Continuous registration of thrombin generation in plasma

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Continuous Registration of Thrombin Generation in Plasma, Its Use for the Determination of the Thrombin Potential

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Summary

A method is described by which the time-course of thrombin generation in plasma can be obtained from a continuous optical density recording of p-nitroaniline (pNA) production in a 2:3 diluted plasma. A chromogenic substrate, methylmalonyl-methylanalyl-arginyl-pNA (SQ 68), is used that is specifically split by thrombin but at a low rate. The thrombin that appears and disappears in the plasma does not split more than 5% of the substrate added, so the rate of substrate conversion is in good approximation proportional to the amidolytic activity in the plasma over the entire period of thrombin generation. The course of the enzyme concentration can be calculated from the amidolytic activity curve. It is shown that the thrombin generation curves obtained in this way are essentially identical to those obtained via the classical subsampling method.

The presence of SQ 68 influences the amount of free thrombin that appears in plasma because it competitively inhibits the inactivation of thrombin by AT III and α_2 macroglobulin. The inhibition of the thrombin peak by heparin, relative to an uninhibited control, remains unaltered by the presence of the substrate.

From the course of thrombin activity and the prevailing decay constants, the course of prothrombin conversion velocity can be calculated. Prothrombin conversion was seen to be inhibited at high (>500 μM) substrate concentrations only, and experimental conditions are found under which the inhibition of the clotting process by the substrate is negligible.

The amidolytic activity is the sum of the activities of free thrombin and of the α_2 macroglobulin-thrombin complex formed. Via a mathematical procedure the amount of SQ 68 that has been split by thrombin alone and not by the α_2 macroglobulin-thrombin complex, can be derived from the course of the optical density.

The total amount of SQ 68 eventually split by thrombin alone is proportional to the surface under the thrombin generation curve, i. e. to the time-integral of free thrombin. This value, that we call the *thrombin potential* (TP), directly indicates how much of any physiological substrate can potentially be split by the thrombin being generated in the plasma.

Introduction

The thrombin generation curve (TGC), i. e. the course of appearance and disappearance of thrombin in triggered plasma, gives information on the process of blood coagulation that is difficult to obtain otherwise. Its determination requires repeated subsampling from the activated plasma sample which, even when automated as far as possible, requires intensive experimentation and is not suited for routine use (1–3).

Optical registration of a signal from thrombin-related amidolytic activity in defibrinated plasma is in principle possible by recording product formation from a chromogenic thrombin-substrate added to the plasma. Current substrates are unsuitable, however, because they will be exhausted long before thrombin generation is over, even when added at the highest concentration that is practically possible. A slow reacting thrombin substrate will therefore have to be used, i. e. the turnover (k_{cat}) of this substrate by thrombin has to be low.

It is inherent to the presence of a chromogenic substrate that the physiological reactions of thrombin are interfered with. The substrate will act as a competitive inhibitor of reactions in which thrombin partakes. In order to minimize inhibition, the substrate should be present at a concentration that is far from saturating the enzyme. By definition the enzyme is half saturated if the substrate concentration equals the Michaelis constant (K_m). The concentration in the experiment therefore should be below K_m. On the other hand the total amount of substrate converted should remain small as compared to the initial amount present, so that the inhibitory effects remain constant during the experiment. This means that relatively high substrate concentrations are required that however remain below K_m, hence K_m should be high.

In this article we show that methylmalonyl-methylalanyl-arginyl-p-nitroaniline (SQ 68) is a substrate with kinetic properties that allow continuous registration of thrombin activity. The turnover is low, but high enough to generate a well measurable signal, even though only a small fraction of the initial amount of substrate is consumed during the experiment. The inhibitory effect on prothrombin conversion remains small.

In clotting plasma, conversion of a chromogenic thrombin-substrate is caused by two different molecular species: free thrombin and the thrombin-α_2 macroglobulin complex (α_2 M-thrombin) (1, 2). We will show that the optical density tracing of p-nitroanilide production can be dissected mathematically in two parts, the one due to the action of free thrombin and the other to that of α_2 macroglobulin-thrombin. The thrombin-dependent curve shows a steady end-level, which represents the total amount of chromogenic substrate that has been converted by free thrombin during the experiment. This amount is proportional to the amount of any other (physiological) substrate that thrombin generated in that sample can potentially split. It is a direct indicator of the amount of enzymatic "work" that thrombin can carry out during its lifetime in the activated plasma sample. Thrombin acts on a great variety of physiological substrates, to the general effect of making it the pivotal enzyme in haemostasis and thrombosis. The amount of enzymatic thrombin-action that can potentially be triggered in a given plasma is therefore likely to be a parameter to indicate the thrombin-mediated anti-bleeding and/or prothrombotic capacity of that plasma. We call this value

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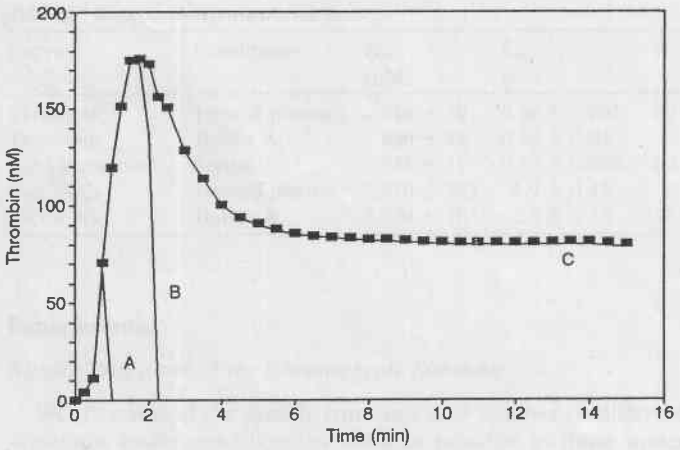

Fig. 1 A simulation of continuous thrombin measurements with different substrates. A given experimental amidolytic activity curve (■) is thought to occur in a reaction medium that contains a chromogenic substrate. Then the course of the enzyme concentration is calculated from the course of the velocity of substrate conversion for substrates at different concentrations and with different kinetic parameters (drawn lines)

	S (μM)	K_m (μM)	k_{cat} (min^{-1})
A (usual substrate)	2,000	10	10^4
B (idem)	200	10	10^4
C (SQ 68 like)	25	800	25

the *thrombin potential* (TP). With the aid of the method described here the TP can be easily determined.

