Two Automated Methods for Plasma Antithrombin III Compared, and the Clinical Significance of the Results

Winfried Prellwitz,¹ Karl-Friedrich Schmitt,¹ Mathias Machner,¹ Carl-Johannes Schuster,² and Ludwig Wellemann²

Antithrombin III (AT III) activity was determined with two different new chromogenic substances—Chromozym-TH (Tos-Gly-Arg-p-nitroanilide; Boehringer Mannheim) and α-N-carbobenzyl-oxy-L-lysine-thiobenzyl ester (Du Pont)—with both a discrete (aca) and a centrifugal analyzer (COBAS BIO). The correlation between the Chromozym-TH/centrifugal analyzer and Du Pont ester/aca methods was good (r = 0.9890). Precision within and between runs was similar to that for typical enzymic determinations. AT III in plasma of 226 healthy men and women ranged from 76.6 to 141.1 % (100 % = “normal”). We found no significant differences ascribable to oral contraceptives. AT III activity was decreased in 27 % of patients with acute thromboembolic diseases (n = 62), in 48 % of patients the first day after abdominal operations without complications (n = 78), and in 100 % of patients with reversible or irreversible shock (n = 58). In patients receiving continuous therapy with heparin (1500 USP units/h) we saw no decrease in AT III within 96 h of beginning treatment. Plasma from 14 of 16 patients with disseminated intravascular coagulopathy showed a decrease in AT III of from 17 to 51 % of normal before and during heparin therapy. We then treated all 16 patients with AT III concentrate. During such treatment, AT III in plasma must be monitored over short intervals to assure that sufficiently high proportions of AT III (>70 % of normal) are reached.

Additional Keyphrases: synthetic substrates · discrete analysis · centrifugal analyzer · coagulation assays · therapy with heparin · reference interval · disseminated intravascular coagulopathy · reversible and irreversible shock · thromboembolic disease · surgery · oral contraceptives · sex-related effect absent

Antithrombin III (AT III), an heptatically synthesized glycoprotein, is an important regulator of intravascular blood coagulation because it can form inactive complexes with coagulation factors IIa, IXa, Xa, and VIIa, and other serinecleaving proteases. Moreover, plasma kallikrein, plasmin, and complement component C1 can bind to AT III. The inactive complex between AT III and these proteases is the result of an esterification between the terminal arginine of the inhibitor and the serine groups of the proteases (1–6).

Without heparin, inhibition of serine proteases, especially thrombin, is very slow. Heparin considerably accelerates the formation of these inactive complexes by binding to lysyl groups of AT III, thereby inducing structural changes in AT III that facilitate rapid esterification with the serine groups of the proteases (6–11). The inactive complexes of AT III and serine proteases have a half-life of 60 h in blood; they are eliminated via the reticuloendothelial system. The functional form of AT III is determined by use of chromogenic substrates.

Bound AT III in AT III–protease complexes and abnormal AT III molecules (e.g., AT III–Budapest) can be determined with immunological methods (12). However, these estimations have no significance in indicating potential inhibition of serine proteases.

Materials and Methods

To determine AT III, we used citrated plasma. Blood samples were collected into Vacutainer Tubes, with one part of sodium citrate (0.11 mmol/L) and nine parts of blood. After centrifugation at 3000 rpm for 5 min, the supernatant fluid was stored for no longer than 2 h at 4 °C, then analyzed. For quality control, plasma of healthy persons was collected in the same manner, mixed, and stored at −25 °C in 300-μL portions.

Centrifugal Analyzer Procedure: Synthetic Substrate

We used Chromozym TH Test Kit for AT III (cat. no. 25377; Boehringer Mannheim, F.R.G.), which includes a synthetic chromogenic substance, Tos-Gly-Arg-p-nitroanilide. The Tris HCl buffer (7.5 mmol/L, pH 8.1) contained heparin and thrombin.

Reaction mixture (final concentrations):
- Tris HCl buffer 7.5 mmol/L
- Heparin 1310 USP units/L
- Aprotinin 5 kilo-int. units/L
- Thrombin 9.2 int. units/L
- NaCl 112 mmol/L
- Chromozym TH 76 μmol/L

Test principle:

AT III + heparin → AT III–heparin complex
AT III–heparin + thrombin (in excess) → AT III–heparin–thrombin + residual thrombin
Residual thrombin + Chromozym TH → Tos-Gly-Arg-OH + p-nitroanilide

Apparatus: COBAS-BIO centrifugal analyzer (Hoffmann–La Roche, Switzerland) with the following settings:
- Temp, °C 37
- Wavelength, nm 405
- Sample vol, μL citrated plasma 10
- Diluent vol, μL 40
- Reagent (buffer) vol, μL 400
- Incubation time, s 300
- Start reagent vol (Chromozym TH), μL 20
- Time of first reading, s 30
- Time interval, s 10
- No. readings 6
- Type of analysis 3

Calculation of sample AT III activities: The calibration curve from AT III standards is prepared from material supplied with the Boehringer kit (we used lot no. 407 143). The “normal” AT III value is that for pooled citrated plasma of

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selected healthy donors, ages 20 to 40 years. The result is expressed as the percentage of this mean value established by each laboratory for "normal" healthy individuals.

