assay of urokinase: mean ± SD for 11 separate assays of each incubation mixture as described in Materials and Methods.](image-url)

| Table 1. Reproducibility of the Assay for Releasable Plasminogen Activator |
|-----------------------------|-----------------------------|-----------------------------|
| Sample | No. analyses | Mean activity CTA units/mL | SD | CV, % |
| 1 | 10 | 0.06 | 0.004 | 6.6 |
| 2 | 16* | 0.21 | 0.02 | 9.5 |
| 3 | 8 | 0.45 | 0.03 | 6.6 |
| 4 | 10 | 0.60 | 0.03 | 5.0 |
| 5 | 12* | 0.25 | 0.03 | 12.0 |

* Two sets of assays were performed on different days. * One sample assayed on 12 separate days.

| Table 2. Effects of Lysine and ε-Aminocaproic Acid (EACA) on Plasminogen Activation by Urokinase |
|-----------------------------------------------|-------------------------------------------------|-------------------------------------------------|
| Urokinase conc, CTA units | 0.1 | 0.01 | 0.001 |
| Control | 2.023* | 0.588 | 0.135 |
| With lysine, 0.1 mol/L | 1.619 | 0.313 | 0.034 |
| With EACA, 10 mmol/L | 0.032 | 0.010 | 0.002 |

* Absorbance of samples at 405 nm.
Pre- and post-occlusion plasminogen activator concentrations were measured and releasable plasminogen activator levels were calculated in 24 individuals by both the radiocasein assay (18, 24) and this colorimetric assay. The correlation coefficient for releasable plasminogen activator (post-occlusion minus pre-occlusion values) is 0.97, with a p < 0.0001 (Figure 2).

The range of plasminogen activator values among 125 healthy subjects, approximately evenly divided by sex and with no history of thromboembolic disease, was 0.02–0.96 CTA units/mL of plasma. The distribution of releasable plasminogen activator is non-Gaussian (Figure 3), as reported in earlier studies (8, 9, 18). Differences between the values for men and women were not significant, in agreement with previously reported data (18). Subjects ranged in age from 25 to 69 years. No attempt was made to group the subjects by age or race because previous studies have demonstrated no difference in releasable activator by age or race (8).

Discussion

The identification of individuals and families who have suffered major thromboembolic events and in whom the only measurable defect is extremely low activities of releasable plasminogen activator highlights the need for a simple, reproducible, and easily standardized assay for the activator. Abnormalities in the fibrinolytic system occur in both congenital and acquired vascular and thrombotic disorders (1-5, 7-9). Currently no widely accepted, standardized method of evaluating releasable activator is available to the clinician. This deficiency not only precludes evaluation of the fibrinolytic potential of a person who suffers from "idiopathic" venous thrombotic disease, but also obviates the possibility of following patient response to drugs or therapies that are known to enhance coagulability or fibrinolysis (e.g., oral contraceptive pills or a planned exercise program).

The colorimetric assay described here has several advantages over previously published assays for both clinical and laboratory use. Like the recently published radiocasein assay (9, 18, 24), the colorimetric assay is superior to the standard fibrin plate assay with regard to simplicity, speed, sensitivity, and reproducibility. Because this assay requires only a standard laboratory spectrophotometer, it can easily be implemented in any clinical chemistry or coagulation laboratory. The assay can measure a large range of human fibrinolytic responses without dilution or concentration of the egulobulin fractions. Figure 2 demonstrates the loss of linearity of the assay above about 0.1 CTA units of activator, probably owing to depletion of the S-2251 substrate. However, the amount of activator exceeds the amount assayed in any of our subjects, because 0.1 CTA unit (in 50 μL) would be equivalent to 2 CTA units/mL of plasma, and none of our subjects exceeded 0.96 CTA unit/mL (Figure 3). The highest reported activity of activator in plasma was 3 CTA units/mL, and this value was obtained after an exercise stimulus rather than by the venous occlusion technique (28). This subject was reported to have a bleeding tendency, believed to be the result of this very high activity. We recommend that several different dilutions of the sample be assayed if the post-occlusion plasma from a subject shows an activity exceeding the linear range described. These values can then be used to obtain a corrected value for the plasma. Pre-occlusion plasma has much lower color yields and it is not likely that any such samples will require dilution for accurate assay.

Results of the new assay correlate well (r = 0.97) with those by radioassay for releasable plasminogen activator and, unlike the casein assay, this one yields data within 6 h and requires no radiolabeled substrates. However, extensive hemolysis and hyperlipidemia do interfere with the determination of the endpoint of the colorimetric assay but not with the radiocasein assay. This is true of all the colorimetric tripeptide assays in which p-nitroaniline is liberated (26). However, extensively hemolyzed samples are generally unacceptable for coagulation studies, and such samples ordinarily would not be studied. Hyperlipidemia is encountered in various familial disorders of lipid metabolism. The egulobulin precipitate from such subjects is cloudy when reconstituted. There is sufficient light scatter from such samples to make it impossible adequately to determine the absorbance. We have found that this problem can be overcome if the final assay mixture is treated with trichloroacetic acid (100 g/L solution) to precipitate all the protein. The pH of the supernatant fluid is then adjusted to neutrality and the color endpoint is determined.

Recently developed fluorogenic substrates (29) may be substituted for S-2251, potentially increasing the sensitivity of the assay. However, this modification would require use of a fluorometer. Moreover, a recent report (30) has questioned the specificity of one of these substrates for the measurement of plasmin. At present, then, the colorimetric assay represents the most practical clinical tool for assessment of releasable activator.

Although EACA and lysine reportedly stimulate activation of plasminogen by urokinase and human plasminogen activator (27), we saw no enhancement of the overall color yield in our equilibrium assay. Furthermore, increasing concentrations of plasminogen failed to increase the color yield of the reaction when the colorimetric substrate was in marked excess, indicating that plasminogen concentrations were not limiting to the reaction sequence studied. This has also been observed with the radiocasein assay (24).

Data on releasable plasminogen activator in 125 healthy volunteers are presented in Figure 3. As previously described, these data are not normally distributed (18). Sex-related differences in values for plasminogen activator are not significant, as was also reported with the radiocasein assay (18).
Moreover, in this previous study we observed no age-related variation (18).

The normal range for releasable plasminogen activator must be defined with some caution. None of the 125 volunteers reported in the present study showed evidence of thrombotic disease. However, in women having oral contraceptive-associated thromboembolic disease and in a pedi-gree with a history of recurrent thrombosis, more than 96% of the patients had concentrations of releasable activator at or below 0.05 CTA unit/mL (5, 31). This activity defines the lowest 5% of the distribution observed in our 125 volunteers, and we believe that such subjects are at higher risk for thrombosis than subjects with higher activator values. This is not to say that all such individuals will experience a thrombotic episode at some time in their lives. The fibrinolytic system is triggered only when coagulation begins; therefore, there must be some thrombotic stimulus before defective fibrinolysis becomes a major factor in thrombosis. Such stimuli would include long-term immobilization with concomitant venous stasis, major surgery, use of oral contraceptives, and increased concentrations of the coagulation proteins. This list is by no means intended to exclude other factors.

Definition of the upper limits of normal is more difficult. As described above, a subject with activator concentration of 3.0 CTA units/mL had an associated bleeding tendency (28). At present, it would seem reasonable to define the normal range of releasable activator concentration as between 0.05 and 1.0 CTA unit/mL.

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References