Antithrombin III (AT3) is the most important inactivator of thrombin, the main enzyme of hemostasis. Its activity is usually measured in >50-fold diluted plasma because fibrin polymerization and thrombin entrapment into nascent fibrin (formerly called “antithrombin I”) is a major obstacle in testing for AT3.1 This could result in falsely-elevated AT3 activities (eg, in intensive-care patients with high plasmatic concentrations of soluble fibrin). Since arginine inhibits fibrin polymerization and thrombin entrapment into nascent fibrin,2 a new functional test for plasmatic AT3 was developed to work with nearly-undiluted plasma.

Material and Methods

Normal Antithrombin III—Kinetic

In duplicate, 25-µL pooled normal plasma (1 part 106 mM citrate + 9 parts of venous blood; centrifuged at 2,800 g [4,000 rotations per minute] for 10 min), AT3-deficient plasma (American Diagnostica, Stamford, CT), 6 % BSA-PBS (Sigma, Deisenhofen, Germany), 0.9% NaCl, 1:2 and 1:4 dilutions of pooled normal plasma with either bovine serum albumin-phosphate buffered saline (BSA-PBS) or 0.9% NaCl, or 1-mL lyophilized 100% of norm plasma (Control Plasma N, Dade Behring) reconstituted in 500 µL H2O and diluted 1:2, 1:4, and 1:8 were pipetted into flat-bottom polystyrol microtiter plate wells (F-wells, Polysorp, NUNC, Wiesbaden, Germany, article nr. 446140). The plate was intensely shaken for 10 s by a microplate shaker. 5 µL thrombin reagent, consisting of 92 IU/ml bovine alpha-thrombin (Dade Behring, Marburg, Germany; 130 IU lyophilized thrombin / vial; 1 mg = 2,525 IU), 10 IU/mL unfractionated heparin (Sarstedt, Nümbrecht, Germany), 1.48 M arginine, pH 7.4 (Sigma), 0.6% human albumin (Kabi Stockholm, Sweden) were added. The citrated plasma is only diluted by 20%. After 0 to 10 min at 37°C, 100 µL 2.5 M arginine, pH 8.6, 0.13% Triton X 100 (Sigma) was added. After 3 min at 23°C, 50 µL 1 mM chromogenic thrombin substrate CHG-Ala-Arg-pNA (Pentapharm, Basel, Switzerland) in 1.25 M arginine, pH 8.7, was added and the linear increase in absorbance (ΔA) at 405 nm was determined with a microtitre plate photometer with a 1-mA resolution (Milenia, DPC, Los Angeles). The maximal ΔA was 1,800 mA; the linear ΔA/t range was up to 40% of maximal ΔA = 720 mA. For the 3 min CRT point, the AT3 test was performed 14-fold for 0.9% NaCl and for AT3 deficient plasma.

50% Inhibitory Concentration (IC50) of Arginine Against Fibrin Polymerization

In 4-fold, 25 µL pooled normal plasma was added to 5 µL 0 to 2.4 M arginine, pH 7.4, in F-wells. 5 µL of a thrombin reagent (Table 1) without arginine, a thrombin reagent without arginine/heparin, or 6% human albumin was added. After 2 min at 37°C and 2 min at 23°C the turbidities of the samples were measured at 405 nm. The same experiment was performed with an experimental dysfibrinogen (pooled normal plasma, pre-oxidized for 10 min (37°C) at 12 mM chloramine-T (Sigma)).

To demonstrate that there is no fibrin polymerization in patient samples in the presence of arginine, the AT3 in n=92 citrated patient samples reacted with the added thrombin as described in Table 1. The turbidity before and 5 min (RT) after the 2 min (37°C) thrombin/AT3 reaction was determined at 405 nm. Both turbidities for each sample were compared. Six percent BSA-PBS instead of plasma was the negative control.

<table>
<thead>
<tr>
<th>Table 1_AT3 Test Scheme</th>
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<tr>
<td>25 µL Plasma (in F-wells)</td>
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<tr>
<td>5 µL 92 IU/mL bovine thrombin, 10 IU/mL heparin, 1480 mM arginine, pH 7.4, 6% human albumin</td>
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<tr>
<td>1 min reaction time at 37°C in water-bath</td>
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<tr>
<td>100 µL 2.5 M arginine, pH 8.6, 0.13 % Triton X 100</td>
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<tr>
<td>3 min</td>
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<tr>
<td>50 µL mM CHG-Ala-Arg-pNA in 1.25 M arginine, pH 8.7</td>
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<td>ΔA/min at 405 nm</td>
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Abstract

Background: Antithrombin III (AT3) in highly diluted plasma may not react like AT3 in circulating blood.

Methods: To avoid artificial changes of the plasma matrix by strong dilution, a new AT3 test was developed: 25 µL citrated plasma reacts with 5 µL thrombin reagent, 92 IU/mL (15.3 IU/mL final) bovine alpha-thrombin, 10 IU/ml (1.7 IU/mL final) heparin, 1.480 mM (247 mM final) arginine, pH 7.4, 6% human albumin for 1 min (37°C). Plasma is only diluted by 20%. Then 100 µL 2.5 M arginine, 0.15% Triton X 100, and 50 µL 1 mM (0.28 mM final) chromogenic thrombin substrate CHG-Ala-Arg-pNA are added and increase in absorbance (∆A/min) is determined.

