Determination of Tissue Plasminogen Activator and Its “Fast” Inhibitor in Plasma

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We describe efficient, accurate methods for specific determination of tissue plasminogen activator (t-PA, EC 3.4.21.31) and its “fast” inhibitor in plasma. In this coupled assay, a sample containing t-PA is incubated with plasminogen, a plasminogen activator (EC 3.4.21.7) substrate of low $K_a$ and high $K_{cat}$, and fibrin as a stimulator. The inhibitor of t-PA is determined by incubating the sample with a known amount of t-PA in excess, then determining the residual t-PA. Both t-PA and t-PA inhibitor can be determined in many samples simultaneously within a few hours. These assays are modifications of procedures described by us (Clin Chim Acta 1983;127:279–88 and Thromb Res 1983;31:427–36). Their accuracy as assessed by analytical recovery of pure t-PA added to blood samples (91 ± 4%) or of partly purified inhibitor added to plasma samples (102 ± 10%) is satisfactory, as is their precision. For the t-PA assay the CV was 1.6% (within run) or 4.1% (between run). The corresponding values for the inhibitor assay were 4.5% (within run) or 8.4% (between run) if the inhibitor concentration exceeded 3 arb. units/mL.

Additional Keyphrases: fibrinolysis · thrombosis · myocardial infarction · pregnancy · reference interval

Tissue plasminogen activator (t-PA), the most important physiological activator of the fibrinolytic system (1), is decreased in thrombotic disease. Sensitive, reliable methods for determining t-PA in blood or plasma samples are therefore important. Several such methods have been developed, but all have some disadvantages, such as long incubation (2) or the need for separation steps such as egulbin precipitation (3) or chromatography on lysine-Sepharose (4).

Recently we demonstrated the presence in plasma of a new “fast” inhibitor of t-PA (5), which rapidly reacts with t-PA, forming a stable inactive complex with an $M_f$ of about 120 000 (6). We also developed a kinetic method for quantifying the inhibitor in plasma (5). Increased concentration of the inhibitor seems to be a major cause for decreased t-PA activity in patients with deep-vein thrombosis (7), in myocardial infarction (8, 9), and in the late phase of pregnancy (10).

Here we report on improvement in the kinetic methods for determining t-PA and its inhibitor in blood or plasma samples. Furthermore, the quality of the new assay procedures is discussed in detail.

Materials and Methods

Reagents and Apparatus

Pure one-chain human t-PA, from melanoma cells, was obtained from KabiVitrum, Stockholm, Sweden (courtesy of Dr. Monica Einarsson) or from Biopool AB, Umeå, Sweden (courtesy of Dr. Mats Rånby). The chromogenic substrates n-valyl-l-leucyl-l-lysyl-p-nitroaniline (n-Val-Leu-Lys-pNA; S-2251) and n-valyl-l-phenylalanyl-l-lysyl-p-nitroaniline (n-Val-Phe-Lys-pNA; S-2390) were from KabiVitrum (courtesy of Dr. Steffen Rosen). Bathroxbin (Defibrinase; a thrombin-like serine protease) was from Pentapharm, Basel, Switzerland. Sephacryl S-200 was from Pharmacia, Uppsala, Sweden, and human fibrinogen from IMCO, Stockholm, Sweden. Other chemicals were of analytical grade and were obtained from Merck, Darmstadt, F.R.G.

We used sodium acetate buffer (1 mol/L, pH 3.9) to acidify plasma and blood samples. For the t-PA assay we diluted the acidified samples with Tris HCl buffer (50 mmol/L, pH 8.8) containing 0.1 g of Tween 80 (polyoxyethylene sorbitan monoooleate) per liter, and we prepared the assay reagent in the same buffer. For the t-PA inhibitor assay we diluted plasma samples with sodium phosphate buffer (20 mmol/L, pH 7.3) containing 0.1 mol of NaCl and 0.1 g of Tween 80 per liter.

For spectrophotometric readings at 405 nm we used a micro-flow spectrophotometer (Model CL-720; Shimadzu, Kyoto, Japan). Silicized Vacutainer Tubes, each containing 0.5 mL of 0.13 mol/L sodium citrate, were from Beckton Dickinson, Stockholm, Sweden.

Procedures

Purification of plasminogen. Human Glu-plasminogen was purified by affinity chromatography on lysine-Sepharose (11), followed by gel filtration on Sephacryl S-200. If necessary in order to eliminate traces of plasmin, we used affinity chromatography on soybean trypsin inhibitor coupled to Sepharose after the last purification step. Plasminogen used in this assay must not contain more than 5 μg of plasmin per gram.