Materials and Methods

Materials

Methylmalonyl-methylalanyl-arginyl-pNA (SQ 68) was synthesized by Serbio Laboratories, France (European Patent 88400304.7).

The chromogenic substrate used for thrombin estimation in subsamples was H.D-Phe-Pip-Arg-PNA (S2238) from KABI, Sweden.

Reptilase was obtained from Laboratoires Stago (Asnières, France), a solution was made according to the instructions of the manufacturer.

As a trigger for coagulation we used relipidated recombinant tissue factor (a kind gift of Dr. Yale Nemerson, Mount Sinai, New York City, USA), supplied with 1.5 μM phospholipid (20% phosphatidylserine, 80% phosphatidylcholine) diluted so as to give a peak of ~250 nM thrombin in normal plasma.

Buffer A contains 0.05 M Tris-HCl at pH 7.35, 0.1 M NaCl, 0.5‰ bovine serum albumin. Buffer B is identical but at pH 7.9 and contains in addition 20 mM EDTA.

Plasma

Blood from healthy donors was collected on 0.13 M trisodium citrate; nine parts of blood to one part of citrate solution. A first centrifugation was performed at 900 × *g*, at 15° C for 15 min. A second centrifugation was done for 15 min at 15° C and 10,000 × *g*. PPP was pooled from at least 10 donors and a third centrifugation was carried out at 4° C, for 1 h at 23,000 × *g*, this plasma was stored at −80° C. It was checked that the clotting factors and the antiproteases were in the normal range.

All thrombin generation experiments were carried out in defibrinated plasma, that was obtained by mixing an aliquot of plasma with 1:50 volume of a reptilase solution, letting a clot form for 10 min at 37° C and keeping the clotted plasma at 0° C for 10 min. It is good to occasionally check that the reptilase-induced clotting time of normal plasma is around 300 s. If it is substantially longer than the reptilase solution has lost activity and incomplete defibrination may result. Clot formation during the experiment and uninterpretable optical signals are the result. The fibrin formed was discarded by centrifugation (10 min, 5,000 × *g*, 4° C) or by winding it on a small plastic spatula. The concentrations of factors II, VII, VIII, IX, X, XI and XII did not significantly change by the reptilase treatment (1).

In order to obtain a plasma in which the kinetic constants of SQ 68 could be determined directly, heated defibrinated plasma was prepared by leaving defibrinated plasma at 60° C for 60 min. Any precipitate that formed was removed by centrifugation for 10 min at 10,000 × *g*. It was checked that thrombin- or factor Xa added to this preparation did not loose activity in the course of 30 min.

Heparin containing plasma samples were obtained from volunteers that received subcutaneously either 5,000 units of unfractionated heparin or 40 mg of Enoxaparin or 1 mg/kg body weight of Enoxaparin. This was done in the course of a study carried out by Dr. A.-V. Bendetowicz in our laboratory, that will be reported on separately. In this article the samples are used only to compare different ways of assessing the effect of heparin on the thrombin potential.

Thrombin

The euglobulin fraction from defibrinated plasma was obtained by acid precipitation at low ionic strength as described by Josso and Prou-Wartelle (4). The precipitate was dissolved in half the original volume of buffer A, containing 0.02 M trisodium citrate, so as to obtain a concentration of clotting factors that was not lower than that in the original plasma. No inhibitor activity could be detected in this solution. Thrombin was generated in this preparation at 37° C by addition of a trace of recombinant tissue factor and $CaCl_2$ to a final concentration of 16.7 mM. The thrombin solution obtained was either used as such, or α-thrombin was prepared according to Pletcher et al. (5).

Factor Xa

Factor Xa was prepared according to Mertens and Bertina (6).

Manual Determination of Thrombin Generation in Plasma

To 240 μl of defibrinated plasma is added 60 μl of buffer A containing SQ 68 at the desired concentration. Thrombin formation is started by the addition of 60 μl of a solution containing 100 mM of $CaCl_2$ and recombinant tissue factor.

At regular intervals 10 μl samples are withdrawn and subsampled into 490 μl of buffer B containing 200 μM S2238. After about 2 min the reaction is stopped with 300 μl of 1 M citric acid. The moment of sampling and stopping directly are recorded on a personal computer with pushbutton-equipped pipettes. The cuvettes are read at 405 nm in a double wavelength (405–546 nm) dedicated instrument prepared in our workshop, using a personal computer for data recording. For full details on the method see ref. 1.

Decay Constants of Thrombin in Plasma

To 120 μl of defibrinated plasma and 40 μl of buffer A, at 37° C, 10 μl of a thrombin preparation were added so as to obtain a final concentration of 100 nM. At suitable intervals (5–10 s) 10 μl samples were drawn and tested for thrombin as described above.

The amidolytic activities (C_t) were fitted to the three parameter curve $C_t = C_\infty + (C_0 - C_\infty) \cdot e^{-k \cdot t}$, where k is the decay constant (k_{dec}), C_0 is the initial amidolytic activity and C_∞ is the residual, steady end-level activity due to the α_2 M-thrombin complex. In our hands, the standard error of a single estimation is below 7% as long as half life times exceed 4 s (k_{dec} = 10 min^{-1}). At shorter half life times it increases proportionally with k_{dec}, with about 1% per min^{-1}.

The decay constant that is obtained in this way is the sum of the decay constants of the reaction of thrombin with the different inhibitors of plasma. We distinguish k_2, the α_2 macroglobulin-dependent constant and k_1, that governs the non-α_2 macroglobulin-dependent reactions, principally (~85%) AT III. We calculated k_2 from the relation $k_2 \cdot f = (C_\infty/C_0) \cdot k_{dec}$ (where f = 0.763, i.e. the ratio of the amidolytic activity of aequimolar amounts of α_2 M-thrombin and thrombin, or the ratio of their k_{cat} values; see refs. 1 and 2 and Table 1). Alternatively $k_2 \cdot f$ can be estimated directly from the optical density traces as described in the annexe.

