

A method for the differential determination of plasma antithrombins

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SYNOPSIS A method for the differential determination of plasma antithrombins, antithrombin III and α_2 macroglobulin, is described. The method is based on the selective inactivation of plasma α_2 macroglobulin by treatment with 0.1 M methylamine for 10 minutes at 37°C and on the observation that antithrombin III and α_2 macroglobulin inhibited in defibrinated plasma low concentrations of thrombin without mutual interference and according to pseudo-first order reaction. In healthy subjects antithrombin III was shown to account for about 70% of the total antithrombin activity. But in patients with liver cirrhosis, where low levels of total antithrombin activity were observed, the relative contribution of antithrombin III was found to be noticeably lower.

Three different thrombin inhibitors have been purified from human plasma, namely, an α_2 globulin called antithrombin III (Abildgaard, 1967; Heimbürger, 1967), the α_2 macroglobulin (α_2 M) (Lanchantin *et al*, 1966; Steinbuch *et al*, 1968), and the α_1 antitrypsin (Rimon *et al*, 1966; Gans and Tan, 1967).

Since thrombin inhibition by α_1 antitrypsin occurs at thrombin concentrations far above those likely to exist *in vivo* (Abildgaard, 1969a, b), the antithrombin capacity of plasma appears to be exerted only by antithrombin III and by α_2 macroglobulin.

The methods available to determine the antithrombin activity of plasma invariably measure the combined inhibition of thrombin by both inhibitors. The antithrombin activity of purified α_2 M has been shown to disappear after exposure to methylamine (Steinbuch *et al*, 1968).

In this paper we examine the possible use of methylamine for the selective inactivation of plasma α_2 M in order to obtain a method for the differential determination of plasma antithrombins.

Material

Nine volumes of whole human blood were collected into plastic tubes containing 1 volume of 3.8%

sodium citrate. Plasma was obtained by centrifugation at 1500 g for 15 minutes.

Defibrinated plasma (d-plasma) was prepared by incubating 3-5 ml plasma samples in glass tubes at 56°C for 3 minutes with intermittent shaking. After cooling, the precipitate was removed by centrifugation at 2000 g for 15 minutes.

Standard reference d-plasma was prepared from a pool of 10 healthy donors and stored in small aliquots at -20°C.

Tris-buffered saline, pH 7.8 (0.15 M NaCl, 0.01 M Tris), was used as diluent throughout.

Human fibrinogen grade L (AB Kabi, Stockholm, Sweden) was dissolved as indicated by the manufacturer and stored in small aliquots at -20°C. Before use it was diluted with buffer to 250 mg/100 ml.

Thrombin (Topostasin, Prodotti Roche SpA, Milan, Italy) was dissolved in buffer to 50 NIH units/ml and stored in small aliquots at -20°C.

Aluminium hydroxide (British Drug Houses, Poole, UK) was suspended in water. The sediment obtained after centrifugation was used for plasma absorption.

Methylamine hydrochloride (Carlo Erba, Italy) was dissolved in buffer at a concentration of 2.5 M and adjusted to pH 7.8 with 1 M NaOH.

Immunochemical quantitation of antithrombin III was determined by radial immunodiffusion on M-Partigen Antithrombin III plate provided by the generosity of Behringwerke AG, Marburg-Lahn, Germany.

Methods

INFLUENCE OF METHYLAMINE ON THE CLOTTING ASSAY OF THROMBIN

In order to rule out a possible effect of methylamine on the clotting assay adopted for the determination of thrombin, the following experiments were performed.

Thrombin solutions of varying strength (range 10-0.25 NIH units/ml) were prepared either in buffer or in buffer containing increasing concentrations of methylamine (range 0.02-0.25 M).

Thrombin activity was assayed by adding at 37°C 0.1 ml aliquots of thrombin dilutions to 0.2 ml aliquots of fibrinogen solution and recording the clotting time. All determinations were performed in duplicate.

INACTIVATION OF α_2 M ANTITHROMBIN ACTIVITY BY METHYLAMINE

Plasma samples showing only α_2 M antithrombin activity were prepared by treatment with $\text{Al}(\text{OH})_3$, which has been shown to be an effective tool to remove antithrombin III (Monkhouse *et al.*, 1955; Abildgaard, 1967).

d-Plasma was added to $\text{Al}(\text{OH})_3$ in a 1:1 ratio. The mixture was allowed to stand, with intermittent shaking, at 37°C for 15 minutes; it was then centrifuged at 3000 g for 10 minutes. The supernatant was tested for residual antithrombin III by heparin cofactor assay, according to Abildgaard (1967), and by immunoassay.

