Development and Analytical Performance of Automated Tests for Antithrombin III and Plasminogen on the Du Pont aca™ Analyzer

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We describe assays for functional antithrombin III (AT III) and plasminogen in plasma with the Dua Pont aca™ discrete clinical analyzer. Both are two-stage kinetic assays, based on synthetic substrate methodologies, and require 20-μL sample volumes. In the AT III assay the sample is incubated with excess thrombin and heparin to form the functionally inactive AT III–thrombin complex. Residual thrombin is measured through its rate of hydrolysis of a lysine thioester and is inversely related to analyte concentration. In the plasminogen assay excess streptokinase is reacted with the sample to form an enzymatically active complex. The substrate hydrolysis rate of this complex is measured, which is linearly related to the concentration of plasminogen in the sample. Reaction conditions for both assays were optimized by univariate and response surface techniques. The assay for AT III has a range of 0 to 150% of the value for normal human plasma (% NHP) with a CV of 3% at 80% NHP. The plasminogen assay is linear from 25 to 200% NHP with a CV of <2% at 80% NHP. No significant interferences with either method by common blood components or drugs were found.

Additional Keyphrases: synthetic substrates • kinetic enzyme analysis • coagulation assays • disorders of hemostasis

Antithrombin III (AT III) and plasminogen are two important components of the hemostatic system. AT III is the primary physiological inhibitor of the coagulation enzymes, particularly thrombin (EC 3.4.21.5), and serves to prevent excessive clot formation (1–3). Plasminogen is the inactive precursor of the fibrinolytic enzyme plasmin (EC 3.4.21.7), which is responsible for dissolution (lysis) of a fibrin clot (4, 5).

Information on plasma AT III and plasminogen concentrations is important in the diagnosis and treatment of hemostatic disorders and other disorders with secondary hemostatic effects. In general, only decreases (to less than approximately 80% of normal) are of clinical significance. Clinical states in which AT III and plasminogen are decreased include thrombosis, disseminated intravascular coagulation, liver disease, thrombotic therapy, trauma, and the nephrotic syndrome (6–11). Frequently, these states are accompanied by circulating complexes of AT III (with thrombin) or α2-antiplasmin (with plasmin) that are enzymatically inactive. Inherited deficiencies are also known in which the protein is functionally abnormal but antigenically similar to the normal protein (12–14). Thus, it is important to measure these analytes with respect to their functional activity rather than to rely on immunological methods, which measure only the absolute protein concentrations.

Electroimmunoassay and radial immunodiffusion are sensitive and selective tests for AT III and plasminogen, but are time-consuming and do not discriminate between functional and nonfunctional antigenic proteins. Measurements of biological activity, such as a clotting time assay for AT III or a caseinolytic assay for plasminogen, are sensitive to only the functional proteins but are generally nonlinear with respect to response to the analyte and have complex kinetics. The recent application of low-molecular-mass synthetic substrates to the analysis of hemostatic components (15, 16) has allowed retention of most of the advantages of other functional assays and has added speed, precision, and the potential for automation.

We describe in this report the first fully automated, functional assays for AT III and plasminogen in plasma. These assays are implemented on the aca discrete clinical analyzer (E. I. du Pont de Nemours & Co., Inc., Wilmington, DE 19898) and are based on the following two-stage reaction schemes:

1. AT III (sample) + thrombin + heparin →
   AT III–thrombin–heparin complex + residual thrombin
   Z-Lys-SBzl + DTNB → residual thrombin → TNB^2− (chromophore)

2. Plasminogen (sample) + streptokinase →
   plasminogen–streptokinase complex
   Z-Lys-SBzl + DTNB → plasminogen–streptokinase complex
   Z-Lys-SBzl + DTNB → TNB^2− (chromophore)

where Z-Lys-SBzl (α-N-carbobenzoxy-L-lysine thio benzyl ester) is a synthetic substrate (17) that is enzymatically hydrolyzed to release benzyl mercaptan. The mercaptan reacts instantaneously with DTNB [5,5'-dithiobis(2-nitrobenzoic acid), Ellman’s reagent] to form a colored product (TNB^2−, 2-nitro-5-thiobenzoate dianion) at a rate that is linearly related to the enzyme activity. In addition, we have evaluated the linearity, range, reproducibility, and the effects of potential interferences on the analytical performances of these methods.