Table 1 Kinetic constants of SQ 68

Enzyme	Conditions	K_m (μM)	k_{cat} (s^{-1})	n
Thrombin	Heated plasma	819 ± 19	0.38 ± 0.002	60
Thrombin	Buffer A	830 ± 48	0.46 ± 0.04	8
α_2 M-thrombin	Serum	788 ± 11	0.29 ± 0.006	60
Factor Xa	Heated plasma	3,210 ± 193	1.9 ± 0.12	8
Factor Xa	Buffer A	3,930 ± 105	2.9 ± 0.16	16

Experimental

Kinetic Properties of the Chromogenic Substrate

We determined the kinetic constants of a number of different substrates under conditions as close as possible to those under which we wanted to use them, i. e. in 2:3 diluted plasma. In order not to be hindered by thrombin inactivation the antithrombin activity was abolished by heating for 60 min at 60° C. We selected SQ 68 as the one which was converted by thrombin sufficiently slow, and yet was sufficiently specific for thrombin. Table 1 gives the kinetic constants of this substrate.

In order to get an impression of what substrate concentrations should be used, we mathematically simulated substrate conversion in a plasma in which thrombin is generated. As input we used the three variables: initial substrate concentration, Michaelis constant (K_m) and catalytic constant (k_{cat}) of the splitting of the substrate by thrombin and a typical thrombin generation curve in normal plasma after triggering with thromboplastin (mean of 5 curves). The program calculated the velocity of substrate conversion according to the classical formula $v_t = k_{cat} \cdot E_t \cdot S_t/(K_m + S_t)$. (The subscripts t indicate the variable at time t, so E_t is the amount of thrombin as given by the thrombin generation curve, S_t is the amount of substrate remaining at time t.) Then the apparent E_t' was calculated as in an actual experiments, i. e. by calculating $E_t' = v_t \cdot (K_m + S_0)/k_{cat} \cdot S_0$. In this formula S_0 replaces S_t, so that E_t' is an approximation of the real E_t.

From the results obtained it can be seen (Fig. 1, curve C) that at SQ 68 concentrations of 25 μM the course of E_t so calculated already fits closely to the actual thrombin generation curve. A conventional substrate would only render the first part of the TGC, even when added at impracticably high concentrations (curves A and B).

The presence of a thrombin substrate in plasma, slows down the action of antithrombins because they act on free thrombin only and not on the thrombin-chromogenic substrate complex. This makes that in actual practice the results with conventional substrates would be even worse than suggested by the simulation experiments. Even if we double the thrombin values of the normal curve, 25 μM of SQ 68 would still give a line hardly distinguishable from the actually measured points (results not shown).

From these simulation experiments it is clear that a substrate with the kinetic properties of SQ 68 will theoretically indicate correctly the amount of free thrombin present when it is added at concentrations of 25 μM and higher. In order to obtain a sufficiently high signal considerably higher concentrations are used in practice (see below).

The kinetic constants of SQ 68 with thrombin, α_2 M-thrombin and factor Xa as a substrate are shown in Table 1.

Continuous Registration of the Thrombin Generation Curve

In a disposable plastic semi-microcuvette we added 400 μl of defibrinated plasma, 100 μl of buffer A, to which substances to be tested (heparin e. g.) can be added and 60 μl of a solution of SQ 68 so as to obtain the required final substrate concentration (200–1,000 μM). The reaction was started at zero time by adding 40 μl of recombinant tissue factor in 0.25 M $CaCl_2$. The reagents were prewarmed to 37° C and the cuvette was thermostated at that temperature during the measurement.

The optical density at 405 nm was recorded at the pace of 2 measurements per second (Fig. 2). We calculated the average of the first derivative of the OD trace at every 24 measuring points, i. e. over 12 s intervals (Fig. 3, upper frame). From the substrate conversion velocities so obtained we calculated the enzyme conditions via the classical formula $dS/dt = k_{cat} \cdot E \cdot S/(K_m + S)$ (Fig. 3, lower frame).

We also determined manually the course of thrombin activity in identical reaction mixtures, including different concentrations of SQ 68, by subsampling at 12 s intervals in cuvettes with the usual thrombin substrate S2238 (Fig. 4). Different concentrations of SQ 68 were included in order to register its effect on the coagulation system. From parallel blank experiments it was seen that the SQ 68 in the reaction mixture did not add more than 2 mOD (equivalent to about 0.1 nM thrombin) to the signal in the subsamples, so that the signal obtained could be interpreted in terms of splitting of S2238. Comparison of the manual data and those obtained from the continuous registration showed acceptable resemblance (Fig. 4).

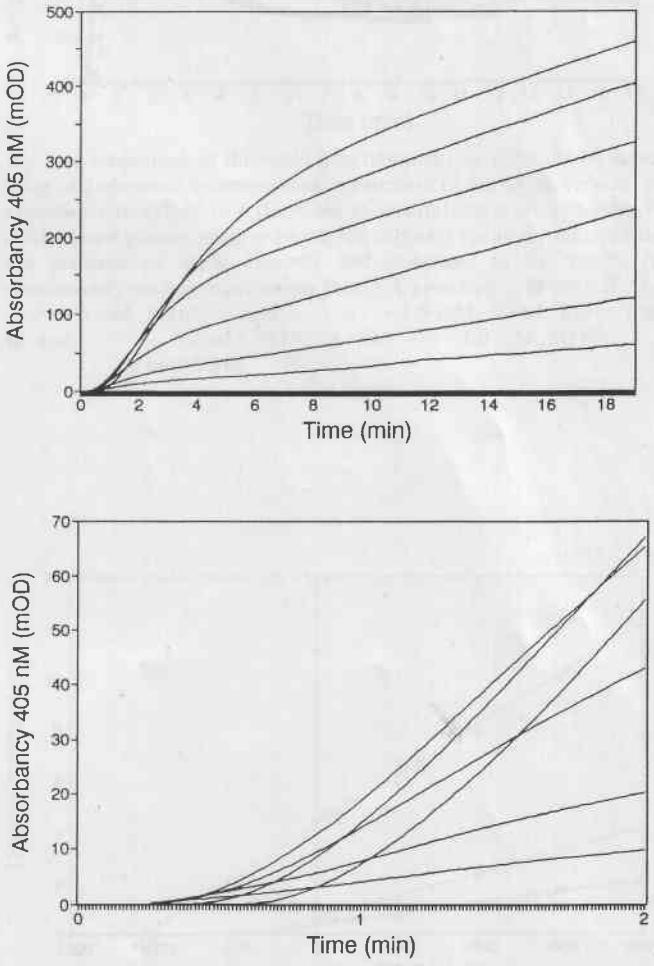

Fig. 2 Continuous registration of OD development due to SQ 68 splitting in plasma triggered with tissue factor. Concentrations of SQ 68, from top to bottom: 2 mM, 1.5 mM, 1 mM, 0.5 mM, 0.2 mM, 0.1 mM. The lower frame represents the first 2 min of the upper frame