Only the samples showing negative results were labelled as α_2 M samples and were subsequently used.

Increasing amounts of methylamine in 0.1 ml aliquots were added to 0.9 ml aliquots of α_2 M sample to give a final methylamine concentration of 0.05-0.1-0.2 M. After 5-10 minutes incubation at 37°C the mixtures were diluted with buffer in order to bring the methylamine concentration below 0.04 M. Their antithrombin activity was tested as follows: 0.1 ml of thrombin (50 NIH units/ml) was added to a 0.6 ml sample at 37°C; after 3 minutes 0.1 ml was removed and residual thrombin was assayed on 0.2 ml of fibrinogen. The results were compared with control assays where 0.6 ml of buffer and 0.6 ml of a correspondingly diluted α_2 M sample were substituted for methylamine-treated samples.

EFFECT OF METHYLAMINE ON ANTITHROMBIN III

Antithrombin III was obtained by elution of the sediment of the $\text{Al}(\text{OH})_3$ -treated plasma with 0.36 M phosphate buffer, pH 7.8.

The eluted solution was treated with 0.25 M methylamine for 10 minutes at 37°C, and antithrombin activity was assayed as above.

KINETICS OF THROMBIN INHIBITION BY ANTITHROMBIN III, BY α_2 MACROGLOBULIN, AND BY BOTH INHIBITORS

Samples containing only antithrombin III (AT III) were obtained by d-plasma treated with 0.1 M methylamine at 37°C for 10 minutes.

According to the above experiments this procedure should result in d-plasma samples practically free of α_2 M activity and containing the original AT III concentration.

Samples containing only α_2 M antithrombin activity were prepared as described above.

AT III and α_2 M samples were then diluted with buffer in order to obtain samples containing the same concentration of inhibitor as a 1/5 dilution of d-plasma. The kinetic analysis of thrombin inhibition was performed as follows: 0.2 ml aliquots of thrombin (50 NIH units/ml) were added at 37°C to 1.2 ml aliquots of AT III, α_2 M, and d-plasma diluted samples; at 1 minute intervals 0.1 ml aliquots were removed from the reaction mixtures, and residual thrombin was assayed on 0.2 ml of fibrinogen.

The initial concentration of thrombin was obtained by substituting buffer for samples.

The results (mean values of three experiments) were expressed by plotting the log of thrombin concentration obtained by the standard clotting curve against the time of incubation.

DIFFERENTIAL DETERMINATION OF PLASMA ANTITHROMBIN

Either 0.1 ml of buffer or 0.1 ml of 1 M methylamine were added to 0.9 ml aliquots of the d-plasma to be tested; after 10 minutes incubation at 37°C the samples were diluted by adding 4 ml of buffer; 0.6 ml of each dilution and 0.6 ml of buffer were pipetted on small test tubes and prewarmed at 37°C for 3 minutes; 0.1 ml aliquots of thrombin solution (about 50 NIH units/ml) were added to each tube, and the mixtures were immediately mixed. After 3-22 minutes of incubation 0.1 ml aliquots were removed from the reaction mixtures and assayed for residual thrombin on 0.2 ml of fibrinogen solution. The clotting time sample/clotting time buffer ratios were calculated, and the results were expressed as percentages of antithrombin activity by reference to a standard curve. This curve was constructed by assaying serial dilutions (1/2.5-1/3.75-1/5-1/10) of the standard d-plasma with no methylamine treatment and by assuming as 100% the 1/5 dilutions.

Results

The clotting assay adopted for the determination of thrombin gave on log-log paper a linear relationship between clotting time and thrombin concentration. Figure 1 shows that methylamine up to 0.04 M does not influence the assay. At larger concentrations non-linear and different curves are observed. Clotting times are prolonged and the clots had a translucent appearance, suggesting possible interference at the stage of fibrin polymerization.

Treatment of plasma samples with 0.1 M methylamine for 10 minutes at 37°C was found to be sufficient to inactivate completely the α_2 M antithrombin (fig 2). This treatment did not change the AT III activity of the sample, even if methylamine was used at 0.25 M (not shown in figure).

