Advanced thrombinoscopy

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Advanced Thrombinoscopy

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Advanced Thrombinoscopy

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit Maastricht,
op gezag van de Rector Magnificus,
Prof. mr. G.P.M.F. Mols,
volgens het besluit van het College van Decanen,
in het openbaar te verdedigen op
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door

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“In mijn kopje had ik dat wereldrecord al gebroken.
Ik moest het alleen nog overdoen in het water.”

FRED DE BURGHGRAEVE

Kleine kinderen denken dat hun ouders volmaakt zijn.
Als ze ouder worden, eisen ze dat hun ouders volmaakt zijn.
Ze zijn volwassen, als ze van hun ouders houden en ze respecteren,
ondanks dat ze niet volmaakt zijn.

Aan mijn ouders

**Voor Sabrina
Lander en Merel**

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List of abbreviations

Activated partial thromboplastin time	aPTT
(Activated) protein C	(A)PC
Alfa-two-macroglobulin	α 2M
α 2M-thrombin-complex	α 2M-IIa or α 2M-T
7-amino-4-methylcoumarin	AMC
Antithrombin	AT
Anti-vitamin K	AVK
Bovine serum albumine	BSA
Calibrated automated thrombogram	CAT
Calibration factor	CF
Calibrator	CAL
Coefficient of variation	CV
Corn trypsin inhibitor	CTI
Desamino-D-arginine-vasopressine (desmopressine)	DDAVP
Dimethylsulphoxide	DMSO
Electro-cardiogram	ECG
Endogenous thrombin potential	ETP
Ethylenediamine-tetra-acetic acid	EDTA
Factor eight inhibitor bypassing therapy	FEIBA
Fluorescence intensity	F
Mixture of fluorogenic substrate and calcium	FluCa
High Molecular weight kininogen	HMWK
Hirudin	H
Innovin	Inn
Lupus anticoagulant	LAC
Melagatran	M
Optical density	OD
Penta-saccharide	Penta-S
Phosphatidyl choline	PhC
Phosphatidyl ethanolamine	PhE
Phosphatidyl serine	PhS
Phospholipids	PL
Platelet poor plasma	PPP
Platelet rich plasma	PRP
Pool of normal platelet poor plasma	NPPP
Prekallikrein	PK
Protein S	PS
Protease activated receptor	PAR
Prothrombin	FII
Prothrombinase	FII-ase or XaVa
Prothrombin-time	PT
(Recombinant) tissue factor	(r)TF
Standard deviation	SD
Tenase	IXaVIIIa
Tissue Factor Pathway Inhibitor	TFPI
Thrombin	FIIa or T
Thrombin activated fibrinolysis inhibitor	TAFI
Thrombin-antithrombin-complex	TAT
Thrombin generation	TG
Thromboelastography	TEG
Thrombomodulin	TM
Unfractionated heparin	UFH
Venous thromboembolism	VTE
Von Willebrand factor	vWF
Whole blood clotting time	WBCT

Chapter I

Introduction

Medical importance of haemostasis and thrombosis

Fortunately, a plug is formed at the site of a lesion when we cut ourselves; otherwise the slightest injury would make us bleed to death. The haemostatic system responsible for this is an extremely intricate physiological mechanism that ensures that haemostasis (normal blood coagulation) occurs promptly at the site of a wound but does not spread unduly outside the wound area and stops as soon as haemostasis is completed [1, 2]. When haemostasis overreacts e.g. if an occlusive plug forms in a vessel (thrombosis), blood flow is hampered and finally stops. In case of arterial thrombosis, the organ fed by that vessel becomes ischemic and ultimately necrotic, while in the case of venous thrombosis the surrounding tissue becomes congested and inflamed. During evolution it was more important to favor adequate haemostasis than to prevent the possibility of thrombosis because bloodshed is a more important issue in the struggle for fertile life than thrombosis is. Without haemostasis there is no chance to survive in nature. Also, life expectancy under primitive conditions must have been such that thrombotic disease hardly occurred. However, nowadays thrombosis being the cause behind common diseases as stroke and myocardial infarction, it has become the second cause of death and disease in the western world. In Western Europe and the USA ~3500 people per day die from thrombotic disease [3] mainly through myocardial infarction and stroke (arterial thrombosis ~90%), or pulmonary embolism (venous thrombosis ~10%). The burden of non-fatal disease is more than twice as high e.g. disability after ischemic stroke and post-thrombotic syndrome after deep venous thrombosis. It is obvious that the medical importance of thrombosis can hardly be exaggerated.

Current tests for clinicians nowadays

Unfortunately, despite decades of investigation in the field of haemostasis and thrombosis, clinicians do still not have one universal test to determine blood coagulation capacity. Clinicians in the old days made their decisions based on clotting-times. Several clotting-time tests were (and are still) available (PT, aPTT, bleeding time, WBCT) each one giving information about another detail of the complicated web that blood clotting is. The Prothrombin-Time (PT) was used to check the extrinsic system, the activated Partial Thromboplastin Time (aPTT) for the intrinsic system. For defects in platelets there was the bleeding-time while the Whole Blood Clotting Time (WBCT) and aPTT were used in haemophiliacs. Depending on which disease or which drug-treatment one wanted to monitor, a different test had to be chosen. None of them could be used as an integral function-test of the haemostatic-thrombotic mechanism. Haemophiliacs show a normal bleeding time and PT but prolonged aPTT or

WBCT. It is possible to monitor heparin-treatment with the aPTT but not with the PT. People treated with oral anticoagulants can be followed-up by PT or aPTT but not with bleeding-time. Thrombasthenias and the action of platelet-drugs are reflected in platelet aggregation and in the bleeding-time. For thrombophilia (hypercoagulability) none of these tests give an indication. The problem with clotting times probably is that they only give a snapshot of the haemostatic process, i.e. the moment that bulk thrombin formation starts.

Therefore, during the last decades new techniques were developed. Attempts have been made to use the developing fibrin as an indicator of further stages in the process. In these methods the development of turbidity is measured or alternatively the mechanical properties are used (thromboelastography). Such fibrinogen-based methods show some important drawbacks which will be discussed later. In general, even though they are certainly useful for the determination of the clotting time, they do not give a full picture of the haemostatic-thrombotic function because all fibrin is converted long before all thrombin has been formed.

Other recently developed techniques used by clinicians nowadays are the 'platelet function analyzer 100' (PFA 100), the 'Verify Now P2Y12 assay' and the 'D-dimer test'. PFA 100 is used to detect defects in the platelets. It is a good screening test for platelet-related diseases as von Willebrand Factor disease and Glanzman and Bernard-Soulier thrombasthenias. Verify Now is designed to measure platelet P2Y12 receptor blockade as induced by clopidogrel. The D-dimer test measures a fragment of fibrin (D-dimer) and is used to detect an abnormal high level of fibrin degradation products in the body. It detects significant clot-formation and breakdown in the body but it says nothing about the location or the cause. It is a good test for excluding venous thromboembolism due to its high sensitivity; however its specificity is very low. In summary, none of these tests give an overall view on the haemostatic-thrombotic function and can serve as universal test to determine blood coagulation capacity.

Therefore, despite the fact that blood is a very important fluid organ, there does not exist yet an adequate function test like e.g. the electro-cardiogram (ECG) for heart rhythm problems. A clotting time is like feeling the pulse. The quest is for the equivalent of the ECG.

TG, a universal function test for the blood coagulation capacity ?

For two reasons we do not have yet such an overall function test available. In the first place because it was not recognized that thrombin generation might fulfill this function. In the second place because it was not yet possible to measure thrombin generation in such an easy and accurate way that it could be used in clinical labs. It was very laborious to perform a thrombin generation experi-

ment with the methods available in the early eighties. However, during the last decade, thrombin generation measurement was automated further resulting finally in the Calibrated Automated Thrombogram (CAT-method) as developed by Hemker et al. (see chapter 2). This method makes it possible to measure thrombin generation at high throughput and with an acceptable experimental error (<5%) which makes it a promising tool for the clinical routine-labs.

As to the suitability of thrombin generation to serve as a function test; during the last years new insights challenged the old paradigm in which a thrombin-independent platelet reaction assures primary haemostasis and thrombin-dependent clotting was responsible for a secondary phase. It appears that thrombin plays a role right from the beginning. Furthermore, thrombin seems to play a pivotal role not only in venous thrombosis but also in arterial thrombosis. These insights, which will be discussed further in this chapter, allow us to make the proposition that the blood coagulation capacity can better be assessed via the thrombin generating capacity of the blood.

The classical clotting-scheme

Blood clotting is a very complicated web of interactions between several clotting factors leading to a burst of thrombin-formation. A few molecules in the beginning of the series create an explosion of the final active enzyme: thrombin. One can compare it with a command that goes from the general to the officers, then to the sergeants and then to the men: in this way it takes little time to activate the whole army. This cascade sequence only explains the explosive formation of thrombin; however, clotting is not as simple as that. In fact, it is a web of positive and negative feedback reactions that finely tunes the output of thrombin and restricts it in time and place to the occurrence of a wound [1, 2] (figure 1).

Coagulation starts at a damaged site in the vessel-wall. The vessel-wall is covered by cells that contain tissue factor (TF). After injury this TF is exposed to blood, which contains a whole series of non-activated clotting factors. TF activates one of these factors (FVII) into FVIIa and binds to it to form the first enzyme: VIIa-TF [4-6]. This activates factor X into FXa, which in turn activates prothrombin (FII) into thrombin (FIIa). This we call the Production Dimension. FXa is not a very efficient enzyme ($k_{cat} = 2-4 \text{ min}^{-1}$); it needs the help of factor Va, which is activated by thrombin itself, to speed up FII activation a thousand fold [7, 8]. Both factors (FXa and FVa) bind to a procoagulant surface and form the prothrombinase complex (XaVaPL) [9]. This procoagulant surface binds also FII and thus favors the meeting of substrate and enzyme [10-12]. The enormous increase in FII activation by the contribution of factor Va and the procoagulant surface explains the burst of thrombin-production once traces of thrombin are formed. This offers also the possibility to regulate the activity of FXa, and hence thrombin production in space and time. This pathway (VIIa-TF $\rightarrow$ XaVaPL) is known as the extrinsic system.

Thrombin-production is a complicated web of positive (blue) and negative (red) feedback loops that allows a controlled explosion of thrombin in time. The elliptical orange shapes represent platelet-derived procoagulant surfaces that are responsible for limitation in space. The activation of protein C via TM-IIa is located on the endothelial cells of the vessel wall.

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molecular weight kininogen (HMWK) and prekallikrein (PK) on a negatively charged surface. The physiological role of this pathway is still a matter of debate. Contact-activation appears often as an artifact during thrombin generation *in vitro* but recent work [17, 18] challenges the view that it is irrelevant for thrombus formation *in vivo*. It thus appears that, although the role of contact activated thrombin generation (the old ‘intrinsic system’) in physiological haemostasis remains uncertain, it may nevertheless play a role in thrombus formation.

Thrombin promotes its own formation by the activation of the helper proteins factor V and factor VIII, but it also inhibits its own formation. The activation of FV and FVIII is direct but its inactivation involves an intermediate step. Thrombin binds to thrombomodulin and this complex converts protein C into its activated form (APC). APC in turn, inactivates FVa and FVIIIa in a process in which also protein S plays a role [1, 19]. This creates a “time-window” in which the complexes prothrombinase (XaVa) and tenase (IXa-VIIIa) are active. This we call the Regulation Dimension.