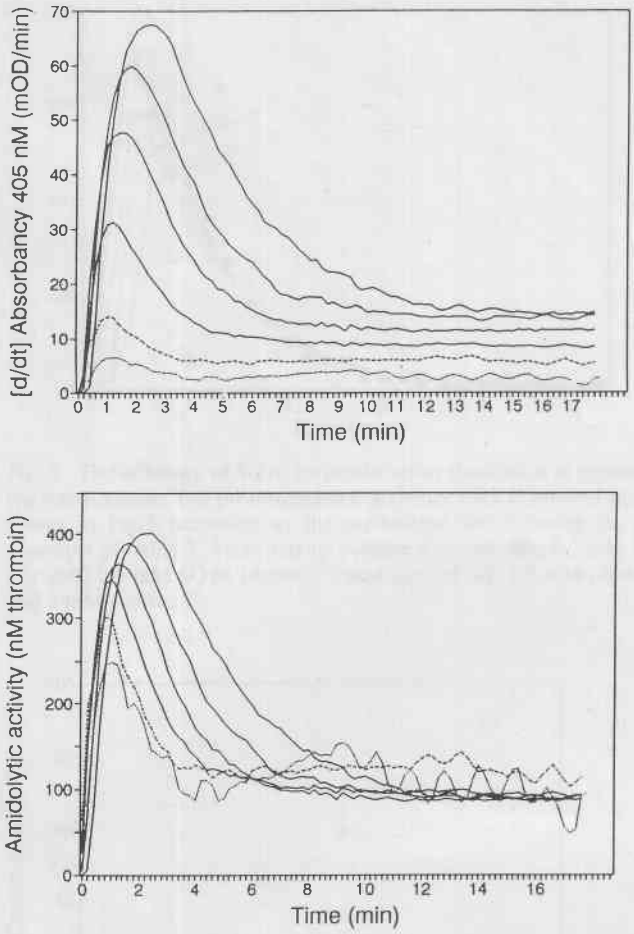

Fig. 3 Continuous registration of reaction velocities of SQ 68 conversion and of enzyme concentrations derived from optical density traces. Upper frame: Reaction velocities of SQ 68 conversion calculated as the first derivatives of the OD traces in Fig. 2. From top to bottom: 2 mM, 1.5 mM, 1 mM, 0.5 mM, 0.2 mM (centre-line), 0.1 mM (dotted line). Lower frame: Enzyme concentrations calculated from the reaction velocities of SQ 68 conversion shown in the upper frame via $E_t = v_t (K_m + S_0)/k_{cat} \cdot S_0$.

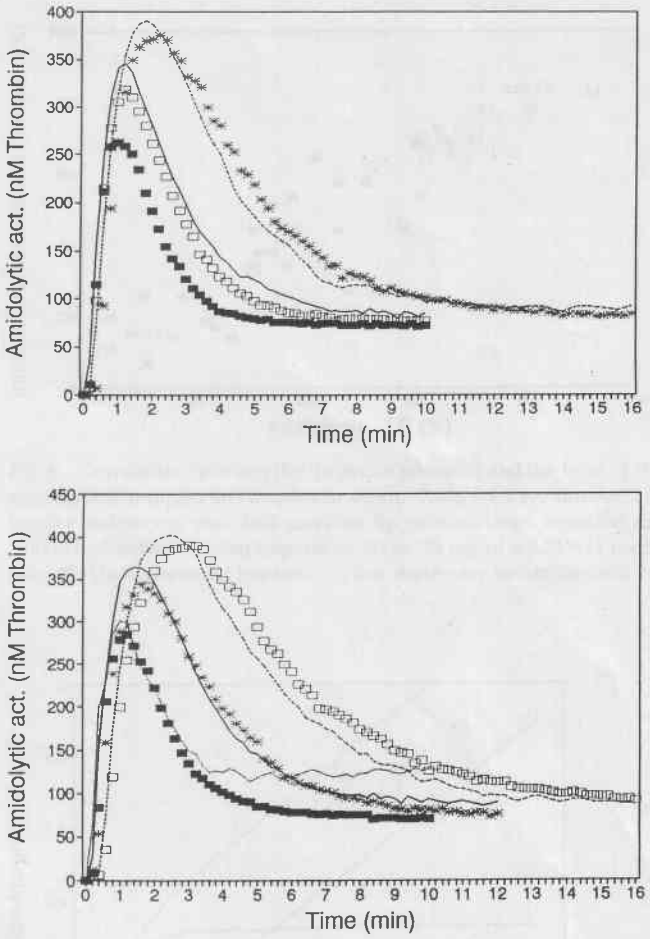

Fig. 4 Comparison of thrombin generation curves obtained by subsampling and obtained by continuous registration of SQ 68 conversion. At a sampling interval of 12 s thrombin concentrations were determined in defibrinated plasma triggered with recombinant tissue thromboplastin in the presence of SQ 68 (points) and compared to the results from continuously reading experiments (lines). Upper frame: ■ control; □ and —— 0.5 mM SQ 68; * and – – – – – – 1.5 mM SQ 68; lower frame: ■ and ····· 0.2 mM SQ 68; * and —— 1.0 mM SQ 68; □ and – – – – – – 2.0 mM SQ 68

Influence of SQ 68 on Thrombin Inactivation

From Figs. 3 and 4 it is seen that both in the continuous and in the manual experiments the presence of SQ 68 causes an increase of the amount of thrombin measured. This is to be expected because the substrate competes for the active centre of thrombin with the natural thrombin inhibitors, such as antithrombin III. Whether α_2 macroglobulin-mediated thrombin inactivation also is influenced is open, because this inhibitor leaves the active centre of thrombin free, at least in the final complex. We determined the AT III and α_2 M-dependent decay constants of thrombin in the presence of different concentrations of SQ 68 (Table 2). It is seen that both constants are decreased by SQ 68 and that $1/k_{dec}$ increases linearly with the SQ 68 concentration (Fig. 5).