Kinetic studies (fig 3) show that the rates of thrombin inhibition by AT III (MA-treated d-plasma), by α_2 macroglobulin (Al(OH)₃-treated d-plasma), and by both inhibitors (d-plasma) follow the rules of a pseudo-first order reaction:

$$\frac{t k_1 \text{ AT III}}{2.303} = \log \frac{T_0}{T_1}$$

$$\frac{t k_2 \alpha_2 \text{ M}}{2.303} = \log \frac{T_0}{T_2}$$

$$\frac{t k_a \text{ A}}{2.303} = \log \frac{T_0}{T_a}$$

where *t* is the time of incubation; *k*₁, *k*₂, and *k*_a are the rate constants respectively for AT III, α_2 M, and A (total antithrombin activity); *T*₀ is the initial concentration of thrombin; *T*₁, *T*₂, and *T*_a are the residual concentration of thrombin of each reaction.

Moreover, it is possible to show (table I) that the inhibition curve produced by both inhibitors (d-plasma) can be obtained by the sum of the curves produced separately by each inhibitor. At each time $\log T_0/T_1 + \log T_0/T_2$ approximates closely to $\log T_0/T_a$, or, in another way, $T_0/T_1 \times T_0/T_2$ approximates to T_0/T_a .

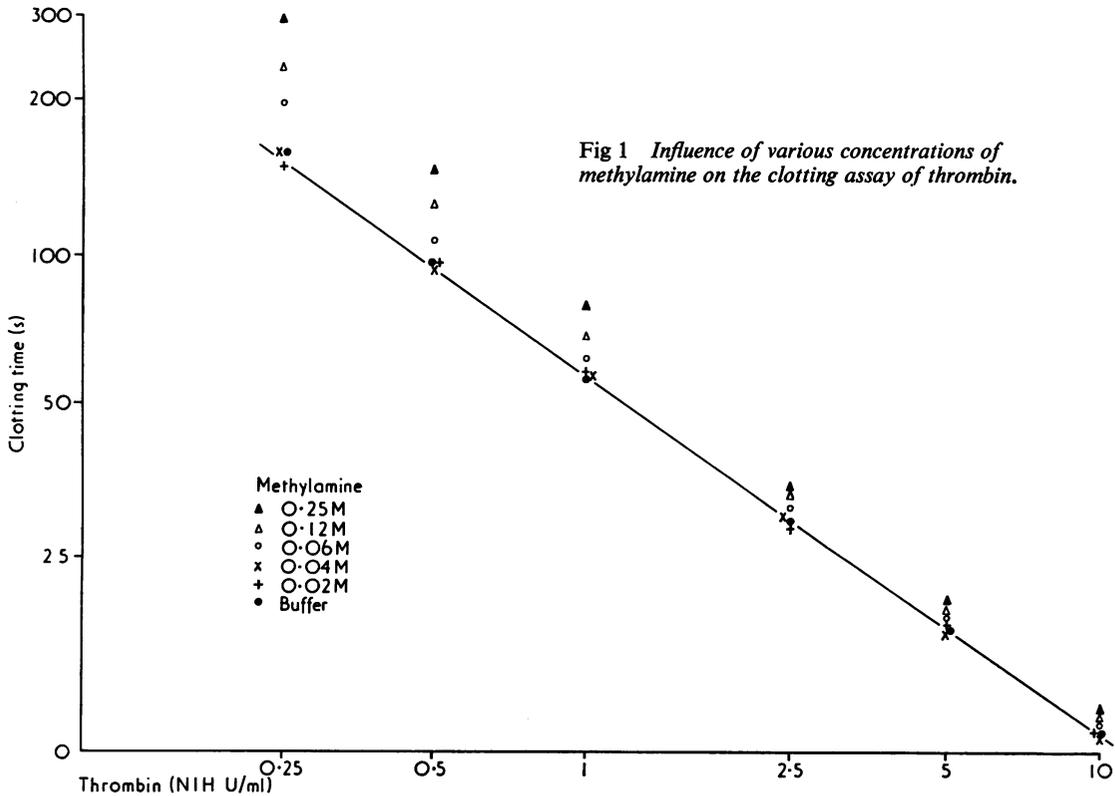


Fig 1 Influence of various concentrations of methylamine on the clotting assay of thrombin.

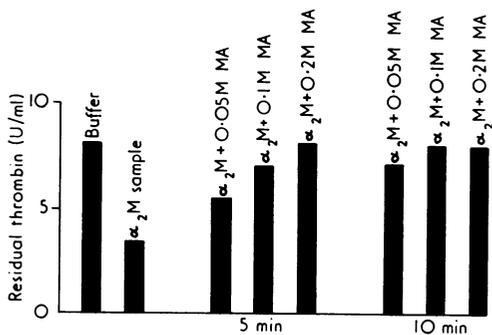


Fig 2 Activity of α_2 M antithrombin after treatment with various concentrations of methylamine (MA) for 5 to 10 minutes.