Discrete Analyzer Procedure: Synthetic Substrate

Reagents: Du Pont de Nemours International Analytical Test Pack AT III, with chromogenic substance: α-N-carboxyanhydrides of L-lysine-thiobenzyl-ester (Z-Lys-SBZL).

Reaction mixture (amount per assay pack):
- Heparin: 20 USP units
- Thrombin: 7 int. units
- Z-Lys-SBZL: 1.4 mg
- 5,5-Dithiobis(2-nitrobenzoic acid) (DTNB): 2.0 mg

Test principle:
- AT III + heparin → AT III–heparin complex
- AT III–heparin + thrombin (in excess)
  → AT III–heparin–thrombin + residual thrombin

Residual thrombin + Z-Lys-SBZL → α-toluenethiol
α-Toluenethiol + DTNB → chromophore

Apparatus: We used an Automatic Clinical Analyzer (aca; Du Pont Instruments, Wilmington, DE 19898), with the following settings:
- Temp, °C: 37
- Wavelength, nm: 452
- Sample vol, µL citrated plasma: 20
- Diluent vol, µL purified water: 4800
- Reaction period, s: 261.5
- Measurement period, s: 17.07
- Type of measurement: rate

Calculation of sample AT III activities: The aca readout is in mAh/min. A calibration curve is obtained for each pack lot by plotting, on the graph paper provided, the mAh/min results (y-axis) and the assigned value (x-axis) of the AT III calibrators (two activities of AT III in lyophilized human plasma). The AT III value is assigned with reference to the World Health Organization's First International Standard of AT III. The results of measurement are expressed in percentage of the mean value for "normal" human plasma.

Patients

We determined AT III in the plasma of 226 adult healthy individuals, 112 men (ages 16–66) and 114 women (ages 15–67), and in
- 62 patients with thromboembolic diseases (as established by 123I scan, phlebography, lung scan);
- 19 patients being treated with heparin;
- 78 patients before and after abdominal operations;
- 29 patients with reversible shock (five patients with exogenous intoxications, eight patients after surgical operations, four patients with acute pancreatitis, two patients with cardiogenic shock, five patients after polytrauma, five patients with hemorrhagic shock);
- 29 patients with irreversible shock (four patients with exogenous intoxications, nine patients after surgical operations, six patients with acute pancreatitis, three patients with hemorrhagic shock, six patients with cardiogenic shock, one patient with septicemic shock). Between the groups with reversible and irreversible shock we saw no differences in mean arterial pressure, cardiac index, arterial oxygen saturation, or lactate concentration in serum. Only the pulmonary vascular resistance increased in the group of non-survivors (400 to 100 dynes...
Table 2. Antithrombin III: Precision Between-Run with the "Boehringer-COBAS" (I) and "aca-Du Pont" (II) Method (n = 20)

<table>
<thead>
<tr>
<th>Control</th>
<th>Method</th>
<th>Mean (%)</th>
<th>SD (%)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PreciChrom I*</td>
<td>I</td>
<td>95.5</td>
<td>4.6</td>
<td>4.86</td>
</tr>
<tr>
<td>Assigned bottle value:</td>
<td>II</td>
<td>93.5</td>
<td>3.8</td>
<td>4.06</td>
</tr>
<tr>
<td>95.0% of &quot;normal&quot; value</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PreciChrom II*</td>
<td>I</td>
<td>47.1</td>
<td>3.1</td>
<td>6.74</td>
</tr>
<tr>
<td>Assigned bottle value:</td>
<td>II</td>
<td>45.0</td>
<td>3.0</td>
<td>6.66</td>
</tr>
<tr>
<td>47.0% of &quot;normal&quot; value</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pooled plasma</td>
<td>I</td>
<td>105.0</td>
<td>9.6</td>
<td>9.20</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>101.5</td>
<td>8.4</td>
<td>8.27</td>
</tr>
</tbody>
</table>

*Boehringer, Mannheim, F.R.G.