Materials and Methods

Reagents

Human thrombin was obtained from Ortho Diagnostics, Raritan, NJ 08869. Streptokinase and fibrinogen were purchased from the Kabir Group, Inc., Greenwich, CT 06830. Z-
Lys-SBz1 was a product of Peninsula Laboratories, San Carlos, CA 94070. Sodium heparin (activity 140 USP units/mg) and α2-macroglobulin were obtained from Calbiochem-Behring Corp., La Jolla, CA 92037. Assayed Reference Plasma® (Helena Laboratories, Beaumont, TX 77704) and pooled normal human plasma were used as analyte sources. De-ionized water was used for all aqueous preparations. Fibrinogen degradation products were prepared by the method of Marder et al. (18) and assayed with the FDP® kit (Burroughs Wellcome Co., Research Triangle Park, NC 27709). All other reagents were of the highest grade commercially available. All studies were performed with Du Pont AT III or plasminogen analytical test packs, processed in a standard aca analyzer.

AT III Test Procedure

The aca analyzer transfers 20 μL of citrated test plasma and 4.980 mL of Du Pont "PHOS" diluent (150 mmol/L phosphate, pH 7.80 at 37 °C) into an AT III analytical test pack. The plasma and buffer are warmed to 37 °C for 75 s, then thrombin, heparin, and DTNB are added. The AT III in the sample reacts with the thrombin and heparin for 187.5 s, after which the substrate, Z-Lys-SBz1, is added. The amount of residual thrombin is determined by the rate of hydrolysis of the thioester. The rate is computed from two measurements, made 17.07 s apart, commencing 29 s after substrate addition. This result is converted to percent of normal human plasma (% NHP) units by using a calibration equation previously stored in the instrument (for the aca III analyzer) or by using linear graph paper (for the aca II analyzer).

Plasminogen Test Procedure

The plasminogen assay is identical to that described above for AT III except that streptokinase is added in the first reagent addition stage instead of thrombin and heparin. The results are directly converted to % NHP units by using a stored calibration equation (for both aca II and III analyzers).

Preparation of Calibration Standards

Assayed Reference Plasma was reconstituted by underhydration with de-ionized water to give stock plasma solutions with AT III and plasminogen contents near 150% NHP. The analyte concentrations for a given lot of Assayed Reference Plasma were determined by reference to the World Health Organization AT III standard (no. 72/1) or to a normal human plasma pool with an assigned plasminogen value of 100% NHP. These stock plasma solutions were diluted with physiological saline (0.15 mol/L NaCl) to provide calibration standards over the assay ranges. All stocks and calibration standards were prepared freshly each day and stored on ice for no longer than 4 h before use.

Normal human plasma was prepared by pooling plasma collected from at least 10 apparently normal men and women who were nonsmokers and were not using oral contraceptives. Sodium citrate (38 g/L) was used as the anticoagulant (one part citrate to nine parts blood), and the resulting plasma was stored in small aliquots at -70 °C.

Results and Discussion

Optimization of the AT III Assay

Reagent concentrations were selected to optimize linearity, sensitivity, and range. The reaction buffer is 150 mmol/L phosphate, pH 7.80 at 37 °C. The choice of buffering agent and the response surface co-optimization of its pH and ionic strength effects upon the kinetic parameters K_m and k_cat are described in an accompanying report (19). The plasma sample volume is 20 μL; the total reaction volume is 5.2 mL, including the volume contributed by dissolved solids in the test pack.