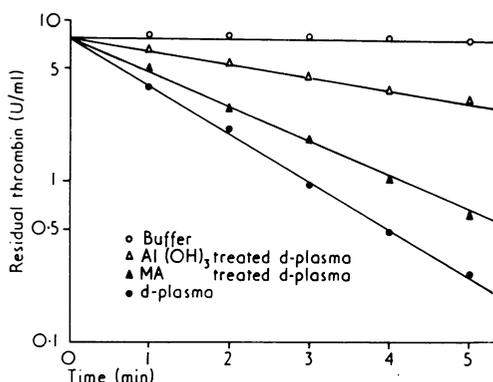


Fig 3 Kinetics of thrombin inhibition by antithrombin III (MA-treated d-plasma), by α_2 macroglobulin (Al(OH)₃-treated d-plasma), and by both inhibitors (d-plasma).

These results permit the development of a method for the differential determination of antithrombins.

The antithrombin activity of a sample can be expressed as the rate of thrombin inhibition, for example, as $\log T_0/T$ at 3 minutes after the start of the reaction. Since a linear log-log relationship was shown to occur between thrombin concentration and

clotting time (fig 1), the expression $\log T_0/T$ could well be substituted by $b \log t/t_0$, where t and t_0 are the clotting times corresponding respectively to T_0 and T , and b is a constant which could be arbitrarily assumed as 1 when the same fibrinogen batch is used. (The value of b depends on the batch of fibrinogen and was shown to vary in our system between 0.70 and 0.88.)

As expected, a linear relationship was observed on a semi-log representation between inhibition rate of thrombin calculated as t/t_0 and antithrombin concentration expressed as dilutions of standard d-plasma (fig 4).

By assaying simultaneously a sample of d-plasma and a sample of d-plasma treated with methylamine we could obtain respectively the total antithrombin activity and the AT III activity. By subtraction the α_2 M antithrombin activity could be calculated.

Results can be expressed as the relative contribution of AT III and α_2 macroglobulin to the total antithrombin activity, assumed to be 100.

Results obtained in healthy subjects and in patients with liver cirrhosis are shown in table II. The relative contribution of AT III to the total antithrombin activity, assumed to be 100, was found to be $72.13\% \pm 5.49$ in healthy subjects and $54.86\% \pm 9.8\%$ in patients with liver cirrhosis.

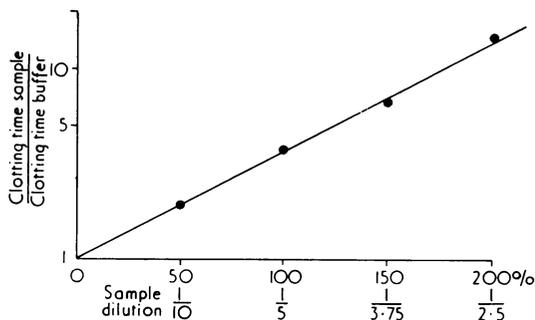


Fig 4 Reference curve for the determination of antithrombin activity as percentage of standard d-plasma.

Time (min)	AT III Sample	α_2 M Sample	d-Plasma	
	T_0/T_1	T_0/T_2	Experimental Value T_0/T_a	Calculated Value $T_0/T_1 \times T_0/T_2$
1	1.6	1.17	2	1.87
2	2.66	1.42	3.72	3.77
3	4.21	1.73	8	7.28
4	7.2	2.10	14.6	15.12
5	11.9	2.42	29.5	28.79

Table I Inhibition of thrombin by antithrombin III (AT III sample), by α_2 macroglobulin (α_2 M sample), and by both inhibitors (d-plasma)

Total Antithrombin Activity % (of standard plasma) Healthy subjects (n = 15)	Antithrombin III Contribution to the total antithrombin activity	α_2 Macroglobulin assumed to be 100
103 ± 15	72.13% ± 5.49	27.87% ± 5.49
<i>Patients with liver cirrhosis</i>		
60	60	40
58	53	47
50	55	45
70	69	31
69	62	38
52	48	58
47	43	57
57.86% ± 8.84	54.86% ± 9.8	45.14% ± 9.8

Table II Total and differentiated antithrombin activities in healthy subjects and in patients with liver cirrhosis

Discussion

According to the law of the pseudo-first order reaction, the method does not require the use of a standardized thrombin solution. The inhibition rate of thrombin by a given antithrombin concentration should not be influenced by minor variations of thrombin concentrations when the antithrombin concentration is in excess with respect to thrombin. Actually it was observed (fig 5) that a 1/5 diluted sample of normal d-plasma produced the same inhibition rate of thrombin when tested with various concentrations of enzyme, ranging from 15 to 60 NIH units/ml. The irrelevance of a precise thrombin standardization greatly simplifies the antithrombin assay.