Influence of SQ 68 on Prothrombinase

With the decay constants of Table 2 we can calculate the course of the velocity of prothrombin conversion, i. e. the course

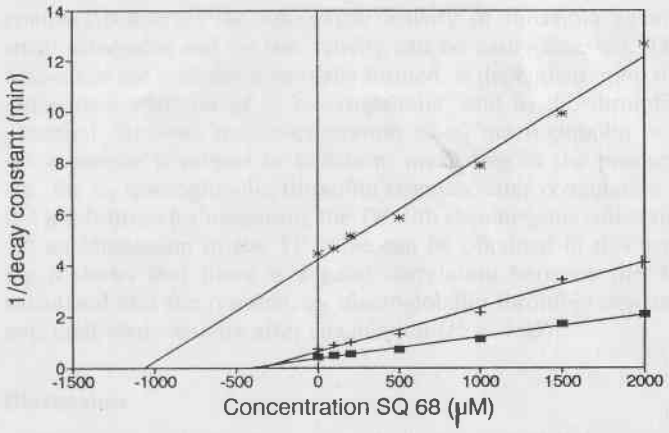

Fig. 5 The influence of SQ 68 on the decay constants of thrombin in plasma. ■ Overall decay of thrombin; * α_2 macroglobulin-dependent decay; + non-α_2 macroglobulin-dependent decay

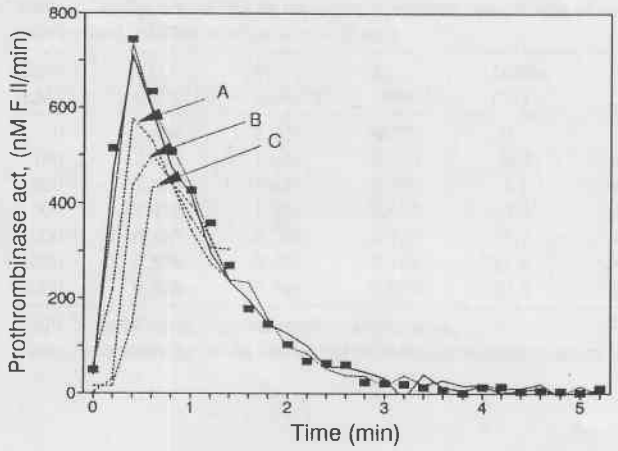

Fig. 6 The influence of SQ 68 on prothrombin conversion in extrinsically triggered plasma. The prothrombinase activities were calculated from the curves in Fig. 3 according to the method of Ref. 1, using the decay constants of Table 2. From top to bottom: Control (■); 0.2 mM SQ 68 (dotted); 0.5 mM SQ 68 (drawn); 1 mM (dotted A); 1.5 mM (dotted B) and 2 mM (dotted C)

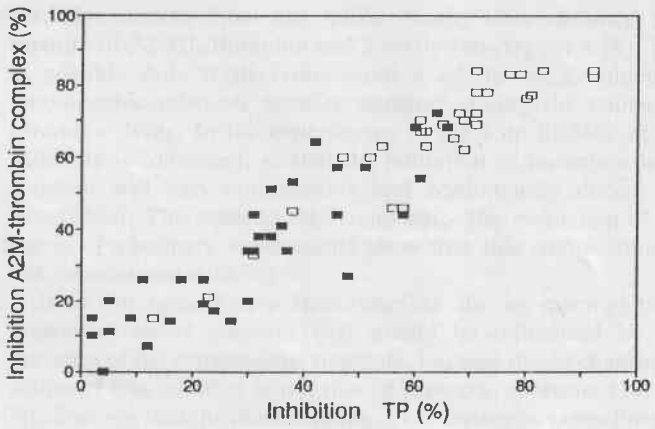

Fig. 8 Correlation between the thrombin potential and the level of the α_2 macroglobulin-thrombin complex in serum. Samples were obtained from healthy volunteers that had received by subcutaneous injection either 5,000 U of unfractionated heparin or 40 or 75 mg of a LMWH preparation. ■: Unfractionated heparin; □: low molecular weight heparin

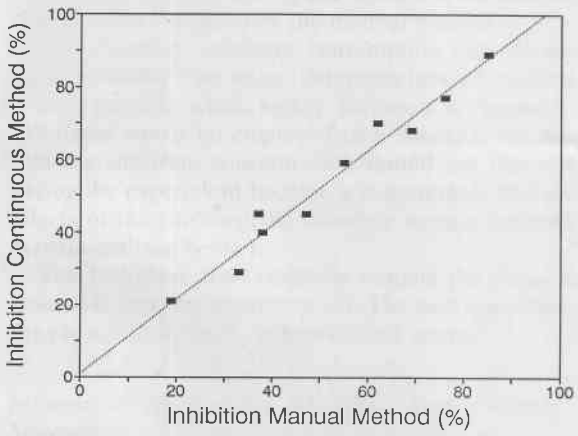

Fig. 7 Comparison of heparin-induced inhibition of the thrombin potential as measured via the subsampling and via the continuous method. Different concentrations (0.01–0.1 U/ml) of unfractionated heparin were added to normal plasma. The inhibition of the thrombin potential obtained was measured by both methods