Preparation of soluble fibrin. Human fibrinogen was dissolved in sodium phosphate buffer (20 mmol/L, pH 7.3, containing 0.3 mol of NaCl per liter) to give a concentration of 20 g/L and subsequently digested with bathroxbin (20 arb. units/g of fibrinogen) for 3–4 h at room temperature, then incubated overnight at 4°C. We dissolved the resulting fibrin gel (des AA fibrin) in an equal volume of freshly prepared 7 mol/L urea solution, to give a fibrin concentration of 10 g/L. This solution can be used, if stored frozen at −70°C in aliquots, for at least a year.

Blood-sampling procedures. After the subject had rested for 10 min, blood was sampled for t-PA inhibitor determination into Vacutainer Tubes containing sodium citrate. The tubes were centrifuged (2000 × g, 20 min) to separate the plasma. We acidified pre-occlusion and post-occlusion blood samples for the t-PA assay immediately after sampling, by mixing two volumes of blood with one volume of acetate buffer and centrifuging the tubes as described above. To assure a sample pH of 4.0–4.1 we added 5 μL of 8.5 mol/L acetic acid to 100 μL of acidified plasma (during blood sampling).
To compare the new assay procedures for t-PA and its fast inhibitor with our previously used methods, we used samples from 40 healthy subjects. The group consisted of 30 men (mean age 43, SD 5, range 34–55 years) and 10 women (mean age 39, SD 12, range 20–60 years). In addition, we used some selected samples from previously analyzed patients.

**Determination of t-PA.** Acidified plasma samples were diluted with Tris HCl buffer, pH 8.8. Pre-occlusion samples were diluted 100- and 200-fold, whereas post-occlusion samples routinely were diluted 100- and 400-fold. Diluted samples were mixed with an equal volume of assay reagent consisting of 0.1 g of plasminogen and 0.6 mmol of n-Val-Phe-Lys-pNA per liter of Tris HCl buffer. Subsequently, solubilized fibrin was added to give a final concentration of 0.1 g/L. A standard curve was prepared by serially diluting (100- to 1600-fold with Tris HCl buffer) an acidified reference plasma sample containing 1 U of t-PA per milliliter and processing as described above. After fibrin was added, the samples were incubated (2 h, 37 °C) and the reaction was then terminated by adding 0.1 volume of 8.5 mol/L acetic acid.

The t-PA concentrations of the standards were plotted vs absorbance at 405 nm, and t-PA concentrations in the unknown samples were evaluated from this standard curve.

For comparison, we also determined t-PA with the previously described method (2, 5).

**Determination of t-PA inhibitor.** Citrated normal plasma samples were diluted fourfold with sodium phosphate buffer. Then human t-PA was added to the diluted samples to give a final concentration of 2 U/mL. The mixture was incubated at room temperature for exactly 20 min and the reaction then was stopped by adding an equal volume of the sodium acetate buffer. We incubated the samples at room temperature for about 15 min and thereafter quickly froze them at acidic pH, in order to efficiently remove the final traces of potential plasmin inhibitors. The acidified samples may be validly stored frozen at −70 °C, or they can be thawed and analyzed directly. For use in preparation of the standard curve a pool of reference plasma was diluted fourfold, acidified as described above, and 2 U of t-PA was added per milliliter. This sample corresponds to 0 arb. units (AU) of inhibitor per milliliter, whereas acidified normal plasma without added t-PA corresponds to 2 AU of inhibitor per milliliter. We define 1 AU of inhibitor as the amount that inhibits 1 U of t-PA during 20 min under the above-specified conditions. Subsequently, residual t-PA activity was determined by a procedure slightly modified from that described above. For this assay all samples were diluted 50-fold with Tris HCl buffer. A standard curve was obtained by mixing the acidified and diluted plasma with t-PA added and the corresponding acidified and diluted plasma without t-PA in different ratios. The diluted samples (250 μL) were mixed with an equal volume of assay reagent, followed by addition of fibrin as described above (see t-PA determination). We incubated the samples for 1 h at 37 °C, and then stopped the reaction by adding acetic acid, and measured the absorbance at 405 nm.

**Results**

**Standard curve for the t-PA assay.** Data for seven consecutive standard curves for t-PA analysis are presented in Figure 1 (each point represents mean ± SD). The increase in absorbance at 405 nm is linear for t-PA concentrations between 0 and 2.5 × 10⁻³ U/mL. This relation can be described by the equation \( y = (9.25 \times 10^{-4}) + 271.3x \). The correlation coefficient for this part of the curve was 0.976. For t-PA concentrations between 2.5 × 10⁻³ and 5 × 10⁻³ U/mL the curve deviates slightly from linearity.

