Increased Concentrations of Heparin Cofactor II in Diabetic Patients, and Possible Effects on Thrombin Inhibition Assay of Antithrombin III

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We compared concentrations of antithrombin III (AT-III) in plasma, as determined by an immunological method and by a functional thrombin inhibition method, in the presence of heparin in 160 blood samples from Type I diabetics. Although the correlation was highly significant (P < 0.001) between the results obtained by the two methods, our data demonstrated that results by the thrombin inhibition assay, 121 (SD 15)% expressed as percentages of the results for a normal plasma pool, were significantly (P < 0.001) higher than by the immunoreactive method, 104 (SD 15)%, indicating an overestimation of functionally active AT-III. Concentrations of functionally active AT-III determined by a factor Xa inhibition assay, 105 (SD 13)%, were in the same range as immunoreactive AT-III. Addition of IgG antiserum to normal pooled plasma quenched only about 90% of the AT-III activity determined by the thrombin inhibition assay, but all of the AT-III activity determined by a factor Xa inhibition assay. These results demonstrate that the factor Xa inhibition assay is more specific for the determination of AT-III than the thrombin inhibition assay. We suggest that the high concentrations of heparin cofactor II, 117 (SD 17)%, might have caused an overestimation of AT III in this group of patients with diabetes Type I, and should not be overlooked in other clinical situations.

Additional Keyphrases: immunoassay and factor Xa inhibition assay compared ; variation, source of ; coagulation

The concentration of functionally active antithrombin III (AT-III) in plasma is usually determined by the capacity of a plasma specimen to inhibit thrombin in the presence of heparin; that is, AT-III is thus measured as a cofactor of heparin (1–4). Another thrombin-binding heparin cofactor in human plasma, heparin cofactor II (HC-II) (5, 6), inhibits thrombin but not blood coagulation factor Xa, and experiments have demonstrated that HC-II affects the total thrombin binding capacity of plasma (7). Although this interference is commonly assumed to be of little significance in routine clinical applications, because the ratio between AT-III and HC-II in plasma is in the same range in healthy volunteers and in patients suffering from conditions related to intravascular coagulation (8–11), this relationship is still incompletely elucidated in other situations.

We report here that patients with Type I diabetes have increased concentrations of HC-II, and that the use of the thrombin inhibition assay for determination of functionally active AT-III in such patients may cause the concentrations to be reported as higher than they actually are. Although most of the recommended and commercially available methods for determining functionally active AT-III in plasma are based on the thrombin inhibition assay, our results suggest that performing the factor Xa inhibition assay of AT-III in the presence of heparin yields more reliable results.

Materials and Methods

Plasma sampling. From 23 fasting Type I diabetics, we collected, in the morning, 3-mL samples of blood into siliconized evacuated glass tubes (Venoject T-273 QS, Terumo Europe SA, Leuven, Belgium) containing 60 μL of 170 mmol/L tripotassium EDTA. During a period of eight months we collected seven samples from each patient. Platelet-poor plasma was prepared by centrifugation in the cold (4 ºC) at 2000 x g for 20 min. All plasma samples were stored at −80 ºC until assay. We also assayed a pool of plasma collected from 40 apparently healthy volunteers (20 women, 20 men) and stored at −80 ºC.

Thrombin inhibition assay of AT-III. Determinations were made by a Cobas Bio centrifugal analyzer (Hoffmann-La Roche, Basel, Switzerland) as described before (3). The concentrations of AT-III are automatically calculated by the analyzer and expressed as percentages of the value of a normal plasma pool. Lyophilized bovine thrombin, 5000 NIH units (Leo Pharmaceuticals, Copenhagen, Denmark), was reconstituted with 200 mL of isotonic saline (150 mmol/L NaCl) to give a stock solution containing 25 units/mL. We stored this solution in 500-μL aliquots at −80 ºC in plastic vials.

The heparin–Tris–EDTA buffer (pH 8.4) contained, per liter, 50 mmol of Tris, 7.5 mmol of EDTA, 3000 int. units of heparin (from porcine mucosa, Leo Pharmaceuticals), and 1.7 g of polyethylene glycol 6000 (Mf 6000). We reconstituted 40 μmol of H-d-Phe-Pip-Arg-pNA substrate (S-2223; KabiDiagnostica, Stockholm, Sweden) with 18.8 mL of isotonic saline containing 0.5 g of Tween 80 (polyethylene sorbitan monooleate) surfactant per liter, to give a stock solution with a substrate concentration of 2.13 mmol/L.