Table 3. Normal Values for Antithrombin III in Plasma of 226 Healthy Persons (Method I = COBAS/Boehringer, Method II = aca-Du Pont)

<table>
<thead>
<tr>
<th></th>
<th>Method</th>
<th>Mean</th>
<th>SD</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total group</td>
<td>I</td>
<td>101.6%</td>
<td>22%</td>
<td>72.6-141.1%</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>100.3%</td>
<td>21%</td>
<td>72.0-140.5%</td>
</tr>
<tr>
<td>Male</td>
<td>I</td>
<td>102.0%</td>
<td>19.5%</td>
<td>72.3-141.4%</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>100.1%</td>
<td>19.0%</td>
<td>71.5-140.6%</td>
</tr>
<tr>
<td>Female</td>
<td>I</td>
<td>101.5%</td>
<td>21.4%</td>
<td>72.8-140.9%</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>100.0%</td>
<td>20.6%</td>
<td>71.1-139.1%</td>
</tr>
</tbody>
</table>

For plasma samples from 62 patients with untreated acute thrombembolic diseases, the mean AT III was 78.1% (range, 49.4–97.2%). In 17 (27%) of these patients the AT III was <70%.

For plasma from eight patients who had thromboembolic diseases but normal values for AT III we determined AT III during therapy with heparin. During 96 h of observation after this treatment was started we did not observe a decrease in AT III; moreover, after the treatment was stopped (four to six days after beginning), AT III activities in plasma in all cases were normal.

Each of 11 patients with acute thromboembolic diseases and decreased values for AT III was treated with 10 mL of AT III concentrate (Behringwerke, Marburg, F.R.G.). These 10 mL of AT III concentrate contained the AT III activity of 500 mL of citrated plasma from healthy individuals. We then treated with heparin. In these cases we saw no decreases of AT III (Table 4).

In plasma collected from 78 patients before and after abdominal surgery the mean AT III was decreased in the first three postoperative days (Table 5): at the first day 48%, the second day 36%, and the third day 27% of the patients had AT III activities corresponding to <70% of the values before surgery.

Table 4. Antithrombin III Levels (Ranges) in Plasma of 19 Patients with Thromboembolic Diseases before and during Therapy with Heparin (28 000 USP units/24 h) *

<table>
<thead>
<tr>
<th></th>
<th>Before therapy</th>
<th>Substitution with AT III concentrate</th>
<th>12 h</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
<th>96 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>11</td>
<td>10 mL</td>
<td>46–66%</td>
<td>45–66%</td>
<td>48–68%</td>
<td>52–72%</td>
<td>50–71%</td>
</tr>
<tr>
<td>8</td>
<td>66–80%</td>
<td>0</td>
<td>61–75%</td>
<td>65–76%</td>
<td>66–74%</td>
<td>68–78%</td>
<td>66–82%</td>
</tr>
</tbody>
</table>

Table 5. Means, Standard Deviations, and Ranges of Antithrombin III in Plasma of 78 Patients before and after Abdominal Operations

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>SD</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-op</td>
<td>96.1%</td>
<td>10.5%</td>
<td>72.2–131%</td>
</tr>
<tr>
<td>1st day post-op</td>
<td>63.3%</td>
<td>17.8%</td>
<td>11.1–100%</td>
</tr>
<tr>
<td>2nd day post-op</td>
<td>71.1%</td>
<td>16.5%</td>
<td>38.8–111%</td>
</tr>
<tr>
<td>3rd day post-op</td>
<td>82.2%</td>
<td>14.2%</td>
<td>47.2–123.2%</td>
</tr>
</tbody>
</table>

We determined AT III in plasma of patients with shock at 8-h intervals, with special interest in the minimal values attained (Table 6). The minimal AT III in plasma of patients with either reversible or irreversible shock was significantly less than the normal range (t-test; p ≤.001). We could not differentiate between the two forms of shock on the basis of AT III measurements.

In plasma of 14 of 16 patients with disseminated intravascular coagulopathy (acute pancreatitis, exogenous intoxication, sepsis) we observed a decrease in AT III activity to final levels of 17 to 51% of normal before and during the therapy with heparin and substitution of coagulation factors. All patients had at the beginning of their coagulopathy fewer than 50 000 platelets per microliter, and coagulation factors, especially Factor VIII, were <20% of the norm. All these patients were infused with AT III concentrate until activities in plasma were nearly 70% of the norm. In four cases a repeated decrease of AT III was observed during the therapy and infusion. Associated with this decline in AT III values, there was a decrease in platelets and coagulation factors. After infusion with adequate amounts of the AT III concentrate (Behringwerke) we observed, on continued treatment with heparin, an increase in AT III, platelets, and coagulation factors (Figure 3).

Discussion

Determinations of AT III with use of chromogenic substances are published (13–15).