Also, FXa regulates its own formation by forming a complex with Tissue Factor Pathway Inhibitor (TFPI). This complex prevents further activation of FX and FIX by VIIa-TF and creates as such a second “time-window”.

It is clear that thrombin has a large number of actions. It regulates its own formation by activating and inactivating other proteins (FV, FVIII, FXI). Moreover thrombin is the main activator of platelets [20] and is responsible for the conversion of fibrinogen into fibrin. It also activates FXIII which in turn is responsible for the covalent binding between fibrin fibers, necessary to form a stable plug at the site of a wound.

Activation of platelets is very important for the localization of thrombin generation. Clotting should not only occur at the desired time but also be restricted to the site of injury so as to avoid thrombosis. This is realized by sticking and subsequent activation of the platelets to collagen exposed at the site of injury or by thrombin-mediated activation (see also below). Activation of platelets causes loss of the natural asymmetry of their plasma membrane and enriches the outside of the platelets with the procoagulant phospholipids phosphatidyl serine and phosphatidyl ethanolamine by a transbilayer movement from the inner leaflet to the outer leaflet of the membrane [21]. Activated clotting factors bind to the membranes of these activated platelets, so as to form the complexes prothrombinase (XaVa) and tenase (IXa-VIIIa). In figure 1 these activated platelets are represented by elliptical brown shapes. These procoagulant surfaces serve as a two-dimensional space where the substrates (FII and FX) are funneled to the enzyme complexes causing an enormous increase of reaction rate [10-12]. Thrombin formation increases by about a thousand fold [7, 8, 22], so without such a surface, no significant thrombin generation will occur. This provides the Localization Dimension: platelets stick in the wound and to fibrin threads and thus clotting only occurs where it is needed and remains restricted to the wounded area.

Thrombin in the blood does not live long; otherwise the slightest wound could make all the blood clot. Its half-life in plasma is less than a minute, due to the fact that antithrombins inactivate thrombin irreversibly. The haemostatic activity that develops in a wound or thrombus is essentially dependent upon the number of “man-hours” of thrombin that can develop in blood. This means that both the amount of thrombin generated and the length of time that it is active count. During haemostasis there is a non-equilibrium situation, comparable to emptying a bucket of water in a sink without a stopper (figure 2). Prothrombinase activates prothrombin into thrombin and thrombin disappears through the action of antithrombins.

Figure 2: Thrombin formation and disappearance (haemostasis)

The dynamics of thrombin appearance and disappearance are like filling a sink without a stopper with a large bucket of water. The level of water will increase and then decrease again depending on how large the opening in the sewer is and how fast water is poured in. Suppose the bucket size is the amount of prothrombin, prothrombinase activity determines how fast it is poured in, and the drain size is the action of antithrombin.

New insights challenge some classical paradigms of haemostasis and thrombosis

1) Primary versus secondary haemostasis

It is a classical paradigm of haemostasis that in a first stage platelets plug the wounded vessel and in a second stage thrombin is formed that cements together this platelet plug. The bleeding time reflects the first phase and the clotting time the second one.

There have always been observations that challenge this ‘textbook view’. Prolonged bleeding times have been detected in cases of severe over-dosage of oral anticoagulants [23] or heparin [24], both drugs acting on blood coagulation rather than on the platelets. Factors Va and VIIIa, products of thrombin action, appear within seconds in the blood flowing from a wound and diminish when heparin is administered [25]. This means that thrombin is formed before a wound stops bleeding. The activity of thrombin in such blood must be dependent upon platelet function because it is inhibited by aspirin intake [26-28]. Early thrombin formation was most convincingly demonstrated in the elegant experiments of the Furie group [29]. They visualized exposed tissue factor, aggregated platelets and fibrin at the site of a micro-trauma in a small vessel in the mouse and saw fibrin appear about 10 – 15 s after the lesion. Plasma clots at the fastest in ~12 seconds when an optimum concentration of TF is added, hence in these experiments thrombin must have appeared almost simultaneously with the platelet activation!

Many experiments that supports the classical two-step view were done in experimental setups in which the interaction between platelets and clotting factors was a priori impossible (washed platelets or plasma without platelets). As soon as platelets and plasmatic coagulation factors are allowed to cooperate, they invariably show early and strong mutual interaction. Thrombin is the most potent platelet activator [20] and the platelet membrane provides procoagulant phospholipids [21] that allow explosive TG [30]. This platelet-clotting-factor interaction is probably the most important positive feedback mechanism in haemostasis and responsible for the sudden arrest of blood flow from a wound. Therefore, it may be clear that thrombin is not only important during clotting, but it also plays a pivotal role for the platelet reaction to injury.

2) Arterial versus venous thrombosis

It is another classical paradigm of thrombosis that arterial thrombosis is a platelet dependent disease that should be treated with anti-platelet-drugs whereas venous thrombosis is a clotting disease that should be treated with anticoagulants.

Classically thrombosis is divided into arterial and venous thrombosis based on the type of vessel that is affected. In venous thrombosis, the role of (genetic) defects in anticoagulant pathways (deficiency of antithrombin (AT) [31], proteins C and S) as well as increased concentrations of coagulation proteins (e.g. prothrombin in association with the 20210 prothrombin gene variant [32]) is well established. Abundant literature data relate increased coagulation activity to a greater risk of venous thrombosis [33]. Several studies demonstrated that individuals with a congenital risk of venous thrombosis, such as factor V_{Leiden} mutation show hypercoagulable plasma, indicated by an increased level of thrombin generation. This is directly reflected in medical practice: treatment and prevention of venous thrombosis require drugs that diminish thrombin ac-

tivity in one way or another: by impeding carboxylation of vitamin K dependent proteins (vitamin K antagonists), by increasing antithrombin activity (heparins) or by inhibiting thrombin directly (hirudin and others).

For arterial thrombosis the role of blood coagulation proteins is much less recognized. An important reason is the absence of strong risk associations between genetic thrombophilic traits such as the factor V_{Leiden} mutation and atherothrombotic complications [34]. The origin of almost all arterial thrombotic diseases is searched in atherosclerotic changes in the vessel wall [35]. It is well known that diet (unsaturated fatty acids, low cholesterol), non-smoking and medication (statins), in short, the way of living, play a crucial role in the development of atherosclerosis which starts at an early age (<30) and continues over the course of decades. It is much less recognized that blood-clotting, more correctly the haemostatic-thrombotic system, pulls the trigger at the moment that the chronic condition of atherosclerosis converts to overt disease.

However, the thrombogenicity of atherosclerotic plaques seems to be related to their TF content [36], which is primarily associated with infiltrating macrophages. TF is exposed to the circulating blood when these plaques are ruptured, thereby initiating thrombus formation and related complications such as acute myocardial infarction [37]. It is being more and more recognized that thrombin plays its part in arterial thrombosis [35, 38]. Excess amounts of FII have been found to correlate with the occurrence of myocardial infarction [39]. Also higher than normal levels of von Willebrand factor (vWF) increase TG and are a risk factor for arterial thrombosis [39-41]. In a sub-population of young stroke patients (~30%) both TG in platelet rich plasma (PRP) and vWF have been shown to be significantly higher than normal [42]. Clinical trials have shown that vitamin K antagonists [43] as well as heparins [44, 45] decrease the reoccurrence rate of myocardial infarction. “Antiplatelet” drugs achieve at least part of their effect by inhibiting TG in PRP [26, 27] and, inversely, aspirin has been shown to be beneficial also in venous thrombosis [46].

The classical paradigm of haemostasis that arterial thrombosis is due to the platelets and venous thrombosis to clotting thus seems to lose ground.

This paradigm was also supported by the old observation that in venous thrombosis the thrombus really looks like a clot while in arterial thrombosis the thrombus doesn't seem to contain much coagulated blood but rather appears as an aggregate of platelets. This however can be explained by the differences in shear rates in veins and arteries (see below).

Nevertheless, the precise role of the plasmatic thrombin generating system in arterial thrombotic disease remains enigmatic.

Thrombin is also needed for thrombus growth

Not only haemostasis and thrombosis but also thrombus growth requires thrombin generation. As soon as experiments are carried out under conditions where thrombin can form, tissue factor and thrombin appear to play an important role, especially at low (venous) and intermediate (large artery) shear rates [20, 47-49]. At high shear rates an effect of thrombin inhibition is not seen on initial thrombus formation but becomes obvious when the experiment is prolonged [48]. One of the functions of the aggregate must be to serve as a niche in which a sufficient local concentration of thrombin can build up. Where the concentration of thrombin is high enough, new platelets will be recruited through interaction of thrombin with platelet-receptors GPIIb α , PAR 1, PAR 3 and PAR 4 [50-51] and more thrombin will form [52, 53]. This activation of the platelets induces a conformational change in their receptors GPIIb/IIIa making them appropriate for interaction with vWF-fibrinogen which induces on its turn aggregation of more platelets.

But thrombin is not the only platelet-activator; several mechanisms of platelet-aggregation or thrombus-growth exist in parallel. Collagen, which as TF is also present in the subendothelial layers of the vessel wall and is exposed at the site of injury, activates the platelets via the platelet-receptor GPVI (directly) and via GPIb with modified vWF. When the collagen-surface is completely covered by platelets the role of collagen is taken over by fibrin, formed in the aggregate, that activates the platelets via the same mechanism (GPIb with modified vWF) [54, 55]. Apparently the fibrin-clot is not only the final product of haemostasis but it also participates as a clotting-factor itself [56, 57]. This explains why thrombin becomes increasingly important when less collagen is exposed. The relative importance of thrombin- and collagen-dependent mechanisms has indeed been shown to be influenced by the type of injury to a vessel wall [58].

The differences in shear rates in veins and arteries and the need of a sufficient concentration of thrombin for thrombus growth also explains the old observation that in venous thrombosis the thrombus really looks like a clot while in arterial thrombosis the thrombus looks like an aggregate of platelets. At low shear rates (veins) a sufficient concentration of thrombin can build up in the aggregate and diffuse into the surroundings converting a lot of fibrinogen. A thrombus in the veins therefore consists of a platelet-rich head of a few millimeters and a long tail of coagulated blood (that can reach over 10 centimeters). In the arteries however thrombin is washed away by the high flow before it is able to convert much fibrinogen. This results in a thrombus consisting mainly of the platelet-rich head.

Thrombin generation and bleeding

Thrombin generation not only plays a pivotal role in thrombosis (venous and arterial) but also in bleeding diseases. It has been demonstrated in haemophili-

acs (deficiency of factor VIII, IX or XI) as well as in all rare clotting factor deficiencies (factor II, V, VII, X) that thrombin generation is diminished and that a clinical bleeding tendency is seen as soon as thrombin generation is below 20% of normal [59, 60]. The objective in antithrombotic therapy is to diminish thrombin activity by the use of vitamin K antagonists, heparins or hirudin, which carries of course a risk of bleeding when thrombin generation is diminished too much. Also in severe thrombocytopenia, as well as in Glanzmann [61] and Bernard-Soulier [56] disorder, thrombin generation is diminished. In von Willebrand's-disease, thrombin generation in PRP is significantly impaired. The defect is more pronounced in PRP than in platelet poor plasma (PPP) [62], which indicates that it can not be explained by the concomitant deficiency of factor VIII.

One of the reasons that low thrombin generation causes a bleeding tendency is likely to be found in the fact that the amount of thrombin formed determines the amount of Thrombin Activated Fibrinolysis Inhibitor (TAFI) that is generated [63]. Low thrombin generation therefore causes increased fibrinolysis. This is likely to be the explanation for the fibrinolytic character of much of the bleeding in haemophiliacs and would explain the beneficial effect of antifibrinolytic agents.