Heparin is added to accelerate the reaction, changing AT III from a slow, progressive thrombin inhibitor to one that acts almost instantaneously (20). This accelerating effect of heparin saturates at activities greater than 1 USP unit/mL (Figure 1). In the absence of AT III, heparin had no effect on the kinetics of the reaction of thrombin with Z-Lys-SBz1. The final heparin activity of 3 USP units/mL ensures sufficient reagent excess.

Z-Lys-SBz1 concentrations exceeding 520 μmol/L suffice to maintain substrate-saturating (V_max) conditions throughout the measurement, a requirement for a valid kinetic assay (21). Under the assay conditions, the K_m is 30 μmol/L and the k_cat is 57 s⁻¹. The initial thioester concentration in the reaction is 663 μmol/L.

The DTNB concentration of 1000 μmol/L provides a 1.5-fold molar excess over the substrate, sufficient to ensure complete reaction of all benzyl mercaptan formed (22) and to prevent interference by any free sulfhydryl in the plasma sample during the rate measurement.

All other pack reagents being in excess in the AT III assay, the reaction is first-order in thrombin activity. Therefore, method precision and sensitivity are direct functions of the thrombin activity. The thrombin activity in the assay was set by optimizations of range and sensitivity. If the thrombin concentration is too low, there will be insufficient enzyme to react with all the AT III in the sample; too much, however, results in absorbance measurements beyond the range of the photometer. Thrombin potency is normally defined in terms of its biological (clotting) activity, but this value does not necessarily reflect its hydrolytic action on synthetic substrates (23); therefore, for this assay system the thrombin activity is defined in terms of its thioesterolytic activity with Z-Lys-SBz1. The results of the optimization study (Figure 2) show that a thrombin concentration sufficient to give a response of 2300 mA/min with a blank (saline) yields consistent assay linearity from 0 through 150% NHP. To assay this amount of enzyme (nominally about 12 NIH units), one must measure the absorbance change at 452 nm because attempts to measure absorbance values at wavelengths nearer the chromophore peak (412 nm) would exceed the photometer's linear range.

Optimization of the Plasminogen Assay

All reagents in the plasminogen assay (streptokinase, Z-
Lys-SBzl, and DTNB) are present in excess and on the plateau regions of their respective concentration–response curves. 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) buffer is optimal for the hydrolysis of Z-Lys-SBzl by the plasminogen–streptokinase complex (19); however, in this buffer the activation step is relatively slow and results in poor assay sensitivity within the timing of the acu analyzer. Phosphate buffer (150 mmol/L, pH 7.80 at 37 °C), the same buffer as used in the assay for AT III, affords sufficient sensitivity for both the activation and hydrolysis reactions. Plasma sample volume is 20 μL, in a total reaction volume of 5.1 mL, including the volume contributed by the solids in the test pack. The Z-Lys-SBzl and DTNB concentrations are the same as used in the AT III test pack. Optimizations for these reagents confirmed that their concentrations are in excess for this reaction and in agreement with calculations based upon the kinetic constants for the reaction: $K_m = 52 \, \mu\text{mol/L}$; $k_{cat} = 85 \, \text{s}^{-1}$.

Streptokinase (1000 int. units/mL$^3$) is in 36-fold molar excess over the normal plasminogen concentration in adult human plasma when diluted into the assay solution. This ensures that the streptokinase concentration falls on the plateau region of the optimization curve (Figure 3). Use of streptokinase in such excess leads to formation of only the plasminogen–streptokinase complex rather than a mixture of kinetically different species such as plasmin–streptokinase or free plasmin (24). This excess will also counteract some of the potential interference caused by antistreptokinase antibodies found in some patients with a history of streptococcal infection or thrombolytic therapy (25). An important advantage of using streptokinase as the activating reagent is that the plasminogen–streptokinase complex is not inhibited by $\alpha_2$-antiplasmin, the fast-acting natural plasmin inhibitor (26).