Results: Antithrombin III is specifically determined with 1 min reaction time; from the third minute onward a second thrombin inactivator significantly acts.

Conclusion: This undiluted AT3 test uses approximately 10 IU/ml (final activity) thrombin, which is the approximate peak activity of thrombin caused by intrinsic hemostasis activation.

Antithrombin III Determination in Nearly-Undiluted Plasma

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Assay Precision, Detection Limit, Calibration, and Normal Range

Pooled normal plasma (100% of norm AT3), AT3-deficient plasma (0%), 6% BSA-PBS (+), 0.9% NaCl (*), pooled normal plasma diluted 1:2 with 6% BSA-PBS (▲) or with 0.9% NaCl (grey △), pooled normal plasma diluted 1:4 with 6% BSA-PBS (●) or with 0.9% NaCl (grey ●) were added incubated with 5 µL thrombin reagent. After 0-10 min coagulation reaction time (37°C) 100 µL 2.5 M arginine, pH 8.6, 0.13% Triton X 100 were added. After 3 min 50 µL 1 mM CHG-Ala-Arg-pNA in 1.25 M arginine, pH 8.7 were added and ∆A/min was determined. Calculation of X²-value at the critical time point 3 min CRT: a 14-fold AT3 determination in 0.9% NaCl results in a mean value of thrombin activity of 279 mA/min 23°C; all 14 results are below the 299 mA/min point; the uncorrected X² – value (derived from the 4-fields-table) is: (0 x 7 - 14 x 12)² / (14 x 14 x 7 x 21) = 9.33; the two-fold Yates-corrected 4-fields-table results in the corrected X² – value of (1 x 8 – 13 x 6)² / (14 x 14 x 7 x 21) = 4.76; i.e. the thrombin activity obtained for AT3-deficient plasma is significantly smaller (P < 0.05) than that obtained for 0.9% NaCl. (B) Plasma dilutions with 0.9% NaCl and a reaction time of 1 min (*), 2 min (●), 3 min (▲), 4 min (◆), 5 min (■). (C) Lyophilized 100% of norm plasma was reconstituted 2-fold concentrated in H₂O and diluted with 0.9% NaCl.

Comparison of the New AT3 Test With a Commercial Test

Unselected citrated patient samples (n=68) were tested with the new undiluted assay (Table 1) and with a routine assay (Berichrom Antithrombin III; Behring Coagulation Timer; Dade Behring), where 1-µL citrated plasma was incubated with 60-µl bovine thrombin-reagent, resulting in a 61-fold dilution of plasma. After 3 min at 37°C, thrombin was chromogenically detected.

Results and Discussion

Table 1 demonstrates the optimized AT3 assay. Arginine inhibits hemostasis activation and fibrin polymerization. Heparin was added at a final concentration of 1.7 IU/mL because strongly-heparinized patients (eg, those undergoing cardiopulmonary bypass-operations) might have 10-fold the usual heparin concentrations in heparinized patients; the intention was to dispose of the same AT3 assay for all patients. Figure 1A shows the normal kinetic of thrombin inhibition by AT3. The inhibitory time point 50% in pooled normal citrated plasma is approximately 2.5 min (37°C). Within the first 3 min coagulation reaction time (CRT), AT3-deficient plasma behaves like 6% BSA-PBS or 0.9% NaCl. Then
thrombin is increasingly inactivated also by AT3-deficient plasma, presumably by heparin cofactor II.4-8 Approximately 10% of added thrombin cannot be inactivated even by reaction times greater than 8 min (37°C). This is presumably the part of thrombin molecules that are complexed with α2-macroglobulin. Plasma dilutions with 6% BSA-PBS (Figure 1B) or 0.9% NaCl (Figure 1C) result in proportionally decreased AT3 activity. The present AT3 assay can be calibrated with purified AT3; AT3 activities up to at least 200% of norm can be detected.

The IC50 of arginine against fibrin polymerization in pooled normal plasma is about 40 mM (Figure 2A) and about 30 mM in pre-oxidized plasma (Figure 2B). In presence of 247 mM arginine during the thrombin/AT3 reaction phase, there is no increase in turbidity (ie, arginine completely prevents the generation of polymerized fibrin in plasma). This is of particular importance because fibrin acts as antithrombin I, which complicates the interpretation of any AT3 assay data.

The intra-assay precision is <5%, and the detection limit is 5% of normal. The normal range is 100 ± 20% (MV ± 2SD). The new AT3 assay correlates with r=0.751 with a functional AT3 assay that works with 61-fold dilution of plasma. AT3 in highly diluted plasma may not react like AT3 in circulating blood. It is suggested that the AT3 assay with arginine in nearly undiluted plasma reflects the physiology better than one in highly diluted plasma. The present AT3 test uses a final plasmatic thrombin activity in the range of approximately 10 IU/mL, which is the approximate peak activity of thrombin that is caused by intrinsic hemo- stasis activation.2 This new AT3 test is a new tool for the hemostasis laboratory to monitor the function of the most important inhibitor of coagulation.