The function of the haemostatic-thrombotic system is better expressed by thrombin generating capacity than by fibrinogen-based methods as e.g. clotting times, thrombelastography.

From the above it may be clear that thrombin plays not only a pivotal role in normal haemostasis but also in thrombosis and bleeding. Thrombin generation is the physiological function that governs thrombosis and it is this function that is pharmacologically manipulated to prevent and cure thrombosis. Since there is not always a close correlation between the thrombin generating capacity of blood and clotting-times (95% of thrombin is formed after clotting [64]), the coagulability of blood is better quantified by its thrombin-generating capacity than by measuring clotting times. Heparins e.g. strongly diminish the amount of thrombin that appears while the PT (TF-induced clotting-time) is not affected. A modified clotting time (the aPTT) has to be used. For anti-Vitamin K treatment a close correlation can be seen between TF induced clotting times as the PT and the amount of FIIa formed while other antithrombotics (e.g. dermatan sulphate) do not affect any clotting time measurement. There are no antithrombotics, however, that do not decrease the thrombin generation curve.

On the other hand, thrombotic tendencies, such as those caused by a congenital defect of protein S (PS) or protein C (PC) or AT, or by an acquired resistance to activated protein C (APC) (e.g. oral contraceptives) [65-68] hardly affect any clotting time but always are accompanied by excessive thrombin formation.

Also, bleeding-diseases do not always affect clotting-time tests. Haemophiliacs for example show a prolonged clotting-time in the aPTT but not in the PT.

Glanzmann thrombasthenia only show a prolonged bleeding time but no prolonged PT or aPTT. Thrombin generation however still is affected because no good procoagulant surface is available [61].

One can only conclude that the thrombin forming capacity of blood can not unequivocally be determined with the aid of a clotting-time test only. Clotting times and the amount of thrombin formed are different things but both merit to be estimated. Measuring a thrombogram has the advantage that one obtains both parameters (lag-time and ETP) at the same time. The lag-time in a thrombogram is the time needed to reach a certain (low) level of thrombin (e.g. 10 nM) which corresponds with the time at which fibrinogen starts to be converted into fibrin, just like a clotting time. It depends on the chosen conditions (e.g. TF-concentration, FIIa-concentration) whether the lag-time will have the character of a PT, a PT with diluted thromboplastin or an aPTT. Therefore measurement of a thrombogram produces more information than the clotting time can give. Measuring a clotting time can be seen as a way to obtain one parameter of the thrombogram, i.e. the lag-time of thrombin formation.

Thrombin generation also yields information that can't be deduced from determination of a (number of) single component(s) of the blood clotting mechanism. Thrombin generation is such a complicated web of interactions between such a large number of factors that there is no simple relation between a single factor concentration and the final outcome of thrombin generation. This is clearly the case in e.g. haemophiliacs: the bleeding tendency of these patients is not necessarily related to the level of the missing factor [69]. The remainder of the clotting system - including the platelets [70] - determines whether or not a given haemophiliac can make sufficient use of the small residual amount of FVIII or FIX.

Particularly interesting is the lupus anticoagulant (LAC). This antibody causes an increase of the clotting time and thus is deemed "anticoagulant" but it also brings about a thrombotic tendency. This is called the 'LAC paradox'. This paradox disappears as soon as one resorts to thrombin generation where it is seen that indeed the lag-time (=clotting time) is prolonged but also that thrombin generation is not inhibited by the activated protein C system [71].

As mentioned before, beside clotting-time tests there are also fibrinogen-based methods that attempt to go beyond the clotting time by measuring the turbidity [72] or mechanical properties of the clot [73]. These are useful for the precise determination of the clotting time but have the important disadvantage that the measured variable (mechanical properties, turbidity) is not unambiguously related to thrombin-concentration. It is a covariable of one of the effects of thrombin formation i.e. fibrin polymerization. This polymerization is related to thrombin formation, or even to the formation of fibrin monomers, in a complicated and as yet incompletely understood manner. Therefore, the fundamental problem with fibrinogen-based methods is that an effect is studied that is dependant in an unknown way on known and unknown variables. No relation is

known between mechanical properties or turbidity and the activity of thrombin, so the information can not be quantified in terms of thrombin concentrations. Further, fibrinogen is consumed long before thrombin formation is over meaning that no information can be obtained on the stages of thrombin generation beyond the very beginning of the production phase.

Sometimes curves are constructed as a result of mathematical transformations that can easily be mistaken for real thrombin generation curves [74, 75], but that do not improve the content of information in any way. In fact, a multitude of different methods allow scores of measurable properties of blood that change as a result of the formation of thrombin and/or fibrin. Often it will be possible to correlate such changes to a clinical picture [76]. Nevertheless, they must be considered surrogate variables that can better be abandoned now that exact quantification of thrombin generation has become available.

How can the thrombin forming capacity of the blood be assessed ?

The thrombogram and the ‘Endogenous Thrombin Potential’ (ETP)

The general form of any thrombogram is independent of the measuring system and the experimental conditions (figure 3). The thrombogram starts with a lag-phase or initiation phase in which hardly any observable traces of thrombin appears [77], followed by a production or propagation phase marked by a sudden burst of thrombin. Clotting occurs at the start of the explosive burst, i.e. at the end of the lag-time [64]. As mentioned before, the clotting-time is therefore a good estimate of the duration of the lag-phase and vice versa. The small traces of thrombin formed during the lag-time activate the clotting factors V, VIII, XI and platelets. As such they prepare the scene for the full blown TG during the production phase. The reaction mechanisms in the lag-phase and the production phase are different, that is why the lag-(=clotting) time does not automatically contain information on the amount of thrombin formed in the following peak. There is no sharp distinction between the production phase and the inactivation phase. The antithrombins present in plasma react with thrombin as soon as it appears. The velocity of inactivation is proportional with the thrombin concentration and the antithrombin concentration. At the peak, thrombin generation and decay are equally fast. Once past the peak, decay gains on prothrombin conversion and somewhere in the down slope the latter will stop [78, 79].

Figure 3: The general shape of a thrombogram.

The most important parameters are indicated. The ETP (nM x min) is a good measure of the thrombin forming capacity of the blood.

Thrombin is an enzyme and acts by converting substrate molecules. The number of molecules that can be converted by thrombin is proportional to both the concentration of this enzyme and the time that it can act i.e. to the area under the thrombin time curve. This area thus reflects the amount of natural substrates that could have been converted under in vivo conditions by the thrombin generated in the sample. It is called the endogenous thrombin potential (ETP) [80] and is a measure of the thrombin forming capacity of the blood.

Timed subsampling on fibrinogen

From the fifties until the early eighties, thrombograms were obtained by timed subsampling of aliquots of clotting blood or plasma onto a solution of fibrinogen, often diluted bovine plasma (without added calcium). By measuring the clotting time in each subsample the thrombin concentration was assessed for each time-point and a thrombin generation curve was constructed. This was very laborious work.

Timed subsampling on a chromogenic substrate

Subsampling onto a chromogenic substrate is the first method in which thrombin is measured directly. The thrombogram is obtained via determination of the

thrombin content in small subsamples taken at short intervals from clotting blood or plasma. Automatic recording of the sampling time and semi-automatic measurement of the thrombin activities became possible, which also allowed executing several parallel experiments [79]. However, it remained laborious: it took about half a day to make six thrombograms with three people requiring great dexterity if parallel curves were to be measured.

Because of the use of low MW-substrates, the total amidolytic activity measured during thrombin generation is the sum of free thrombin and the $\alpha 2$ -macroglobulin-thrombin-complex ($\alpha 2$ M-IIa). Thrombin bound to $\alpha 2$ M is sterically impeded to react with any physiological substrate because it is bound in a niche of this big molecule. The small chromogenic substrate however can still be attacked. This means that one has to correct for the contribution of this $\alpha 2$ M-IIa complex to the total amidolytic activity if one wants to know the concentration of free FIIa alone. The velocity of the formation of the complex is in good approximation proportional to the concentration of free thrombin. Therefore the amount of $\alpha 2$ M-IIa in serum is proportional to the integral of the thrombogram i.e. to the ETP. This makes it easy to correct for this contribution of $\alpha 2$ M-IIa by using a simple algorithm [80].

Adding a chromogenic substrate directly to the clotting plasma

In an attempt to further automate the TG-measurement a chromogenic substrate was added directly to the clotting plasma. This cannot be done with high affinity substrates because they interfere with all thrombin driven feedback reactions and the clotting mechanism during the lag-phase is seriously disturbed. They bind thrombin so tightly that they act as potent thrombin-inhibitors. The decay phase seen in these thrombogram-like curves when using such substrates is due to substrate exhaustion and not to thrombin-disappearance because also the inactivation of thrombin by antithrombin is strongly inhibited.

However, suitable slow reacting thrombin substrates were found with such kinetic properties (high K_m and low k_{cat}) that the velocity of product formation is in good approximation proportional to the prevailing concentration of FIIa [81]. Of course, the presence of a chromogenic substrate implies that the physiological reactions of thrombin are interfered with. Even a slow reacting substrate will compete with the antithrombins of the plasma for the active site of thrombin. This results in an artificially increased ETP because the thrombin decay will be slowed down. The magnitude of this effect can be easily calculated and corrected for.

This method was restricted to defibrinated plasma. Clot formation had to be avoided when using a chromogenic substrate because the light-scattering caused by the developing clot would disturb the OD-measurement. Defibrination of the plasma was carried out with suitable snake venom enzymes like Arvin which convert fibrinogen into fibrin without affecting any clotting factor of the coagulation system. However, when removing this fibrin-clot out of the plasma, plate-

lets were removed as well. This means that this method could only be used in defibrinated, platelet poor plasma.

Adding a fluorogenic substrate directly to clotting plasma.

To solve the drawback of defibrination, the logical next step was the use of a fluorogenic substrate since fluorescence can be measured in turbid media. This makes it possible to measure thrombin generation in PRP as well as in PPP [82]. However, other drawbacks appeared notably substrate-depletion and the inner-filter effect. As a result of these effects, the same amount of thrombin activity causes a far greater increase of signal at the beginning of the experiment than towards the end. This means that the calibration factor is not constant but increases during the experiment which makes it necessary to measure the calibration factor continuously. Another problem is the varying color of the plasma-samples which introduces serious differences in the absorption of light. Therefore, the reaction velocities measured in one plasma-sample can not be standardized by comparison to another plasma-sample. This would introduce serious experimental errors.

Outline of this thesis

The three drawbacks of fluorogenic methods mentioned above can be dealt with by the use of continuous individual calibration as in the Calibrated Automated Thrombogram (CAT-method). This method is presented in **chapter II**. A description is given of the standard conditions as well in PPP as in PRP and also the influence of added APC and TM is shown. Further the normal values and variability – experimental variation, intra-individual variation and inter-individual variation – are presented.

The mathematical procedure required to convert raw data (fluorescent signal versus time) into TG curves is explained in **chapter III**.

In **chapter IV** it is shown how the time-course of prothrombinase-activity during thrombin generation can be calculated starting from the TG curve if the initial concentration of AT is known.

What happens if the effect of plasma-color, inner-filter effect and substrate-consumption are neglected is seen in **chapter V**. Certain commercialized methods use a fixed calibration factor measured in buffer instead of continuous individual calibration. We investigated the effect of both calibration methods on the reproducibility and compared the experimental and intra-individual variation of both methods.