**Linearity**

Both the AT III and plasminogen assays were calibrated with saline dilutions of stock plasma containing concentrations of AT III and plasminogen equivalent to 200% NHP each. Final sample concentrations ranged from 0 to 200% NHP. The standard curve for AT III (Figure 4, top) has a negative slope, because the method is based on an inhibition reaction. Ionic attraction between the plasminogen–streptokinase complex and the interior surface of the analytical test pack may be the cause of the low-end curvature in the plasminogen standard curve (Figure 4, bottom). Standard curves for both assays made by diluting stock plasma with bovine serum albumin (50 g/L) or with AT III- or plasminogen-depleted plasma showed no difference from the curves obtained on saline dilution, which indicates there is no protein or plasma matrix bias in the assays.

The standard curves for both assays show excellent linearity, as judged by low biases at the medical decision level of 80% NHP (1.5% bias for AT III and −1.4% bias for

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**Fig. 2.** Effect of thrombin on the sensitivity and linear range of the assay for AT III

Standard aca AT III analytical test packs were used except that the thrombin activity was varied as indicated. Plasma samples or saline were used to generate the standard curves. Thrombin activity in the final design is nominally 12 NIH units per pack.

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**Fig. 3.** Effect of streptokinase concentration on the plasminogen assay reaction rate

Standard aca plasminogen analytical test packs were used except that the streptokinase concentration was varied as indicated. Normal human plasma was used as the sample. Streptokinase concentration in the final design (1000 int. units/mL) is indicated by the arrow.

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**Fig. 4.** Representative standard curve for the aca AT III (top) and plasminogen (bottom) analytical test packs

Calibration samples were prepared as indicated in the text.
plasminogen) and coefficients of determination ($r^2$) of 0.999 for AT III and 0.998 for plasminogen.

**Reproducibility**

Method reproducibility for each assay was studied by analysis of within-day and day-to-day variation. A within-day trial consisted of 20 consecutive analytical test packs run for a single sample. Day-to-day testing involved daily assay, for 20 consecutive testing days, of a single plasma sample, aliquots of which had been stored at $-70^\circ$C.

Table 1 gives results of these studies. For all cases, assay reproducibility was excellent. Continued monitoring of the methods showed consistent performance for at least three months before recalibration was required. This is in agreement with standard guidelines for the acc analyzer. Test packs for both methods have a shelf life of one year.

**Anticoagulants**

We examined the effect of various anticoagulants on method performance by drawing replicate blood samples from the same donor and using five different anticoagulants: sodium citrate (12.9 mmol/L, final concentration in blood sample), sodium oxalate (111.1 mmol/L), heparin (28.6 USP units/mL), EDTA (4.0 mmol/L), and fluoride (102 mmol/L). We did this with three different donors. Citrate was used as the reference treatment and is the recommended anticoagulant for these assays, in accordance with current coagulation testing practices (27). Samples drawn with heparin, EDTA, or fluoride as anticoagulants all showed a statistically significant bias (at the 95% confidence level) when compared with the citrate treatment. Oxalate gave results similar to those with citrate but was not consistent for all samples tested.

**Effects of Potentially Interfering Substances**

Thirty common plasma components and drugs, dissolved in physiological saline, were tested for potential interference with the assays for AT III and plasminogen. An aliquot of a given test substance was added to a normal human plasma sample. The control was an equal volume of saline added to the normal human plasma sample. Five replicate determinations were performed on both the test and control samples, and the results were analyzed by use of the paired $t$-test. Table 2 lists all substances tested and their test concentrations. None of these substances caused a significant bias at the 95% confidence level.

In conclusion, fully automated methods for determination of AT III and plasminogen in plasma have been developed for use with the Du Pont acc discrete clinical analyzer. These methods have excellent linearity and reproducibility. Examination of 30 common plasma components and drugs showed no significant interference with either method. Evaluations of the clinical performance of these methods correlate well with alternative methods; they will be published as separate reports.

*Manuscripts in preparation by Fareed, Walenga, and Kelly and by Ito and Statland*.

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**References**