Table 6. Antithrombin III Levels (Ranges) in Plasma of 19 Patients with Thromboembolic Diseases before and during Therapy with Heparin (28 000 USP units/24 h) *

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<tbody>
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<td>10 mL</td>
<td>46–66%</td>
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<td>52–72%</td>
<td>50–71%</td>
</tr>
<tr>
<td>8</td>
<td>66–80%</td>
<td>0</td>
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<td>65–76%</td>
<td>66–74%</td>
<td>68–78%</td>
<td>66–82%</td>
</tr>
</tbody>
</table>

* 11 patients with decreased AT III and substitution with AT III concentrate; eight patients with normal AT III and without substitution with AT III concentrate.
We determined the biological activity of AT III with two different new chromogenic substances and the aca OR COBAS-BIO analyzer. Both instruments are commonly used for emergency analysis.

A good correlation was found between the two methods, regardless of whether we used patients' plasma, pool plasma, or lyophilized standards. The precision, both within and between assays, is similar to typical findings for enzymatic determinations. Lipemia, bilirubinemia, and hemolysis have no effect on the assays.

Recently an international reference preparation of AT III has been established by the WHO (16), providing a good basis for future intralaboratory comparisons.

Routiney, we used for quality control, in addition to lyophilized standards, a plasma pool collected from healthy individuals in the same manner as the patients' samples. In the group of healthy controls we observed no sex-related differences nor were there differences between women who were or were not taking oral contraceptives. The latter finding is probably due to the use of preparations containing predominantly progesterone (estrogen content, <20 μg per dose). Although a decrease in AT III is described in the literature (17-19), the decrease depends on the estrogen content of the medication. With a high estrogen content of the pill (30 μg), it is significant, but progesterone alone did not induce any decrease in AT III (19).

Today the association between thromboembolic disease of inborn or acquired depression of AT III is accepted (20-27). Lechner et al. (23) observed, in plasma from 290 patients with acute thromboembolic disease, 27 cases (9.3%) of significant reduction in AT III. In 71 of 69 patients (27%) we measured a decrease of AT III activity of <70%. These individuals would be candidates for inhibitor infusion.

It still is unclear whether AT III decreases in plasma during continuous heparin therapy. Macniniak and Gockerman (28, 29) described a decrease in AT III during treatment with heparin. Kakkar et al. (30), however, observed this decrease only during intravenous infusion, but not after subcutaneous administration. We saw no decrease in AT III during continuous therapy with heparin, either in plasma of patients with normal values or in those with hereditary decreased activities following administration of the inhibitor.

Likewise, in plasma of normal persons we did not observe any decrease of AT III during intravenous or subcutaneous administration of heparin (mean heparin concentration: 0.45 USP units/mL of plasma, observation time: 96 h).

One possible reason of these differences might be the different methods used to determine AT III activity.

After abdominal operations without any complications we observed, especially in the first day after operation, a decrease in AT III, reaching <70% of the norm in plasma of 48% of all patients. Schipper et al. (31) described similar results, especially in the presence of bacterial complications.

It seems possible that the relative frequency of postoperative thromboembolic episodes (35%, or 46% after polytrauma or orthopedic operations) is connected with a decrease in AT III (32). Whether this frequency of complications can be reduced by infusion of antithrombin III must be investigated.

A deficiency of AT III in plasma of patients with shock is described as an early indicator, especially for septicemia (33, 34). In our patients with shock we only observed one case with urosepsis; all other patients had no septicemia. Therefore we believe that the decrease of AT III in plasma is a general biochemical symptom of shock. Discrimination between the reversible and the irreversible form on the basis of AT III data is not possible.

In disseminated intravascular coagulopathy, we observed in nearly all cases a reduction of AT III in plasma, values being between 17 and 51% of the norm, as also described by Schipper et al. (35). Ten for these patients had decreased activity of AT III immediately after the beginning of the disseminated intravascular coagulopathy. They were administered 20–35 mL of AT III concentrate during 3–5 h until AT III activities were nearly 70% of the norm. In four cases a repeated decrease of AT III was measured during therapy with heparin and substitution with fresh blood. In these cases, a simultaneous reduction of platelets and coagulation factors was observed, because heparin is nearly ineffective without AT III and therefore platelets and clotting factors will be consumed further.

During treatment with AT III concentrate, monitoring of plasma for AT III at short intervals (60–90 min) is necessary
to assure that its activity is sufficiently high to minimize complications of disseminated intravascular coagulopathy.

References


2. Abildgaard, U., Evidence that antithrombin III is the main physiological inhibitor of coagulation enzymes. Ibid., pp 31-34.