Measuring a system always means disturbing it. The addition of an artificial substrate that binds to thrombin, although it is a slow reacting substrate, will interfere with the physiological reactions of thrombin and as such influence thrombin generation. In **chapter VI** we show what this disturbance amounts to.

We discussed already two classical paradigms. A third current paradigm is that thrombin formation is the result of chemical interactions only [1]. In accordance with this view the velocity of thrombin generation is uniquely determined by the initial concentrations and reaction constants of the partaking components [83]. In **chapter VII** we show evidence that as clotting takes place thrombin generation velocity is co-determined by diffusion.

It is impossible to measure thrombin generation in plasma without diluting it. Several methods are available using different dilutions of plasma. This poses the question what the influence of dilution is on thrombin generation. This is the subject of **chapter VIII**.

In **chapter IX**, finally, protocols are presented for measuring thrombin generation. Not only of the most advanced method up to now, the CAT-method, but also of the ones preceding the CAT-method. As such an overview is given of measuring thrombin generation during half a century. However, nostalgia is not the reason why these 'old' techniques are raked up. As will be discussed in the preface of this chapter, each technique has its benefits and its specific use in specific situations.

A general discussion of this thesis with a summary of the conclusions is given in **Chapter X**.

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Chapter II

Calibrated automated thrombin generation measurement in clotting plasma (CAT)

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Abstract

Calibrated automated thrombography displays the concentration of thrombin in clotting plasma with or without platelets (PRP / PPP) in up to 48 samples by monitoring the splitting of a fluorogenic substrate and comparing it to a constant known thrombin activity in a parallel, non-clotting sample. Thus, the non-linearity of the reaction rate with thrombin concentration is compensated for, and adding an excess of substrate can be avoided. Standard conditions were established at which acceptable experimental variation accompanies sensitivity to pathological changes. The coefficients of variation of the surface under the curve (ETP) are: within experiment ~3%; intra-individual: <5% in PPP, <8% in PRP; interindividual 15% in PPP and 19% in PRP. In PPP, calibrated automated thrombography shows all clotting factor deficiencies (except factor XIII) and the effect of all anticoagulants (AVK, heparin(-likes), direct inhibitors). In PRP, it is diminished in von Willebrand's disease, but it also shows the effect of platelet inhibitors (e.g. aspirin and abciximab). Addition of APC or thrombomodulin inhibits thrombin generation and reflects disorders of the APC system (congenital and acquired resistance, deficiencies and lupus antibodies) independent of concomitant inhibition of the procoagulant pathway as for example by anticoagulants.

Introduction

The thrombin generation curve (thrombogram) is an old and established tool in blood coagulation research [1–4]. It reflects much, if not all, of the overall function of the blood clotting system [5]. In PPP, it shows all clotting factor deficiencies with the exception of factor XIII [6–8], and it is sensitive to the action of oral anticoagulation, heparins of all types [9], direct thrombin inhibitors [10] and in fact all anticoagulant drugs tested. Hyperprothrombinaemia [11] and lack of AT [12], PS or PC as well as APC resistance increase thrombin generation. Thrombin generation and a derivative test, the end level of $\alpha 2M-T$ in the 'serum' of defibrinated plasma, have been shown to be sensitive indicators of congenital [13] and acquired [14–16] defects in the APC pathway.

In platelet-rich plasma, the thrombogram also reveals the part played by platelets and hence the effect of von Willebrand factor [17], hypofibrinogenaemia [17], thrombo(cyto)penia [18] and Glanzmann's [17] and Bernard-Soulier's thrombopathies [19] as well as the effect of anti-platelet drugs [20, 21], GPIIb/IIIa antagonists included [22]. Agents that increase platelet reactivity (e.g. arachidonic acid, epinephrine and collagen [23]) accelerate thrombin generation in PRP.

Despite its potential value for clinical diagnosis, drug monitoring and epidemiology, thrombin generation has not found universal application, primarily because of technical reasons: with the established subsampling techniques it takes about 1 h for a skilled laboratory worker to acquire one to four curves. The use of a slow-reacting fluorogenic substrate enables continuous measurement of thrombin generation in fibrin(ogen)-containing media which makes it possible to use PRP and to test the influence of platelets on thrombin generation [24, 25]. However, it raises the question of how to convert the velocity of fluorescence change (dF/dt) into thrombin concentration [26]. For various reasons, during the course of the experiment, the relationship between dF/dt and thrombin concentration varies as fluorescence increases. We calculate thrombin activity as a function of time by comparing the fluorescent signal from the thrombin-generating sample to that from a known stable concentration of thrombin activity measured simultaneously in a parallel sample. We developed software to combine the data from the two experiments and display the concentration of thrombin in the coagulating sample in real time during the experiment. Using a 96-well plate fluorescence reader, up to 48 samples can thus be run.

Materials and Methods

Chemicals

Recombinant relipidated tissue factor not containing polybrene or Ca^{2+} was a kind gift from Dade Behring (Marburg, Germany). Phospholipid vesicles consisted of 20 mol% phosphatidylserine, 20 mol% phosphatidylethanolamine and 60 mol% phosphatidylcholine. Hepes-buffered saline comprised 20 mM Hepes, 140 mM NaCl, pH 7.35 (prepared as described [12]).

Fluorogenic substrate, Z-Gly-Gly-Arg-AMC, was obtained from Bachem (Bubendorf, Switzerland). Upon splitting by thrombin, it releases the fluorophore AMC, which is measured using a 390-nm-excitation and a 460-nm-emission filter set.

A fresh mixture of fluorogenic substrate and $CaCl_2$ was prepared for each experiment as follows: to 875 μ l of buffer (Hepes 20 mM, pH 7.35) containing 60 g/l BSA (Sigma, A-7030), 100 μ l of 1 M $CaCl_2$ was added. At 37 °C, 25 μ l of a 100 mM DMSO solution of the fluorogenic substrate was squirted in and immediately vigorously mixed. The resulting clear solution, referred to as FluCa, thus is 2.5 mM in fluorogenic substrate and 100 mM in $CaCl_2$.

The chromogenic thrombin substrate was S2238 (Chromogenics, Mölndal, Sweden). Heparin was unfractionated heparin from LEO (Copenhagen, Denmark).

APC was purified according to Regnault et al. [27]. As a TM-preparation we used recombinant human TM, a kind gift of Asahi (Japan).

Blood and Plasma

Blood was obtained through antecubital venipuncture (1 volume trisodium citrate 0.13 M to 9 volumes blood) from healthy individuals who consented to participate in this study. Free flow or minimal suction was employed; vacuum containers were avoided. PRP was collected from the upper 3/4 volume of plasma supernatant, after centrifugation at 265 g for 10 min at room temperature. The platelets were counted (Beckman Coulter counter) and PRP was adjusted to 150×10^9 platelets/l with its own PPP, prepared by centrifuging twice at 2900 g for 10 min at room temperature. PRP was always used within 1 h after venipuncture. In order to avoid contamination with procoagulant microparticles from aging platelets, PPP is preferably prepared within 30 min after venipuncture.

Preparation of the Calibrator

In order to obtain a stable thrombin-like activity in plasma, we used α 2M-T. A crude α 2M-containing fraction was isolated from citrated bovine plasma by precipitation with 12% (w/v) PEG-20000; the pellet was dissolved in 100 mM NaCl, 20 mM Hepes, pH 7.9 (1/10 of the original plasma volume) (modified from Barrett [28]). To this solution we added 12 μ M bovine prothrombin, 6 mM CaCl_2 , 50 μ M phospholipid vesicles (20% brain phosphatidylserine, 80% egg yolk phosphatidylcholine), 5 nM bovine factor Xa and 0.78 nM bovine factor Va. The mixture was stirred for 30 min at room temperature and then kept overnight at 4 °C. The formed fibrin was removed and the preparation was applied to a Sephacryl column equilibrated with 100 mM sodium citrate, 20 mM Hepes (pH 7.4) and 0.02% NaN_3 . The α 2M-T that had formed eluted as a sharp peak before contaminating proteins.

The concentration of α 2M-T is measured by its activity towards S2238 and adjusted to the activity of 600 nM human thrombin; 100 nM bovine AT and 2 U/ml heparin are added in order to prevent coagulation of the plasma sample upon use. The material is lyophilized and used as a calibrator after reconstitution with sterile water.

Automated Fluorogenic Measurement of Thrombin Generation under Standard Conditions

The thrombograms are measured in a 96-well plate fluorometer (Ascent reader, Thermolabsystems OY, Helsinki Finland) equipped with a 390/460 filter set (excitation/emission) and a dispenser. Immulon 2HB, round-bottom 96-well plates (Dynex) are used.

Each experiment needs two sets of readings, one from a well in which thrombin generation takes place (TG well) and a second one from a well to which the calibrator has been added (CAL well). The color of the plasma can influence the fluorescence intensity and therefore each plasma needs to be compared to its own calibrator measurement. If the same plasma is used under different condi-

tions (different triggers, addition of APC or TM, addition of substances to be tested), the experiments can share the same calibrator. PPP and PRP from the same individual can also share the same calibrator. Usually experiments are carried out in quadruplicate, i.e. a set of 4 TG wells is compared to a set of 4 CAL wells. A dedicated software program (Thrombinoscope, Synapse BV, Maas-tricht, The Netherlands) enables the identification of the (sets of) wells and determines the duration of the experiment and the sampling rate (usually 4 wells / minute).

To each well, 80 μ l of plasma is added. The TG wells receive 20 μ l of buffer, containing the trigger but no Ca^{2+} , and the CAL wells 20 μ l of the α 2M-T-solution. For PPP, the trigger is 30 pM of rTF and 24 μ M phosphatidylserine/posphatidylcholine/phosphatidylethanolamine vesicles in Hepes-buffered saline. For PRP the trigger is 20 μ l of 3 pM of rTF without added PL.

The plate is placed in the fluorometer and allowed to warm to 37 °C (minimally 5 min). The dispenser of the fluorometer is flushed with warm 100 mM CaCl_2 solution, emptied, and then flushed with warm FluCa. At the start of the experiment, the instrument dispenses 20 μ l of FluCa to all the wells to be measured, registers this as zero time, shakes them for 10 s and starts reading. During the measurement, the program uses a default calibrator to calculate tentative thrombin concentrations and displays the course of thrombin concentration in time for each group. Once the measurement is finished the program recalculates the TG-curves using for each group the readings of the relevant CAL wells.

Experimental Results

From Fluorescence Intensity to Thrombin Activity

Free thrombin activity rapidly decays in plasma, so, in order to investigate the effect of constant thrombin activity on substrate consumption in plasma we used the α 2M-T complex that splits the signalling substrate like thrombin does but that is not affected by plasma inhibitors. Under test conditions, its amidolytic activity in plasma, as tested by subsampling on S2238, remained constant for over 3 h (results not shown). The kinetic constants of α 2M-T were compared to those of thrombin (table 1). The K_m of thrombin and α 2M-T is identical within the limits of experimental error. Therefore, the variation in the reaction velocity with decreasing substrate concentration will be practically identical. The turnover velocity of α 2M-T (k_{cat}) is half that of thrombin. To compensate for this difference, we express henceforward the concentration of α 2M-T in 'thrombin equivalents', i.e. as the concentration of free thrombin that gives the same reaction velocity (i.e. at half the actual concentration).