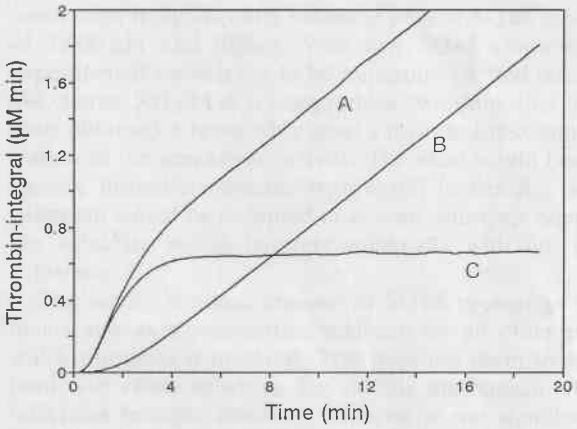

Fig. 9 Mathematical dissection of the optical density trace from p-nitroaniline production from SQ 68 in clotting plasma. A: Original OD tracing; B: contribution of the α_2 macroglobulin-thrombin complex; C: contribution of free thrombin; TP: amount of substrate eventually converted by free thrombin, i.e. steady level of C

of prothrombinase activity from the curves of Fig. 4. The results are shown in Fig. 6. It is evident that significant inhibition of prothrombinase activity only appears at the higher (>1,000 μM) concentrations of SQ 68.

Comparison of Inhibitions Found with the Old and the New Method

In order to compare the continuous and the subsampling method as to their susceptibility to inhibition, we determined the inhibition of the area under the TGC, i.e. the thrombin potential, brought about by different amounts (0.01–0.1 U/ml) of unfractionated heparin both with the subsampling method and with the continuous method (Fig. 7). A good correlation is found between the two methods.

The α_2 Macroglobulin-thrombin Complex as an Indicator of the TP

It did not escape our attention that plasma contains a natural pseudo-substrate of thrombin that is not exhausted during the clotting process, i.e. α_2 macroglobulin. This inhibitor reacts with thrombin to form the α_2 macroglobulin-thrombin complex. The complex conserves the amidolytic activity of thrombin against small substrates and by this activity can be easily assessed. The amount of the complex eventually formed, is dependent upon the initial concentration of α_2 macroglobulin, and to the thrombin potential. Because the concentration of α_2 macroglobulin in a given sample is subject to variation, measuring of the product, i.e. the α_2 macroglobulin-thrombin complex after coagulation is not a substitute for measuring the TP with chromogenic substrate, yet an impression of the TP value can be obtained in this way. Fig. 8 shows that there is a good correlation between the TP calculated and the residual, α_2 macroglobulin-thrombin dependent, amidolytic activity after coagulation ($r^2 = 0.95$).

Discussion

The chromogenic substrate, methylmalonyl-methylalanyl-arginyl-pNA (SQ 68) has kinetic properties that allow the entire course of thrombin-related amidolytic activity in triggered plasma

Table 2 Influence of SQ 68 on decay constants, end-levels of amidolytic activity and inhibition of prothrombinase

Conc. (μM)	k_{dec} (min^{-1})	k_1 (min^{-1})	k_2 (min^{-1})	Inhib. (%)	Cons. (%)
0	2.446	2.223	0.223	0	–
100	2.072	1.858	0.214	0.5	8.2
200	1.863	1.669	0.194	1.1	8.1
500	1.425	1.254	0.171	3.2	6.6
1,000	0.885	0.758	0.127	5.7	4.5
1,500	0.578	0.477	0.101	13.8	3.7
2,000	0.478	0.399	0.079	20.5	3.2

Inhib. = Inhibition of prothrombin conversion.
Cons. = Percentage of the initial SQ 68 concentration consumed in 10 min

to be followed in a spectrophotometer. The reaction velocity of substrate conversion can be obtained in real time from the first derivative of the OD-tracing. It is in good approximation proportional to the amidolytic enzyme activity because maximally 8% of the substrate is consumed during the first 10 min of the reaction. The proportionality factor is obtained from classical enzyme kinetics: $E = v\ (K_m + S)/k_{cat} \cdot S_0$. Fig. 4 shows that indeed in practice the curves found by the automatic method are acceptably near to those obtained by the manual method.

The effect of substrate consumption can be accounted for mathematically and exact determination of enzyme activity is readily possible when higher precision is required or if other substrates would be employed (see annexe). Yet it is important that the substrate concentration should not diminish too much during the experiment because it is preferable that any inhibitory effects of the chromogenic substrate remain constant during the experiment (see below).

The technique thus faithfully renders the time course of the amidolytic enzyme concentration. The next question is in how far the presence of SQ 68, influences this course.

Influence of SQ 68 on the Amidolytic Activity Curve, Selection of a Suitable Substrate Concentration

Part of the amidolytic activity must be attributed to factor Xa because of the relatively high turnover-number of SQ 68 with that enzyme (Table 1). This is counteracted by the high K_m, which also assures little inhibition of physiological factor Xa activity by the substrate. Indeed no important inhibition of prothrombinase activity is found (Fig. 6). The peak activity of factor Xa in clotting plasma is around 10 nM (7). With the kinetic constants of Table 1 one can calculate that this amount of factor Xa will cause a pNA production that, at a SQ 68 concentration of 500 μM, is less that 10% of that of the peak activity of thrombin.

AT III is a pseudosubstrate that attacks the active centre of thrombin. A chromogenic substrate will compete with AT III for this active centre and therefore competitively inhibits AT III action (8). From standard enzymology it follows that, because the AT III (~2.5 μM) and the SQ 68 (>100 μM) are in excess over the thrombin (<200 nM), the pseudo first order constant of AT III dependent thrombin decay (k_1) in good approximation decreases by a factor $K_m/(K_m + S)$ (S = substrate concentration, K_m = Michaelis constant). This explains why increasing concentrations of SQ 68 cause increasing amounts of thrombin to be generated (Figs. 3 and 4).

Because the concentration of SQ 68 hardly diminishes during the experiment, its effect on the decay of thrombin is essentially constant. The effect of an additional inhibitor, like heparin, remains unaltered when expressed as a percentage of an uninhibited control (Fig. 7).

It has been proposed to calculate the decay constant of thrombin directly from the optical density trace obtained in a mixture of AT III, thrombin and Tos-Gly-Pro-Arg-pNA (9). This is possible only if the concentration of the decay-inhibiting chromogenic substrate remains constant during the course of thrombin decay. In the experiments of ref. 9 up to 34% of the substrate is consumed, so that the inhibition of thrombin decay constant will vary considerably and continuously during the experiment. This considerably complicates the evaluation of the curves. Preliminary experiments show that this complication is not encountered with SQ 68.