Factor Xa inhibition assay of AT-III. The factor Xa inhibition of AT-III in plasma was determined by the automated method as described below. Lyophilized bovine factor Xa, 10 nmol (KabiDiagnostica), was reconstituted in 40 mL of the heparin–Tris–EDTA buffer to give a stock solution containing 0.25 mmol/mL. We reconstituted 38 μmol of Bz-Ile-Gly-Gly-Arg-pNA substrate (S-2222; KabiDiagnostica) with 16.8 mL of sterile water containing 0.5 g of Tween 80 per liter, to give a stock solution with a substrate concentration of 2.26 mmol/L.

The assay was modified for use in a Cobas Bio centrifugal analyzer as follows: The analyzer further dilutes 12 μL of the diluted plasma sample (1 + 19 in isotonic saline) with 48 μL of distilled water and mixes this with 200 μL of the factor Xa stock solution. After incubation for 300 s at 37 ºC, this solution is mixed with 60 μL of the S-2222 substrate solution, and initial rates are obtained from repeated determinations of the absorbance at 405 nm, the first reading after 0.5 s being followed by readings every 10 s. Dilutions of plasma from the normal pool are used to construct a
various percentage reference was the presence of the various dilutions of plasma are determined. The factor Xₙ inhibition (representing functionally active AT-III) is automatically calculated by the analyzer and expressed as a percentage of the value for the pooled plasma. Precision studies of routine assays (run over 60 days) of the same pool of reference plasma gave an estimated CV of 3%.

**Thrombin inhibition assay of heparin cofactor II.** Again using a Cobas Bio centrifugal analyzer (14), we determined the concentration of functionally active HC-II in plasma as the capacity of plasma to inhibit human thrombin in the presence of dermatan sulfate. Lyophilized human thrombin, 126 NIH units (Boehringer Mannheim, Mannheim, F.R.G.), was reconstituted with 12.6 mL of sterile water to give a stock solution containing 10 NIH units/mL. This solution was stored in 300-μL aliquots at −80 °C in plastic vials. Dermatan sulfate was prepared from chondroitin sulfate, type B (from porcine skin; Sigma Chemical Co., St. Louis, MO) (13, 14). A Polybrene—Tris—EDTA buffer, pH 8.4, was prepared to contain per liter, 50 mmol of Tris, 7.5 mmol of EDTA, 4 mg of Polybrene (hexadimethrine bromide), 1.7 g of polyethylene glycol 6000, and 400 mg of dermatan sulfate. We reconstituted 30 μmol of the Tos-Gly-Pro-Arg-pNA substrate (Chromozym TH; Boehringer Mannheim) with 21.1 mL of sterile water containing 2 g of Polybrene and 0.5 g of Tween 80 per liter, to give a stock solution with a substrate concentration of 1.43 mmol/L.

**Other assays.** "Norpartigen" plates (Behringwerke, Marburg, F.R.G.) were used for the determinations of immunoreactive AT-III. We determined the residual activities of AT-III (factor Xₙ inhibition, thrombin inhibition) after adding increasing amounts to pooled normal plasma of rabbit IgG antiserum against human AT-III (Dakopatts, Glostrup, Denmark). The protein content of the antiserum was 12 g/L.

**Statistical analysis.** Paired data were evaluated by means of Student's t-test. The regression line of best fit was constructed by the method of least squares. Analysis of variance was used to estimate the predicted y-values around the regression line.

**Results**

Comparison of the concentration of immunoreactive AT-III (y) with the concentration of functionally active AT-III determined by the thrombin inhibition assay (y) yielded a regression line expressed by the equation $y = 0.73x + 46\%$ (Figure 1), with $r = 0.73$ ($P < 0.001, t = 13.4, df = 158$). Analysis of variance demonstrated that the intercept of the regression line on the y-axis (Figure 1) was significantly different from zero ($P < 0.001$).