Kinetic constants	α 2M-T in buffer	α 2M-T in plasma	Thrombin (buffer)	Thrombin (plasma)
K_m (μ M)	292 ± 44	310 ± 34	305 ± 46	280 ± 57
k_{cat} ($M^{-1}s^{-1}$)	0.95 ± 0.1	0.89 ± 0.1	1.86 ± 0.26	ND

Table 1. Kinetic constants of thrombin and α 2M-T

n=12. The K_m of thrombin in plasma was measured indirectly as the K_i of the inhibition of thrombin decay. (ND = not done)

Figure 1A demonstrates that constant α 2M-T activities do not cause a constant rate of fluorescence increase. The initial rates, measured as the tangents to the curves at $t = 0$, are proportional to the enzymatic activity added (figure 1B). The curvature is due to substrate consumption and to the non-linearity of fluorescence intensity (F) with increasing concentration of fluorogenic molecules (the ‘inner filter effect’) [29]. Both effects increase with the amount of product formed and hence with the level of fluorescence. We calculated the difference between the ideal fluorescence intensity (F_i), i.e. the value that would have been obtained experimentally if the initial velocity would have been maintained and the experimentally measured fluorescence (F_e), i.e. the vertical distance between the bold and the thin lines in figure 1A. In the plot of $F_i - F_e$ against F_e (figure 1C) the lines obtained at the six different thrombin activities superimpose, demonstrating that the deviation from the initial velocity is indeed only dependent on the level of fluorescence. Therefore, every fluorescence level requires a different calibration factor to convert the velocity of the fluorescence increase (dF/dt) into thrombin activity (in nM). This calibration factor can be read from the graph obtained with a known calibrator concentration.

The calibration factor is not only a function of the fluorescence level attained, it also depends upon the instrument used, the age of lamp and filters, and the color of the plasma; therefore, each group of experiments that is carried out with the same plasma requires its own calibration experiment.

Influence of the Fluorogenic Substrate on the Course of Thrombin Generation

The more substrate is present, the more thrombin molecules are occupied by the substrate and the less are they available for interaction with the natural antithrombins [30]. This effect is not corrected for by calibration, because the calibrator does not react with antithrombins. Therefore, the measured thrombogram increases with increasing concentrations of the fluorogenic substrate (figure 2A). When the amount of free thrombin is calculated ($= [\text{total thrombin}] \times K_m / (K_m + [\text{substrate}])$), the influence of the substrate concentration becomes negligible (figure 2B), showing that the fluorogenic substrate has little or no influence on the mechanism of prothrombin conversion.

Figure 1. The effect of constant thrombin activity on the fluorescent signal production in plasma.

A. The fluorescent traces of a range of $\alpha 2$ M-T concentrations with the tangents at $t=0$ (initial velocity). B. Relation between initial velocity and $\alpha 2$ M-T concentration. C. Difference between tangents and curves of the top frame plotted as a function of the measured fluorescence.

Figure 2. Thrombograms obtained at different substrate concentrations (PPP, standard conditions).

A. Total thrombin. B. Free thrombin.

Substrate concentrations (from top to bottom): 0.4; 0.3; 0.2 and 0.1 mM.

The Effect of Fibrinogen

Previous techniques for automatic determination of the thrombogram used chromogenic substrates and required defibrination with coagulating snake venom enzymes in order to prevent turbidity changes [30]. We observed that the removal of fibrinogen has a profound influence on the thrombogram: in the absence of fibrinogen, the fluorogenic technique shows a lower thrombin peak and

a higher α 2M-T end level than in its presence (figure 3A). By adding increasing concentrations of fibrinogen to afibrinogenemic plasma, fibrinogen dose-dependently decreases the amount of thrombin found in a sub-sampling experiment and increases the amount of thrombin in a fluorometer experiment (figure 3B).

Figure 3. The influence of fibrinogen on the thrombogram.

A. Course of amidolytic activity (expressed as thrombin equivalents) in normal pooled plasma before (A) and after (B) defibrination. Means $\pm$ 2 SD, n = 24.

B. ETP of the plasma from an afibrinogenemic patient with increasing amounts of fibrinogen added. ● = Fluorogenic method; ○ = subsampling method.

Standard Conditions

In PPP we chose a final concentration of 5 pM of rTF together with 4 μ M of procoagulant phospholipids. At this concentration of rTF, thrombin generation is dependent upon factors VIII and IX (figure 4) but not on factor XI, the influence of which is only seen at TF concentrations below 1 pM [8, and results not shown]. In the 3-pM range and above, thrombin generation in PPP is not critically dependent on the rTF concentration, whereas around 1 pM it is. rTF does not normally carry enough procoagulant phospholipids to ensure optimal prothrombin conversion. Thrombin generation is phospholipid dependent in the range of 0 μ M to 2 μ M and reaches a plateau at $\sim$ 3 μ M. At $\sim$ 10 μ M, the contact activation properties of negatively charged PL start to play a role [8], we therefore used 4 μ M PL.

In the absence of traces of rTF, the lag phase of thrombin generation in PRP is long and variable, probably influenced by endogenous TF and contact activation. Adding 0.5 pM (final concentration) of rTF largely abolishes this variability. It is essential that no PL are added to make the test critically dependent upon the PL coming from the activated platelets. Under these conditions,

thrombin generation is dependent upon the platelet number in the range of $0-100 \times 10^9/l$; at a platelet-number $>200 \times 10^9/l$ dependence is minimal. In our experience, at $150 \times 10^9/l$ a useful equilibrium establishes between the independence of small fluctuations in the platelet count and the sensitivity to platelet inhibitors.

Figure 4. Influence of TF concentration on thrombin generation in normal and haemophilia A and B plasma.

a. Normal plasma.
b. Haemophilia A plasma.
c. Haemophilia B plasma.
rTF concentrations in decreasing peak height: 15, 10 and 5 pM rTF.

In PRP, under these conditions, thrombin generation is dependent upon platelet function (and hence on the level of von Willebrand factor [7]) as well as upon the concentration of factors VIII, IX and XI; the effect of activated factor VII is readily seen (results not shown). The thrombogram reflects the action of all anticoagulants tested, alone or in combination. In the case of heparin, release of platelet factor 4 causes the inhibitory effect to be significantly smaller in the presence of platelets than in PPP (figure 5). In the case of oral anticoagulation, the concomitant inhibition of the protein C system is readily seen.

Figure 5. Influence of heparin administration on thrombin generation in PPP and PRP.

At $t=0$, 7000 aXa units of enoxaparin were injected subcutaneously.

Thin line = PPP, $t=0h$

Bold line = PPP, $t=4h$

++ = PRP, $t=0h$

o o = PRP, $t=4h$.

Time axis truncated at $t=30$ min.

Normal Values and Variability

The experimental variability in the main parameters of the thrombogram is shown in table 2. (The lag time is arbitrarily defined as the moment that 10 nM thrombin is formed.) The variation measured in identical parallel experiments in one 96-well plate is low (3–5%) and even lower when an occasional apparently aberrant curve, usually due to the presence of a floating bubble or accidental miss-pipetting, is left out.

		Average (n=36)	SD	CV Inter- individual	CV Intra- individual	CV expe- rimental
PPP	ETP (nM.min)	1879	284	15	4.5	2.5
	Peak (nM)	458	60	13	5.5	4.5
	Lag-time (min)	3.1	1.4	47	8.1	5
PRP	ETP (nM.min)	1648	315	19	7.4	3
	Peak (nM)	118	41	34	10.3	3.5
	Lag-time (min)	8.1	1.9	24	9.4	7

Table 2. Normal values and variability

The intra-individual day-to-day variation was measured on 9 different days in 4 individuals. Experimental CV was determined on 36 identical samples measured simultaneously.

Figure 6. Intra- and inter-individual variability in the PRP thrombogram.

From the experiments used to establish the intra- and inter-individual variability, the curves are given of three subjects, determined four times at 2-week intervals. The line type is varied per individual. ○ = Mild von Willebrand factor deficiency.

The intra-individual CV was measured by making thrombograms of 4 healthy subjects once in 9 consecutive weeks. The CV of the ETP in PRP for each individual was around 8% (7.4, range 6–11). The interindividual variability determined based on the means of the 9 individual experiments was 17.5%. The combination of relatively stable day-to-day values and large interindividual variation is further illustrated in figure 6 (a representative selection is shown so

as not to overload the figure). Part of the variation in PRP must be due to the variation in von Willebrand factor. The lower curve in figure 6, for example, is from an individual with a mild von Willebrand factor deficiency (ristocetine cofactor activity 49%, factor VIII:c 72%).

Influence of Added APC and TM

Figure 7 demonstrates that thrombin generation is considerably decreased by the addition of APC or TM. PRP is significantly less sensitive to APC than PPP. The sensitivity to TM does not show this large difference between PPP and PRP. The degree of inhibition observed is dependent upon both the TF concentration and the concentration of APC and TM. Under our standard conditions the inhibitions shown in table 3 are observed. Because the effect of APC or TM is compared to the same plasma to which no activator of the PC system is added, its effects can be observed independent of concomitant inhibition of thrombin generation as for example under antithrombotic treatment or in the presence of lupus anticoagulants [16].

Figure 7. Effect of the protein C system on normal plasma.
A. PPP. B. PRP. Drawn line = Control; ++ = TM added (10 nM); ○○= APC added (10 nM in PPP, 6 nM in PRP). Inhibition of the ETP: PRP-TM: 64%; PRP-APC: 29%; PPP-TM: 76%; PPP-APC: 87%.

Plasma	Addition	Concentration (nM)	Inhibition (%)	SD (%)
PPP	TM	3	34.4	4.9
	APC	6.5	47.5	5.1
PRP	TM	10	69.7	5.8
	APC	25	71.8	5.5

Table 3. Effect of TM and APC supplementation

Discussion

Measuring the haemostatic function of the blood is important both in bleeding and thrombotic disorders. Nevertheless, there is no universal test of this function available to the clinician. Clotting times of different types do not indicate a thrombotic tendency of unknown origin or mild haemostatic disorders. Different clotting assays are needed for different types of anticoagulants (oral anticoagulation/heparin). And for some types of antithrombotic therapy no adequate function test is available (e.g. heparin likes).

The thrombin generation test is a time-honored tool in coagulation research [1–4], and its wide scope is easily understood from the focal place of thrombin in the haemostatic-thrombotic mechanism [5]. On the one hand, scores of factors influence its formation, and on the other hand, it has numerous actions on blood and vessel components. The ensemble of pro- and anticoagulant factors determines thrombin generation in PPP, where a standard amount of procoagulant phospholipids is normally added. In PRP, thrombin generation is also dependent upon platelet function because platelets provide the necessary phospholipids, clotting factor V and, presumptively, clotting factor receptors.

Thrombin generation in PRP thus reflects the major part of the physiological clotting system. Ideally, the contribution of white and red cells and the vessel wall should be included, and flow conditions created. To date, this is not yet technically possible. In PRP, the vessel wall can be partly represented by adding TF and TM.

In the older literature thrombin generation is used both as a diagnostic test and as a tool in basic research [1–4, 18]. Its use in modern, large-scale, clinical, epidemiological or pharmacological studies is hampered by its extreme labor-intensiveness. With the conventional subsampling techniques, even when optimally streamlined [31], it takes about 1 h of a skilled technician to obtain one to four thrombograms. Therefore, we developed the test presented here, which allows a throughput of up to 48 single thrombin-time graphs per hour in an automated procedure.

In this test, the thrombin concentration in the sample is calculated from the conversion velocity of a slow-reacting fluorogenic thrombin substrate. The principle of this approach has been developed with chromogenic substrates [30]. Unlike optical density measurements, the fluorescent signal is not influenced by the turbidity of the emerging clot, so fibrinogen-containing plasma and PRP can be used.