It is not immediately clear whether the α_2 macroglobulin dependent decay constant (k_2) should be influenced by the presence of the chromogenic substrate, because the mechanism of action of this inhibitor is not that of a pseudo substrate (10). In Fig. 5 we see that the inverse of the decay constants varies linearly with the concentration of substrate, i. e. that the inhibition is competitive. This may be caused by the fact that, even though in the final enzyme-inhibitor complex the active centre of thrombin is free, the first step in inhibition by α_2 macroglobulin is proteolytic cleavage of the "bait" region of the inhibitor (10).

From the amidolytic activity curves we calculated the velocity of prothrombin conversion according to refs. 1 and 2, using k_1 and k_2 as obtained in the presence of SQ 68 (Fig. 6). Prothrombin conversion is significantly inhibited only at SQ 68 concentrations of 1,000 μM and higher. With low SQ 68 concentrations the experimental noise tends to be important (dotted lines in Fig. 3). We choose 500 μM as a compromise. We think that in SQ 68 we have obtained a reasonably good substrate for continuous registration of the amidolytic activity. The ideal would be an entirely specific thrombin-substrate with a still higher K_m, so that few thrombin would be occupied in enzyme-substrate complexes and the substrate would interfere minimally with other thrombin substrates.

The nearly constant amount of SQ 68 present in the experiments acts as a competitive inhibitor on all other reactions in which thrombin is involved. This does not seem to alter importantly the relations within the clotting mechanism. At least the inhibition brought about by heparins is not significantly influenced by the presence of SQ 68 (Fig. 7).

Calculation of the Thrombin Generation Curve and the Thrombin Potential (TP)

Fig. 1 shows the general form of a thrombin generation curve as it is known from previous work. The amidolytic activity measured is caused by the simultaneous activity of thrombin and the α_2 macroglobulin-thrombin complex. The latter builds up during the test with a velocity proportional to the amount of free thrombin available (1). It is responsible for the thrombin-like amidolytic activity found in serum, i. e. for the non-zero end-level of the TGC. In TGCs that are determined with fibrin as the substrate for the thrombin in the subsamples, this phenomenon is not seen because α_2 M-thrombin does not act on fibrin (11). A simple mathematical treatment of the data allows to obtain the course of free thrombin from the amidolytic activity curve if we know $k_2 \cdot f$, i. e. the pseudo-first order constant of the interaction between α_2 macroglobulin and thrombin (k_2) multiplied by f, the ratio of the k_{cat} of α_2 macroglobulin-thrombin over the k_{cat} of thrombin (1, 2). The introduction of the constant f in the calculations accounts for the difference between k_{cat} of free thrombin and α_2 macroglobulin-thrombin. The differences between K_m are not accounted for but the error that is thus introduced remains below 2.5%. A calculation analogous to that giving the thrombin curve from the amidolytic activity curve can be carried out on the experimental OD trace (Fig. 9A). This

allows to dissect the OD development due to the action of the free thrombin (Fig. 9C) from the OD generated by α_2 M-thrombin (Fig. 9B, see further annexe). The steady end-level of the thrombin-trace represents the thrombin potential.

The k_2 term implicitly incorporates the α_2 macroglobulin concentration, in fact the plasma to plasma variations of k_2 must be attributed to variations in α_2 macroglobulin concentration. The algorithm of the annexe seeks k_2 (multiplied by the constant f) for each individual experiment and therefore eliminates this variation as a source of error. When SQ 68 is used as a substrate then $f = 0.763$.

If k_2 and f have been determined before, then the colour development due to free thrombin can be calculated in real time. From this the concentration of free thrombin can be calculated, again in real time, because the reaction velocity (first derivative of the OD trace) is dependent on the thrombin concentration and a number of known constants: initial substrate concentration (S_0), turnover number (k_{cat}) and Michaelis constant (K_m) (see annexe).

With an unknown plasma sample, $k_2 \cdot f$ can be obtained from the experimental curve after the experiment is finished and the concentration curve of free thrombin can then still be calculated (see annexe). Also $k_2 \cdot f$ can be guessed in order to obtain an approximately correct thrombin generation curve on line, that can be corrected after the experiment is finished. We reported before that k_2 equals 0.232 ± 0.004 min^{-1} (SEM; $n = 25$) (3), in the presence of 500 µM SQ 68 this reduces to 0.136 min^{-1}. This value is a safe guess unless patients are studied in which an increase of α_2 macroglobulin is to be expected.

The steady end-level of the curve that represents the amount of substrate split by free thrombin (ETP in Fig. 9), is proportional to the thrombin potential. Knowing the kinetic constants of SQ 68 we can calculate how much thrombin has been acting on the substrate and for how long. The dimension of this figure is thrombin concentration × time (i. e. nM min). The normal value of the thrombin potential is 487 ± 21 nM min ($n = 12$) (12).

The TP directly indicates how much of any physiological substrate present in, or in contact with, the plasma can be converted by thrombin if the plasma is triggered. The TP is identical to the time-concentration integral of the formed thrombin, i. e. the surface under the thrombin generation curve. The surface under the thrombin generation curve has been introduced very early in tests for prothrombin estimation (11, 13). It indeed can be traced back into the 19th century (14). In the more recent literature it is not frequently encountered, a. o. because it requires time consuming experimentation.

Use of the Thrombin Potential (TP)

The overall activity of the clotting system is usually assessed by measuring a coagulation time. This type of test comes in many varieties, but it always essentially measures the time that elapses until a level of 10–20 nM of thrombin is formed in the reaction mixture (15). Spectrophotometric tests that are meant to replace clotting tests also measure the moment of onset of explosive thrombin generation. At that moment, the peak of thrombin formation (100–300 nM) is still to come. Often, e. g. in plasma that clots via the extrinsic pathway in the presence of heparin, the lag-phase of thrombin formation is hardly prolonged even when the peak amount is significantly inhibited (2, 3). In general it can be said that alterations of the clotting system are not always necessarily reflected in the lag-time of thrombin formation, i. e. in the clotting time.