The concentrations of AT-III determined by the thrombin inhibition assay were higher than both the concentrations of immunoreactive AT-III ($P < 0.001; t = 20.1, df = 159$) and the concentrations determined by the factor Xₙ inhibition assay ($P < 0.001; t = 19.1; df = 159$) (Figure 2). The mean values were as follows: the thrombin inhibition assay 121 (SD 15)%; the immunoreactive assay 104 (SD 15)%; and the factor Xₙ inhibition assay 105 (SD 13)% (Figure 2).

We added increasing amounts of antiserum against human AT-III to normal plasma and measured the residual antithrombin activity by the thrombin inhibition assay and the factor Xₙ inhibition assay. While about 90% of the antithrombin activity measured by the thrombin inhibition assay was quenched by the antiserum, all the activity in the factor Xₙ inhibition assay was quenched (Figure 3).
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Residual antithrombin III activities were determined by the thrombin inhibition assay (II) and the factor Xₐ inhibition assay (III).

(a) if the binding of AT-III to thrombin in the diluted plasma sample is enhanced with moderate amounts of heparin (9), or (b) when bovine thrombin is used instead of human thrombin (15), or (c) when a brief incubation of the mixture of thrombin and the diluted plasma is used (7). Our results demonstrate that these modifications are not always sufficient to ensure specific determinations of functionally active AT-III. We observed a marked overestimation of the concentration of functionally active AT-III in patients with Type I diabetes when we compared the results with the concentrations of immunoreactive AT-III (Figures 1 and 2). This was unexpected, because a possible non-enzymatic glycation in diabetes reportedly impairs the function of AT-III (e.g., 16). Hence, another inhibitory compound present in human diabetic plasma might have influenced the thrombin inhibi-

tion assay. Determined by the factor Xₐ inhibition assay the concentrations of functionally active AT-III were much lower than those obtained by the thrombin inhibition assay (P < 0.001) and in the same range as the concentrations of immunoreactive AT-III (Figure 2). Further experiments showed that addition to normal plasma of IgG antiserum against AT-III resulted in a residual antithrombin activity of ~10% determined by the thrombin inhibition assay (Figure 3), whereas IgG antibodies against AT-III completely quenched the plasma antithrombin activity determined by the factor Xₐ inhibition assay. These results indicated the existence of a significant effect of HC-II on the thrombin inhibition assay, because HC-II activated by heparin selectively inhibits thrombin and has no significant effect on the inhibition of factor Xₐ (5, 6, 9).

Note that the mean value for the functionally active HC-II in this group of 22 Type I diabetics was 117 (SD 17)% of that for the pool of normal plasma (Figure 4).

The ratio between the concentrations of AT-III and HC-II in plasma has often been reported to be in the same range in healthy normal persons as in individuals suffering from such pathological conditions as hepatic failure or disease states related to intravascular coagulation (8–11, 14). It has therefore commonly been assumed that the interference of HC-II with measurement of AT-III by the thrombin inhibition assay is of minor importance if the concentrations of functionally active AT-III are expressed as percentages of that in a normal plasma pool. However, it has recently been reported that the concentrations of HC-II in plasma increase with increasing age, whereas the concentration of AT-III remains constant (17), and our results definitely demonstrate that there is no correlation between concentrations of AT-III and HC-II in a group of Type I diabetics (Figure 4). Therefore, the possible influence of HC-II on the thrombin inhibition assay cannot be neglected in all situations. We found the factor Xₐ inhibition assay to be more specific for determination of functionally active AT-III in plasma than the thrombin inhibition assay, and we have described an automated method that is convenient for use in the routine laboratory.

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References
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Fig. 3. Residual activities (%) ordinate of antithrombin III in normal human plasma after adding increasing amounts (µL, abscissa) of IgG antiserum against antithrombin III per milliliter of plasma

Residual antithrombin III activities were determined by the thrombin inhibition assay (II) and the factor Xₐ inhibition assay (III).

Fig. 4. Correlation between the plasma concentrations of immunoreactive antithrombin III (abscissa) and heparin cofactor II (ordinate) expressed as percentages of values for a pool of normal plasma.

The coefficient of correlation was not statistically significant (P > 0.05)

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