With fluorogenic substrates, however, constant thrombin activity does not cause a constant increase in the output signal, and thus the first derivative of the signal produced does not directly reflect thrombin concentrations. As seen in figure 1, the velocity of the fluorescence increase diminishes as the level of fluorescence increases. It is also seen that (i) the initial velocities are proportional to the amount of enzyme added (figure 1B) and (ii) that the decrease in signal produc-

tion is a function of the level of fluorescence but not of time or of the thrombin activity per se (figure 1C). The two phenomena that cause this non-linearity are (i) non-linearity of fluorescence with the concentration of fluorogenic molecules [29] and (ii) substrate consumption.

In practice, the effect of substrate consumption – with any substrate – cannot be overcome by adding the substrate in excess. The more substrate is added, the more thrombin molecules are bound to it and thus protected from the action of plasmatic antithrombins. This not only prolongs the experiment beyond reasonable limits but also increases substrate consumption and consequently the inner filter effect.

Moreover, the substrate is a competitive inhibitor of physiological feedback reactions and thus may interfere with the development of prothrombinase: figure 2 shows that at the concentration of substrate used in our standard procedure, no significant inhibition of thrombin formation is observed.

The effect of non-linearity cannot be solved by comparing thrombin generation in an experimental sample to that in standard plasma. In an inhibited sample less product is formed than in the standard so substrate consumption and the inner filter effect are low and more signal will be obtained per unit of thrombin activity than in the control. Thus inhibition is underestimated. Inversely, in samples where the activity is higher than normal, the signal is more reduced by substrate-consumption and by the inner filter effect than in standard plasma so that higher activity is also underestimated. From the data in figure 1 we calculated the magnitude of such under-estimations, both as a percentage of the standard plasma and as a percentage of the actual value and found that they transgress acceptable limits as soon as the activity deviates significantly from that in the standard plasma (figure 8).

Figure 8. Under-estimation induced by comparison with a standard plasma

Underestimation induced by calculating inhibition or activation by comparison of the uncorrected ETP to that in a standard plasma. The thin line gives the deviation in percent of the standard plasma, the bold line gives the deviation in percent of the measured value.

Fibrinogen

In subsampling experiments, where the thrombin concentration in the fluid phase is determined, 30–50% more thrombin is found in defibrinated plasma than in plasma from which the fibrin has not been removed [32]. In fluorogenic experiments, significantly more thrombin activity is found in the presence of fibrin than in its absence (figure 3). Therefore, thrombin bound to fibrin must be co-estimated in the fluorogenic experiment. This is not without importance because it has been shown that thrombin bound to fibrin can activate factors V, VIII and XI as well as platelets [32]. Also, antithrombin (and hence heparins) is reported not to inhibit fibrin-bound thrombin, whereas heparin cofactor II (and thus dermatan sulfate) and direct thrombin inhibitors do [33–35]. Our previously reported study showing that in PRP the presence of fibrinogen is essential for normal thrombin generation [17] has been confirmed with the fluorogenic technique presented here (results not shown).

Another important effect of fibrin(ogen) is that it diminishes the amount of $\alpha 2M$ -T formed during the experiment (figure 3). The high end level attained in defibrinated plasma (or in the presence of polymerization inhibitors, results not shown) suggests that polymerizing fibrin prevents the interaction between thrombin and $\alpha 2M$. Therefore, in the presence of fibrin(ogen), correction for the $\alpha 2M$ -T end level is minimal, and the traces of amidolytic activity obtained during the experiment practically represent the course of free thrombin. This implies that the use of the $\alpha 2M$ -T end level as a means to estimate ETP [30, 15] is not only dependent on the level of $\alpha 2M$ in the plasma but also on the level of fibrinogen. For unknown reasons, the experimental error in defibrinated plasma is higher than in non-defibrinated plasma (figure 3A).

Standard Conditions

The form and magnitude of the thrombogram is dependent upon the trigger used. An excess of tissue factor (11 nM, as in a thromboplastin time estimation) will cause the mechanism of prothrombinase formation to take the extrinsic pathway, i.e. involve factors VII, V, X and TFPI. Decreasing the TF concentration changes the pathway of thrombin formation to include first factors VIII and IX and then factor XI [for a review, see ref. 37]. In the absence of any TF but with sufficient procoagulant phospholipids added, contact activation ('intrinsic pathway') will cause a burst of thrombin formation after ~10 min. Even though plastics are used throughout, contact activation cannot be entirely avoided unless blood is taken on corn trypsin inhibitor (CTI) containing citrate (results not shown). In samples with a high content of circulating TF [38], thrombin generation can be seen even in the presence of CTI (results to be published).

If in PPP the test is run without added phospholipids, thrombin generation becomes dependent upon non-sedimentable PL present in the plasma, i.e. upon the presence of circulating procoagulant microparticles, which in the first place helps to check pre-analytical conditions. Variable amounts of PL can be intro-

duced by the use of vacuum tubes, by insufficient centrifugation, by storing PRP, especially in the cold. Consequently, the conditions of centrifugation as given in the methods section should be strictly followed.

Thrombin generation is dependent upon the concentrations of PL and TF added. In the lower ranges, the relation is practically linear, to approach an upper limit at higher values. The concentrations chosen in our standard procedure are such that the upper ranges of thrombin generation are attained but not excessively. Thus, the method is insensitive to small experimental variations in trigger, whereas an excess of trigger is avoided to not overrule modest inhibitions. The number of platelets was chosen on the basis of the observation that between 0 and 75×10^9 platelets/l there is an almost linear increase in thrombin generation with increasing platelet count, which levels off subsequently to reach a plateau near 150×10^9 platelets/l. Higher platelet numbers hardly increase thrombin generation [18, 39], but thrombin generation is less sensitive to the influence of platelet-based mechanisms and their inhibitors.

Normal Values, Normal Effects of Addition of APC or TM

The normal values under our standard conditions are shown in table 2. Of note, the thrombin peak is much lower in PRP than in PPP, whereas the ETP is not significantly different. This is most likely explained by the fact that platelet procoagulant activity is gradually noted in the course of the activation process, whereas in PPP the added procoagulant phospholipids are present at recalcification, i.e. at zero time.

The inhibitory effect of APC and TM on the thrombin generation curve (table 3) is significantly diminished in dysfunctions of the APC system, such as the use of oral contraceptives or factor V_{Leiden} [to be published, 40]. A comparison of thrombin generation in the presence or absence of TM or APC enables to assess the function of the APC system even if the coagulation process is impaired. In figure 9, for example, thrombin generation is diminished by oral anticoagulation, which concomitantly affects the APC mechanism, resulting in a diminished response to the addition of TM. It also has been shown that in the presence of the lupus anticoagulant severe resistance to APC and TM is accompanied by a considerably prolonged lag time (clotting time) [16].

The experimental variation is within acceptable limits (table 2) and similar between the three instruments at two locations used in the experiments reported here. In our experience, the fluorescence intensity of an identical product solution (AMC) can differ severalfold between fluorometers, even if they are of the same type. This variation is compensated for by the use of the calibrator.

The variation in the population is much higher than the day-to-day variation per individual. Thus, some individuals generate more thrombin than others. A correlation between the clotting factor level and the occurrence of venous and/or arterial thrombotic disease has been reported for various clotting factors [41,

42], which strongly suggests a relationship between the thrombin generation capacity and thrombotic disease. Measurement of overall thrombin generation enables the assessment of simultaneous effects of numerous small changes, which may either reinforce each other (e.g. in pregnancy [43]) or compensate each other (e.g. a bleeding tendency and factor V_{Leiden} [44–46]).

Figure 9. Thrombogram in a patient under stable oral anticoagulant treatment (INR 2.5).
A. PPP. B. PRP. + = Normal plasma: top curve (black) without TM, lower curve (gray) with TM; full line = patient plasma: top curve (black) without TM, lower curve (gray) with TM. Concentrations as in figure 7. Time axis truncated at $t = 30$.

Hemorrhagic diatheses

It has been shown before [for a review, see ref. 5] that thrombin generation is diminished in haemophilia and rare clotting disorders [6–8]. Also, in PRP, it is diminished if von Willebrand factor is low [17] and increases upon DDAVP treatment [7]. As can be seen from figure 4 and 6, calibrated automated thrombography shows these effects as well as subsampling techniques. In an accompanying article by Turecek et al. [47] it is shown that in haemophiliacs, the effect of inhibitor bypassing therapy can be seen in uncalibrated fluorogenic experiments, thus enabling the quantification by calibrated automated thrombography.

Anticoagulant Treatment

We hypothesize that in anticoagulant therapy diminished thrombin generation is of the essence. All anticoagulant drugs have this effect in common although they may achieve it in completely different ways: decreased prothrombin and prothrombin conversion (AVK), increased thrombin inactivation (heparin) or direct inhibition (hirudin, argatroban and melagatran). There is no single clotting test that reacts similarly to these different types of anticoagulants. Clotting tests measure the length of the initiation stage of thrombin formation, i.e. the

time during which no significant amount of thrombin (<5 nM) is formed. Clotting occurs at the end of this initiation phase and is complete when $>95\%$ of all thrombin is still to appear. That the amount of thrombin formed in the clot is essential for thrombosis and haemostasis can be derived from comparisons of laboratory and clinical observations. Heparin, for example, has a minimal effect on the TF-induced clotting time ('quick time') but has an undeniable antithrombotic effect. Defects in the protein C system have a recognized prothrombotic effect; nevertheless this system only comes into action after APC is formed by the TM-thrombin complex, i.e. in the later stages of thrombin formation, long after the clot has formed. In figures 7 and 9, the effect of added TM is seen in the second half of the thrombin generation curve. In fact the use of clotting times is restricted to those cases where the length of the initiation phase indicates the amount of thrombin to come, which is far from being a general rule.

Some observations that are liable to be of clinical importance will readily escape our attention unless thrombin generation curves are made. It is logical, for example, that AVK treatment impairs the function of the APC system as well as of the clotting system. A net anticoagulant effect must remain to explain the antithrombotic effect, but assessment of the combined effect remained difficult. In figure 9 it is not only seen that TM resistance accompanies oral anticoagulation, the effect can also be quantified.

Another example is seen in figure 5. Thrombin generation in PRP is far less influenced by heparin than thrombin generation in PPP. This must be ascribed to the effect of platelet factor 4 [31]. We recently demonstrated that in PPP all heparins have the same mode of action, independent of their molecular weight. Nevertheless, there may be important molecular-weight-dependent differences among heparins when tested in PRP – and hence in vivo.

As a third example (not illustrated here), we mention that there are essential differences between irreversible (hirudin) and reversible (argatroban and melagatran) direct thrombin inhibitors in that the first act primarily on the lag time, but the second also have a strong inhibitory effect on the thrombin peak.

Alternative Methods Depending on Clot Formation

Several methods exist that measure the moment of clot formation or changes in the mechanical or optical properties of the clot, such as thrombelastography [48], resonance thrombography [49], roTEG coagulation analysis [50], the sonoclot analyzer [51], measurement of platelet-mediated force development during clot formation [52] and free oscillation rheometry [53]. The results of such methods sometimes show a superficial similarity to thrombin generation curves [54]. These methods can often be applied to whole blood, which is an important practical advantage.