**Reliability of the t-PA assay.** The reproducibility of the t-PA assay was assessed for t-PA activities between 1.5 and 2.0 U/mL. The t-PA activity concentration in 10 samples of the same plasma assayed simultaneously was determined as 1.58 (SD 0.03) U/mL, corresponding to a CV of 1.6% "within-run". To obtain the "between-run" CV, we analyzed another plasma sample on five different occasions, finding a mean t-PA concentration of 2.01 (SD 0.08) U/mL, corresponding to a CV of 4.1%.

To investigate the accuracy of the procedure we carried out the following analytical-recovery experiments. Blood (pre-occlusion samples from five individuals, post-occlusion sample from one individual) was thoroughly mixed with t-PA (1 U/mL) and immediately acidified with 0.5 mL of acetate buffer. Alternatively, 1 mL of blood from the same persons was acidified by addition of 0.5 mL of acetate buffer, and then 1 U of t-PA was added. The blood samples were then processed and assayed for t-PA as described. The same results were also analyzed before the addition of t-PA and these results were subtracted. Thus, the analytical recovery of t-PA added to blood before acidification was 1.72 (SD 0.08) U/mL (range 1.59–1.82) corresponding to 90.8 (SD 4.2)% if a mean hematocrit of 0.45 is assumed. In the samples with t-PA added after acidification, the yield was slightly higher: 1.75 (SD 0.06) U/mL (range 1.64–1.79), corresponding to an analytical recovery of 92.4 (SD 3.2)%.

**Comparison between the new t-PA assay and the previously used procedure.** The correlation between results by the improved method for t-PA determination and the previously used procedure is demonstrated in Figure 2. For this purpose we used acidified post-occlusion samples from 28 subjects (19 healthy individuals and nine patients). As can be seen, the correlation is very good (r = 0.993) and the corresponding equation is \( y = 0.18 + 0.936x \).

**Standard curve for the t-PA inhibitor assay.** Figure 3 shows a standard curve for t-PA inhibitor determination. These results were obtained from 16 consecutive analyses and the points represent mean ± SD. A linear response in absorbance against t-PA concentration was obtained, the corresponding equation being \( y = 0.54 - 0.456x \) (r = -0.992).

**Reliability of the t-PA inhibitor assay.** The reproducibility
of the t-PA inhibitor assay was determined on two different plasma samples with different concentrations (Table 1). The "within-run" CV was <5%, the "between-run" CV about 8%, if the inhibitor concentration was >3 AU/mL. If it was <2 AU/mL the CV was considerably higher (data not shown).

In an attempt to study the accuracy of the assay procedure, we added partly purified inhibitor (about 100-fold purified from plasma) to plasma samples from six different subjects with different starting concentrations of the inhibitor (range 1.0–7.9 AU/mL), giving an increase in the inhibitor concentration of 3.50 AU/mL. The concentrations then were determined in the samples before and after addition of inhibitor (Table 2). The mean recovery was 3.58 (SD 0.36) AU/mL, corresponding to 102 ± 10.3%.

In another experiment we added the partly purified inhibitor to a plasma sample from one healthy subject whose inhibitor concentration was low (1.0 AU/mL) to give different concentrations ranging between 1.0 to 12.0 AU/mL and assayed. The results showed that the relation between the theoretically expected concentrations of the inhibitor and the experimentally obtained concentrations was linear, represented by the equation \( y = -0.28 + 1.14x \) (\( r = 0.933 \)).

Comparison between the new method and the previously used procedure to measure t-PA inhibitor. The inhibitor concentration was measured in plasma samples from 68 subjects (patients and healthy subjects) with the earlier-used method and the results compared with those by the present improved method. A linear relationship was obtained, described by the equation \( y = -0.16 + 1.01x \) (\( r = 0.946 \)). Figure 4 illustrates the distribution of the t-PA inhibitor concentrations found in plasma from 40 healthy individuals (individuals with overweight or increased levels of triglycerides in plasma were not included). The mean concentration was 0.89 (SD 0.83) AU/mL (median 0.6, range 0–3.5 AU/mL).

Discussion

Decreased fibrinolytic activity is often due to a combination of decreased release of t-PA upon provocation, and increased concentrations of the recently described inhibitor of t-PA in plasma (7–9). To investigate the role of the fibrinolytic function in various pathological conditions it is important to have access to sensitive, reliable assay proce-

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<th>Table 2. Analytical Recovery of Partly Purified t-PA Inhibitor Added to Plasma</th>
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*To various plasma samples we added 3.50 arb. units of t-PA inhibitor per milliliter.