Recent research tends to stress the central position of thrombin in the pathogenesis of thrombosis (16, 17, see also 18 and 19 for an overview). Thrombin appears as the pivotal enzyme in haemostasis and thrombosis. Its effects are due to its action on a number of physiological substrates (e. g. the tethered ligand of the platelet membrane, fibrinogen). The magnitude of these effects is determined by the amount of substrate that thrombin converts. This amount is always proportional to the thrombin level combined with the time that the thrombin can act, i. e. to the time integral of the concentration of free thrombin, i. e. to the surface under the TGC, i. e. to the TP. It is easily seen that the amount of the artificial substrate that is converted by thrombin is necessarily proportional to the amount of any physiological substrate that can be converted by this thrombin. It therefore is a direct indicator of the haemostatic- and thrombotic action that this plasma can potentially exert via thrombin.

It is our conjecture that the influence of an anticoagulant on the thrombin potential is a direct indicator of its efficiency. It therefore is important that inhibitions observed by the continuous method correlate well with those seen in the direct method (Fig. 7). Preliminary experiments indicate that the continuous method lends itself well to adaptation to a laboratory automation. It may be interest to those that cannot adopt the SQ 68 method that the α_2 macroglobulin-thrombin level in serum is a direct indicator of the thrombin potential, be it with individual variations in α_2 macroglobulin level as a confounding factor (Fig. 8). In cases where one wants to investigate the influence on the thrombin potential of inhibitors added to a plasma sample the α_2 macroglobulin level is equal in all samples and the α_2 macroglobulin-thrombin level after coagulation directly indicates inhibition of the thrombin potential relative to the control.

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Annex

Calculation of the Thrombin Potential from Integral Thrombin Generation Curves

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The method described in this article records a range of optical density readings together with corresponding time values. These discrete optical density-time pairs reflect both the integral of the thrombin concentration and the integral of the α_2 M-thrombin complex concentration. Since the formation of the α_2 M-thrombin complex adheres to simple first-order kinetics, the integral of the thrombin concentration can easily be obtained as described below.

Abbreviations used are:

m_t: α_2 M-thrombin concentration at time t
M_t: integral of α_2 M-thrombin concentration at time t
e_t: thrombin concentration at time t
E_t: integral of thrombin concentration (thrombin potential) at time t
O_t: optical density at time t
S_t: concentration of chromogenic substrate SQ 68 at time t
k_2: second order rate constant of α_2 M-thrombin complex formation
k_{cat1}: turnover number of SQ 68 by thrombin
k_{cat2}: turnover number of SQ 68 by the α_2 M-thrombin complex
f: turnover number of SQ 68 due to α_2 M-thrombin relative to the turnover number due to thrombin
K_m: Michaelis constant for the conversion reaction of SQ 68 by thrombin and by the α_2 M-thrombin complex

At any moment the rate of formation of the α_2 M-thrombin complex is proportional to the concentration of thrombin:

$$\frac{dm_t}{dt} = k_2 e_t \tag{1}$$

integration between 0 and t gives:

$$\int_0^t \frac{dm\tau}{d\tau}\, d\tau = k_2 \int_0^t e_\tau\, d\tau \tag{2}$$

since $m_0 = 0$ this is equal to:

$$m_t = k_2 \cdot E_t \tag{3}$$

At any moment, the conversion velocity of the substrate is the sum of the conversion velocities by thrombin and the α_2 M-thrombin complex:

$$-\frac{dS}{dt} = k_{cat1} \cdot e_t \cdot \frac{S_t}{S_t + K_m} + k_{cat2} \cdot m_t \cdot \frac{S_t}{S_t + K_m} \tag{4}$$

which resolves into:

$$E_t = \frac{S_0 - S_t + K_m (\ln S_0 - \ln S_t)}{k_{cat1}} - f \cdot M_t \tag{5}$$

with f equal to k_{cat2}/k_{cat1}.

S_t can be obtained from:

$$S_t = S_0 - O_t/\varepsilon \tag{6}$$

ε being the molar absorption coefficient of para nitro aniline at a wavelength of 405 nm.

Since measurement is carried out at discrete time points, M_t can be derived from (3) as follows:

$$\frac{\Delta M_t}{\Delta t} \approx m_t = k_2 \cdot E_t \tag{7}$$

so that:

$$\Delta M_t \approx k_2 \cdot E_t \cdot \Delta t \tag{8}$$

and:

$$M_{t_i} \approx M_{t_{i-1}} + k_2 \cdot E_t \cdot (t_i - t_{i-1}) \tag{9}$$

Using equations (5), (6) and (9) the time course of the integral of the thrombin concentration can be calculated from the time course of optical density and k_2. An integral thrombin curve goes to a steady end-level which directly represents the value of the thrombin potential.

The value of k_2 is often not known in advance. It can be determined in separate experiments as reported earlier (ref. 1). Alternatively, if it is assumed that after a certain timepoint t_e prothrombinase conversion has stopped and the concentration of free thrombin is 0, then k_2 can be estimated directly from the optical density vs time curve in a manner analogous to subsampled thrombin generation curves as described above. A relatively simple way to do this is to calculate the time courses of the thrombin potential for a range of assumed values κ for $f \cdot k_2$, and determine the slope of these curves from time t_e on using linear regression. In case the κ value is bigger than the true k_2 value, the thrombin potential will decrease after t_e. If, on the other hand, the value is smaller than the true value, the slope will be positive. The true k_2 value is that value where the slope of the thrombin potential curve after time t_e is zero. It can be obtained by plotting the obtained slopes versus their corresponding κ values and determining the intersect of the resulting curve with the x-axis. It proved practical to estimate this intersect by fitting the slope vs κ curve to the following exponential:

$$\text{slope} = ae^{b\kappa} + c \tag{10}$$

so that k_2 can be obtained from the parameters a, b, and c of this exponential:

$$f \cdot k_2 = \frac{\ln(-c) - \ln(a)}{b} \tag{11}$$

A computer program (for IBM PC compatibles) that both estimates k_2 and calculates the time course of the integral thrombin curve as described above, can be obtained from the authors.