The disadvantage of clot-based methods is that they depend upon thrombin formation as well as on properties such as fibrinogen content and quality, fibrin polymerization, crosslinking and fibrinolysis [50]. These two aspects may coun-

teract each other, e.g. in a febrile patient (high fibrinogen) under anticoagulant or other antithrombotic treatment (e.g. aspirin). Clot formation is only one of the many functions of thrombin and not necessarily the most important one, as can be judged from the mild bleeding syndrome in hypofibrinogenaemia. Through selection of the right substrate and comparing the fluorescent signal to that of a calibrator with fixed activity we can obtain a correct estimate of the course of thrombin concentration in clotting plasma. Fibrinogen as a substrate is quickly exhausted and the signal from its product – i.e. one or the other mechanical or optical property – cannot be unequivocally related to the amount of thrombin formed.

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CHAPTER II

53. Ramstrom S, Ranby M, Lindahl TL: *The role of platelets in blood coagulation – Effects of platelet agonists and GPIIb/IIIa inhibitors studied by free oscillation rheometry*. Thromb Res 2002;105:165–172.
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Chapter III

Mathematical procedure to obtain the thrombin generation curve starting from raw data (time course of fluorescent signal).

This chapter is based on a manuscript in preparation that will be submitted to Computers in Biology and Medicine:

Mathematical procedure to obtain the thrombin generation curve starting from raw data.

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Introduction

In the introduction of this thesis we discussed the relevance of measuring TG and in chapter 2 we presented the CAT-method as a practical solution for measuring TG in plasma. We did not yet discuss the mathematical procedures required to convert raw data (fluorescent signal versus time) into TG curves. In the CAT-method as it is available on the market these procedures are part of the software that is not accessible to the client. Furthermore, these procedures are continuously being adapted in order to be able to convert raw data in correct TG-curves in an automated way as efficiently as possible what results in new versions of the software.

Since it is important for the investigator to understand what is behind the software and to give him the opportunity to calculate a TG-curve manually if the software does not succeed a procedure is presented in this chapter of how to calculate a TG-curve starting from the raw data. This procedure is not necessarily the same as in the software, but it is accurate and can be applied on any raw data.

To calculate a TG-curve is not as simple as it might seem. It does not suffice to take the derivative of the raw TG-data and to transform these data with a fixed calibration factor obtained from the initial rate of fluorescence production by a known thrombin activity. In any fluorogenic method, TG is monitored via added fluorogenic substrate and the thrombin generated converts this substrate into a product that gives a fluorescent signal which is recorded in time. In an ideal situation the rate of fluorescence increase would be proportional to the enzyme-concentration during the entire process. In an actual experiment this proportionality is disturbed by substrate-consumption and by the inner-filter effect. This means that the raw data have to be corrected for the influences of these two phenomena and this is one of the reasons why continuous calibration is required (see also chapter 2 and chapter 5).

Another complication is that the signal generated in clotting plasma is the result of both the transient activity of free (α -)thrombin and the permanent activity of α 2M-T. For the TG curve we only need the course of free thrombin, so the signal generated by α 2M-T has to be subtracted from the total signal. This is done by a mathematical procedure that requires that the moment can be estimated at which free thrombin no longer contributes to the over-all activity, i.e. when only the activity of α 2M-T is present. We here describe a procedure to determine objectively this end of the TG curve ('end of tail').

Methods and materials

Thrombin generation was carried out in the presence of 416 μM ZGGR-AMC in a pool of normal platelet poor plasma using 5 pM TF and 4 μM PL as described before (chapter 2 or [1]). In parallel the fluorescence was measured generated by 200 nM of bovine $\alpha 2\text{M-T}$ (calibrator) in plasma in which no thrombin generation was triggered. All measurements were carried out in quadruplicate. The raw fluorescence data from the TG experiment and from the calibrator experiment were exported to SIGMAPLOT version 9.0 (Systat Software Inc., Point Richmond, CA, USA) for further mathematical operations.

Calculation of the TG curve from raw data

Correction for substrate-depletion and inner-filter effect

Both the calibrator- and the TG-data are influenced by substrate-depletion and inner-filter effect to an extent that is only dependent upon the amount of substrate converted, i.e. to the amount of product formed, i.e. to the level of fluorescence measured. Therefore we first determine the corrections that have to be carried out on the measured calibrator curve so as to produce the straight line that would be obtained if substrate depletion and the inner filter effect would not play a role. Next, these corrections will be applied to the TG-data and this will render the “ideal” course of fluorescence, i.e. the fluorescence curve that would be produced if the disturbing phenomena would not occur. In figure 1 the mean raw data are shown of the calibrator wells (gray curve) and the TG-wells (thick black line). The raw calibrator data are fitted with equation i (thin black line in the gray curve).

$$y = y_0 + at + bt^2 + ct^3 + dt^4 + et^5 + gt^6 \quad (\text{i})$$

This is a polynomial of the sixth order in which y is the fluorescent signal, t is time, y_0 the fluorescent signal at time 0 and a , b , c , d , e and g constants that are found by fitting equation (i) to the experimental data. The first terms of this equation ($y = y_0 + at$) gives a straight line which is the tangent of the curve at time 0 (dotted line in figure 1), i.e. the “ideal” calibrator curve that would have been measured in the absence of substrate depletion and inner filter effect.

Figure 1. Conversion of raw data into ideal raw data.

The reaction mixture contained normal platelet poor plasma in a 2/3 dilution, calibrator with an equivalent activity of 100 nM human thrombin, 16.7 mM CaCl_2 and 417 μM ZGGR-AMC. The gray curve shows the measured raw data of the calibrator and the thin black line gives the fit using equation (i). The dotted straight line represents the ideal calibrator data after correction. The continuous thick black line and the short dash thick black line represent respectively the measured and the ideal raw TG data.

Figure 2. Relation between measured and ideal raw data

Plot of ideal raw data against measured raw data of the calibrator of figure 1 (gray curve). The thin black line in the gray curve is the fit using equation (ii).

In a next step, the measured raw data and the ideal raw data of the calibrator are plotted against each other (figure 2, gray curve) and this plot is fitted with equation ii (thin black line in the gray curve).

$$y = y_0 + ax + bx^2 + cx^3 + dx^4 + ex^5 + gx^6 + hx^7 + ix^8 + jx^9 + kx^{10} \quad (ii)$$

This is a polynomial of the tenth order in which x is the measured fluorescent signal and y is the ideal fluorescent signal and a, b, c, d, e, g, h, i, j and k are constants. Using equation (ii) and the constants of this fit the measured TG-data (x) can now be converted into ideal data (y) (figure 1, thick black short dash line).

Determination of the thrombin generation curve

*From fluorescent signal to [thrombin]*time (progress-curve)*

These corrected TG-data are still expressed as fluorescent signal against time and so these data are multiplied with a calibration factor. This calibration factor is obtained from the calibrator concentration divided by the initial slope of the experimental calibrator line (constant a of equation (i)), which is the same as the slope of the corrected calibrator data. The calibrator concentration (~ 200 nM α_2 M-T) is here expressed as thrombin concentration (100 nM thrombin) (see also [1]). As such, the fluorescent signal is converted into the progress variable: product of thrombin concentration and time ([Thrombin]*time). The obtained progress curve (figure 3, gray circles) represents the workload or the man-hours of thrombin (nM.min) responsible for the generated fluorescent signal.

Fit procedure

The progress-curve in figure 3 is not the result of thrombin only but is built up by two enzymes: the man-hours of transient present thrombin and the man-hours of ongoing present α_2 M-T. Both enzymes hydrolyze ZGGR-AMC. We want to know the course of free thrombin, so the progress-curve has to be split mathematically in its thrombin-related part and its α_2 M-T-related part. As shown in [2] this can also be done after one has taken the first derivative of the progress-curve, but this necessarily introduces inconvenient experimental scatter. We therefore prefer to first fit the experimental data (progress-curve) to a mathematical equation and to continue with this fit for further calculations. The essence of the fit procedure is described in [3]. In this article the W-function is presented which fits TG curves to a four parameter equation.

$$T = W(a, b, c, t) = a \cdot b \cdot c \cdot e^{(-b \cdot c \cdot t)} \cdot (e^{(b \cdot t)} - 1)^{(c-1)} \quad (iii)$$

In which T is thrombin concentration, t is time and a, b and c are constants (> 0). (In some cases the sum of two W-functions is required for a good fit, which does not change the principle of the procedure however.)

Figure 3. Fit on the product-curve (thrombin-minutes against time)

The gray circles show the progress curve, the ideal TG-data of figure 1 after they were converted into $[\text{thrombin}] \cdot \text{time}$. This curve is fitted with equation (vi) (thin black line).

This original W-function describes only the course of free thrombin in time. The $\alpha 2\text{M-T}$ part is not included. The concentration curve of $\alpha 2\text{M-T}$ (concentration of $\alpha 2\text{M-T}$ against time) has the shape of a sigmoid and $\alpha 2\text{M-T}$ forms with a velocity proportional to the concentration of thrombin [2]. The concentration of $\alpha 2\text{M-thrombin}$ therefore raises proportional with the integral of the W-function. Equation (iv) describes the course of free thrombin and $\alpha 2\text{M-T}$ in time:

$$AA = W(a, b, c, t) + k \cdot \int (W(a, b, c, t)) \quad (\text{iv})$$

In which AA is the sum of the free thrombin and $\alpha 2\text{M-T}$ concentration and k is an additional constant.

The progress-curve in figure 3 which represents the man-hours of thrombin and $\alpha 2\text{M-T}$ can now be fitted with the integral of equation (iv), i.e.

$$P = \int (W(a, b, c, t) + k \cdot \int (W(a, b, c, t)))$$

Substituting the W function into this equation gives:

$$P = a \cdot (1 - e^{(-b \cdot t)})^c + a \cdot k \cdot \sum ((1 - e^{(-b \cdot t)})^c) / s \quad (v)$$

In which P is the sum of [thrombin]*time and [α 2M-T]*time or the total man-hours of thrombin and α 2M-T, t is the time and a, b, c and k are constants; s is the time between each subsequent reading in the fluorometer.

Equation (v) makes it possible to fit most of the progress-curves but not all. It was already mentioned that in ref [3] in some cases (e.g. in PRP) it was necessary to fit with two waves instead of one (two W-functions with two sets of constants). We also found conditions (Von Willebrand patients) which had to be fitted with three waves (results not yet published). More waves however were not found to be necessary. Therefore it was decided to fit progress-curves with a three wave-function (equation vi) as is shown in figure 3 (thin black line in the gray curve).

$$P = a \cdot (1 - e^{(-b \cdot t)})^c + a \cdot K \cdot \sum ((1 - e^{(-b \cdot t)})^c) / s \\ + d \cdot (1 - e^{(-e \cdot t)})^g + d \cdot K \cdot \sum ((1 - e^{(-e \cdot t)})^g) / s \\ + h \cdot (1 - e^{(-i \cdot t)})^j + h \cdot K \cdot \sum ((1 - e^{(-i \cdot t)})^j) / s \quad (vi)$$

In which P is the sum of [thrombin]*time and [α 2M-T]*time or the total man-hours of thrombin and α 2M-T; t is the time; $K = k/(a+d+h)$; a, b, c, d, e, g, h, i, j and k are constants; s is the time between each subsequent reading in the fluorometer.

Determination of the end of tail

At the end of a thrombin generation experiment no free thrombin is left and only a fixed amount of α 2M-thrombin is left. Therefore the corrected progress-curve should increase linearly once thrombin generation is over. In practice this part of the curve sometimes shows slight deviations from a straight line, which can affect the outcome of the fitting procedure. Especially in those cases where the “straight” part is very long, slight deviations from a straight line can lead to rather big changes in the found ETP. The other parameters - peak height, lag-time and time to peak - are hardly affected by this phenomenon. This poses the question how the end-point of the fit i.e. the ‘end of tail’ can be determined correctly. To objectify the fitting procedure we designed the following protocol.