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<th>Table 1. Precision of t-PA Inhibitor Assay for Two Concentrations of Inhibitor</th>
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<td>t-PA inhibitor, arb. units/mL</td>
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Fig. 2. Correlation between t-PA determined by the previously described method with D-Val-Leu-Lys-pNA and by the present method, with D-Val-Phe-Lys-pNA as plasmin substrate, for samples from 19 healthy subjects (●) and nine patients (○).

Fig. 3. Standard curve for the t-PA inhibitor assay (mean ± SD of 16 consecutive analyses).

Fig. 4. Concentration of t-PA inhibitor in 40 healthy subjects.
ured for both these components. Here we have significantly improved the methods we previously described (2, 6).

The standard curves obtained with the present methods are almost linear. The main reason for this is our use of a better plasmin substrate, which protects the fibrin used as a stimulator from degradation by plasmin (12). Another effect of the better plasmin substrate, in combination with increased pH and a decreased ionic strength during incubation, is that the reaction rate, measured in terms of p-nitroaniline liberated, is increased by more than 50-fold in the t-PA assay compared with the previous procedure (2). This has made it possible to shorten the incubation times considerably for both the t-PA assay and the inhibitor assay. We have also subjected the new procedures to thorough quality control. Precision has been improved by about 50% for both assays. The between-run CV was determined to be 4.1% for the t-PA assay, about 8% for the t-PA inhibitor assay. The limit of detectability for the t-PA assay was found to be about 0.05 AU/mL when used an incubation time of 2 h, about 0.01 AU/mL if the incubation was prolonged to 4 h. In this way, t-PA can be accurately determined in most plasma samples taken when the subject is at rest. However, it is absolutely essential that potential inhibitors of t-PA be neutralized as quickly as possible after blood collection, if the analysis is expected to reflect the situation in vivo (5). This is especially important if the patients have an increased concentration of the t-PA inhibitor, as is frequently found with patients suffering from conditions associated with thrombotic disease (7, 9).

The accuracy of the t-PA assay, as judged from analytical recovery experiments, exceeded 90%. The specificity of the t-PA assay was ascertained already with the previous procedure, because antibodies against t-PA completely abolished the activity (2). Furthermore, if fibrin was omitted in the present procedure, no activity was detected after 4 h of incubation (data not shown).

With regard to the new procedure for t-PA inhibitor determination, the detectability limit is about 0.5 AU/mL. However, in the present version with use of a fourfold dilution of plasma samples, the precision is considerably better for concentrations above 2 AU/mL. If a value exceeding 6 AU/mL is obtained, a further dilution of the plasma sample must be assayed, preferably so that 25 to 75% of the added t-PA becomes inhibited.

From the theoretical point of view, the above-described procedure for measuring t-PA inhibitor can never become completely specific and accurate at the same time, because the reaction time must be prolonged in order to complete the reaction between t-PA and its inhibitor. On the other hand, a prolonged reaction time would lead to a slight overestimation, owing to the presence of α2-antiplasmin, a slow inhibitor of t-PA (13, 14). To demonstrate the accuracy of the method, we added different amounts of partly purified t-PA inhibitor to samples of plasma for which the inhibitor content was known. A very good correlation (r = 0.993) was obtained between the theoretical and found concentrations, suggesting that the procedure, from the practical point of view, indeed is accurate. Furthermore, the partly purified inhibitor was added in a single concentration to different plasma samples differing in their basal concentrations of the inhibitor. The recovery (1.02 ± 10%) was quite satisfactory and also demonstrates the accuracy of the assay. The inhibitor assay can be performed on many samples simultaneously and the results may be obtained in a few hours. No problems have been seen with blood sampling. In the present work we have used citrate as anticoagulant, but EDTA- or heparin-treated blood gives very similar results (data not shown). The concentration of the t-PA inhibitor in a reference population was determined as 0.98 (SD 0.83) AU/mL, when overweight individuals or those with increased values for plasma triglycerides were not included. Such individuals frequently have increased concentrations of the inhibitor (9).

The new methods for determination of t-PA and its fast inhibitor in plasma samples have already proven to be of great value for evaluation of the fibrinolytic potential in patients. In view of the findings of increased t-PA inhibitor concentrations in patients with deep-vein thrombosis (7), in pregnancy (10), in septicemia (15), and in young patients with myocardial infarction (8, 9), a great demand for this type of analysis will probably develop.

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References