Many substances may inhibit the thrombin-fibrinogen reaction in human plasma. It is important to make a distinction between antithrombin activity produced by specific substances and a number of aspecific antithrombin reactions which work either by an absorption mechanism (fibrin-antithrombin I), by a kinetic effect (antithrombin IV), or by inter-

ference with the fibrin polymerization (abnormal protein found in hypergammaglobulinaemia or in gamma myeloma-antithrombin V-and fibrin degradation products-antithrombin VI) (Mammen, 1971).

It is rational to use the term antithrombin only for well-characterized substances such as AT III, α_2 macroglobulin, and α_1 antitrypsin, which act specifically on thrombin. Two different procedures have been used to determine the antithrombin activity of plasma (Mammen, 1971). The first type of assay reflects the total antithrombin capacity of plasma; it is performed by incubating the plasma with an excess of thrombin and measuring the residual thrombin after completion of the reaction. The other type of assay, which uses small concentrations of thrombin which are likely to occur *in vivo*, would appear to be more relevant from a physiological and clinical point of view.

The contribution of α_1 antitrypsin to the total antithrombin activity of plasma seems to be irrelevant when using small concentrations of thrombin. Individuals with congenital α_1 antitrypsin deficiency showed, with this assay, a normal antithrombin activity (Abildgaard, 1969); moreover, no correlation has been found between immunochemical α_1 antitrypsin and antithrombin activities (Abildgaard *et al*, 1970).

The kinetics of thrombin inhibition by AT III and α_2 macroglobulin appear to be similar; a second-order reaction was observed with each purified inhibitor in systems containing large concentrations of thrombin (Abildgaard, 1969a, b; Lanchantin *et al*, 1966; Steinbuch *et al*, 1968). By using small concentrations of thrombin, pseudo first-order reactions should be expected. This was shown to be the case in our system. In addition, we were able to show that in plasma both inhibitors were acting on thrombin without mutual interference. Our studies showed that the addition of 0.1 M methylamine to plasma produced in 10 minutes the selective inactivation of α_2 macroglobulin, the AT III activity remaining unchanged. On the basis of these results we developed

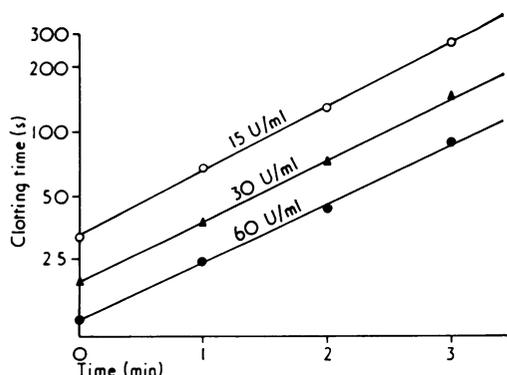


Fig 5 Inhibition rates of various thrombin concentrations produced by a 1/5 diluted sample of d-plasma.

a method which would permit the differential determination of plasma antithrombins.

Fagerhol and Abildgaard (1970), by removing α_2 macroglobulin by selective immunoprecipitation, were able to show that AT III accounts for about 70% of the total antithrombin activity of a plasma sample. These findings were confirmed by our results in healthy subjects, but in patients with liver cirrhosis, in whom a low total antithrombin activity has been found (Hedner and Nilsson, 1973), we found that the relative contribution of AT III may be much lower.

The clinical meaning of this shift in the AT III/ α_2 macroglobulin ratio remains unknown. Antithrombin III is considered to be one of the most important physiological inhibitors of coagulation, acting as a powerful inhibitor of activated factor X (Biggs *et al*, 1970) and as heparin cofactor (Rosenberg and Damus, 1973).

An increased tendency to thrombosis has been shown to occur in subjects with congenital AT III deficiency (Kaulla and Kaulla, 1972). Our method could be useful for a sensitive detection of minor changes of AT III concentration as it provides a differential determination of antithrombins.

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