First the corrected progress curve is fitted with equation (vi) (thin black line in figure 3) and the derivative of the fit is determined. The part of the derivative after the peak is a sigmoid that starts high and decreases to a level determined by the residual α 2M-T activity. The difference between peak and end-level we call Δ . Then the times (t_{75} , t_{25}) are determined at which the value of the curve is 75% and 25% of Δ above the end level. The difference between the two time points ($\delta = (t_{25} - t_{75})$) is taken as an estimate of the half-life time of thrombin in-activation. Because thrombin generation probably is still ongoing it can be an

overestimation but not an underestimation. The TG curve is considered to be finished at $7 \times \delta$ after t_{75} . This time point we call 'end of tail'. The amount of thrombin left then is less than 1% of the amount present at the t_{75} time. This procedure was applied on the data of figure 3 and it was found that TG was completed after 36 minutes. Next the fit procedure with equation (vi) was repeated taking only time points between 0 and 36 minutes into account. This fit is shown in figure 4.

Correction for $\alpha 2M$ -T activity

Now that the end of tail is determined and the progress curve is fitted, it can be divided in its two partaking enzymes: thrombin and $\alpha 2M$ -T (figure 4). This involves splitting formula (vi) in its two constituent parts, i.e. the right parts represent the contribution of $\alpha 2M$ -T to substrate hydrolysis (short dash thick black line), while the left parts represent the hydrolysis of the substrate by thrombin (continuous thick black line).

The constant end-level due to the activity of free thrombin (continuous thick black line) is a good estimate of the ETP. The ETP is the surface under the thrombin generation curve and can also be calculated as the sum of a , d and h (constants of equation (vi)). If the final slope of the $\alpha 2M$ -T activity curve is calculated this gives the $\alpha 2M$ -T end-concentration which equals the constant k of equation (vi) (or K times the ETP). So, the final $\alpha 2M$ -T concentration is proportional to the developed thrombin activity or ETP.

Figure 4. Splitting the corrected progress curve into the parts due to free thrombin and to $\alpha 2M$ -T.

The gray circles show the corrected progress curve of figure 3. The thin black line in the gray curve is the fitted curve (see text). The continuous thick black line shows the contribution of free thrombin and the short dash (thick black) line that of $\alpha 2M$ -T.

Calculation of the thrombin generation curve

The first derivative of the contribution of free-thrombin to the corrected progress curve (continuous thick black line in figure 4) gives the TG curve. Instead of taking the derivative of the calculated data (based on the fit) it is also possible to take the derivative of the measured data of the progress-curve (and then subtract the $\alpha 2\text{M-T}$ -part). This derivative however has a tendency to scatter widely, especially towards the end of the curve, because its calculation involves the measurement of small differences between large fluorescence values. We therefore prefer to use the first derivative of the fitted curve, which can be constructed because from the fitting procedure the constants that describe this curve are known. In the case of the example shown in figure 5, where the fitting was done on the basis of the sum of three W-functions, this amounts to constructing the curve:

$$T = d(a \cdot (1 - e^{(-b \cdot t)})^c + d \cdot (1 - e^{(-e \cdot t)})^g + h \cdot (1 - e^{(-i \cdot t)})^j) / dt \cdot s \quad (\text{vii})$$

in which T is thrombin concentration, t is time, s is the time in between two readings in the fluorometer. It is the derivative of equation (vi), but without the $\alpha 2\text{M-T}$ -parts. To be able to determine the parameters of the TG curve with sufficient accuracy the TG curve was calculated in steps of 0.02 min.

Figure 5. Thrombin generation curve

The open circles show the derivative of the measured progress curve of figure 4 (grey curve) after correction for $\alpha 2\text{M-T}$ activity. The black line is calculated with the mathematical description of equation (vii) using the constants of the fit in figure 4 (equation vi).

From figure 5 it is clear that curve fitting to the corrected progress curve avoids the scattering inherent to working with the first derivative of experimental val-

ues. This is the more important when the concentration of the formed thrombin is low. Since the noise is always of the same order, the signal to noise ratio decreases in this case. This is illustrated in figure 6. In this figure thrombin generation is measured in PRP of a Von Willebrand patient. Although there is low noise in the measured progress curve, this noise is blown up enormously when taking the derivative.

Figure 6. Thrombin generation curve of a Von Willebrand patient in PRP

The upper figure shows the measured progress curve corrected for the $\alpha 2\text{M-T}$ activity (gray symbols) and the three wave fit (thin black line in the gray curve). The lower figure shows the derivative of the two curves shown in the upper figure. The open circles show the measured data and the black line the fit.

Parameters of the thrombin generation curve

The fit of figure 5 is used to calculate the parameters of the thrombin generation curve: ETP, lag-time, time to peak and peak-height. The ETP is the sum of the constants a , d and h and represents the area under the TG curve (nM.min). The lag-time is defined as the time at which the thrombin concentration reaches 10 nM. Both the lag-time, the time to peak and the peak-height have to be read from the fit, because they cannot be determined mathematically.

Discussion and conclusion

It is shown how two fitting-procedures are needed for the conversion of the raw data into a thrombin generation curve. First the experimental fluorescence data obtained with the calibrator have to be fitted, so that it is possible to convert them into ideal fluorescence data, i.e. data that would have been obtained if substrate consumption and inner filter effect would not occur. The same procedure is then applied to the experimental data from the parallel thrombin generation experiment. In other words, the fluorescence data from the thrombin generation experiment are transformed into “ideal” data.

The second fit-procedure is needed to split the progress-curve into its thrombin-part and α_2 M-T-part. By fitting the progress-curve we avoid the large experimental scatter that arises from working with the first derivative of experimental data. Instead of first determining the first derivative of the ‘ideal’ experimental data and then mathematically split it into its thrombin-part and α_2 M-T-part, we first fit the ‘ideal’ experimental progress curve and then carry out the calculations on the fitted curve.

The fit on the progress-curve is based on the W-function, i.e. a four parameter function that describes a simple thrombin generation curve (free thrombin only). The course of free-thrombin- and α_2 M-T-concentration in time (the AA-function) is obtained by adding to the W-function a curve that is proportional to its own integral, thus introducing an extra parameter. The integral of the AA-function then represents the progress-curve (P-function). In this way a theoretical function is obtained to which the ‘ideal’ progress curve is fitted.

With the constants found, the W-function, i.e. the thrombin generation curve is then constructed. In practice it is sometimes required to use the sum of two or three W-functions to obtain an acceptable fit. This does not change the principle of the approach. It does raise the number of fitting parameters to a maximum of ten which is still small compared to the number of measuring points (> 120).

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Chapter IV

Mathematical procedure to calculate prothrombinase-activity during thrombin generation.

This chapter is based on a manuscript in preparation that will be submitted to Computers in Biology and Medicine:

Mathematical procedure to calculate prothrombinase-activity during thrombin generation.

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Introduction

In the previous chapter it was shown how thrombin generation curves can be obtained from raw data. The amount of thrombin formed during the clotting process is - roughly spoken - dependent on three parameters, the prothrombin concentration, the activity of plasmatic antithrombins and the activity of the prothrombinase complex (FII-ase). The FII-ase activity is the final step in the formation of thrombin and is dependent on the components of the FII-ase complex: factor Xa, factor Va and phospholipids, which in their turn are dependent upon the function of the other clotting factors and regulatory systems and the platelets. The FII-ase activity therefore reflects the activity of the clotting system but not that of thrombin breakdown. In this chapter it is shown how the time-course of FII-ase activity during thrombin generation can be calculated starting from the thrombin generation curve if the initial concentration of AT is known.

Methods and materials

Thrombin generation was measured in quadruplicate in a pool of normal platelet poor plasma using 5 pM TF and 4 μ M PL according to [1]. The TG curve, lag-time, time to peak, peak height and ETP were obtained by mathematical operation of the raw data as described in chapter 3. Further mathematical operations for the calculation of the FII-ase curve were carried out in SIGMAPLOT version 9.0 (Systat Software Inc., Point Richmond, CA, USA).

Human AT in plasma was determined by adding a fixed amount of thrombin and heparin to a dilution series of plasma. The remaining thrombin-concentration after 10 min at 37 °C was measured in each dilution with the chromogenic substrate S2238 and these values were plotted against the amount of plasma in each dilution (μ l plasma). The slope of this linear curve gives the concentration of thrombin that has been inactivated per μ l plasma. This corresponds with the concentration of AT present in this μ l plasma.

The k_{dec} of the thrombin-AT reaction was determined in a reaction mixture that contains purified AT, a chromogenic substrate and the reaction is started by addition of thrombin. By using the formula $k_{dec} = (k_{cat} \cdot S) / (K_m \cdot P_{end}) \cdot \ln[A / (A - E)]$ the k_{dec} can then be calculated (S = substrate concentration, P_{end} = end-level hydrolyzed chromogenic substrate, A = AT concentration and E = enzyme (thrombin) concentration).

Calculation of the prothrombinase activity

Principle

To determine the FII-ase activity we first calculate the amount of thrombin generated by FII-ase at each time-point during thrombin generation. This is the course of (total) thrombin concentration against time. The derivative of this curve gives then the FII-ase activity or the thrombin forming velocity (nM/min) at each time-point during thrombin generation.

Thrombin generation in plasma is the sum of two processes, thrombin formation by the action of FII-ase and thrombin inactivation by plasmatic inhibitors. To be able to calculate the amount of thrombin generated by FII-ase at each time-point we need to know the amount of thrombin-inhibitor complex formed at each moment besides the not yet inactivated thrombin (TG-curve). The TG curve is obtained as described in chapter 3. The course of the thrombin-inhibitor-complexes we still have to calculate. The most important inhibitor is AT while α_2 M and α_1 -antitrypsin play a minor role in the inhibition-process.

Course of [TAT] against time (TAT-curve)

The course of [thrombin-AT] or [TAT] in time can be calculated by numerical integration because the increment over the time interval between two readings of the thrombin generation curve can be calculated. If s is the time interval between two readings, than the increment is

$$\Delta[\text{TAT}]_t = s \cdot k_{\text{dec}} \cdot [\text{AT}]_t \cdot [\text{T}]_t$$

So to construct the course of [TAT] in time, three parameters are required: the decay constant of the thrombin-AT reaction (k_{dec}), the initial AT concentration in the plasma ($[\text{AT}_0]$) and the thrombin concentration at each time point ($[\text{T}]$). The decay constant of the thrombin-AT reaction was found to be 12000 (M.s)^{-1} (see methods). Also $[\text{AT}_0]$ was determined independently (see methods). In the plasma we used it was found to be $2.15 \text{ }\mu\text{M}$. The thrombin concentration can be read from the TG-curve (see chapter 3). So in principle the amount of formed TAT can be calculated step by step, essentially in the same way as done earlier for the subsampling method [2, 3].

However, there is a complication due to the presence of the thrombin substrate in the reaction mixture. This makes that part of the thrombin is bound to the substrate and thus unavailable for reaction with AT and other plasmatic inhibitors. The free fraction of thrombin follows from Michaelis-Menten kinetics and is $[\text{T}] \cdot K_m / (K_m + [\text{S}])$, in which $[\text{T}]$ is the thrombin-concentration, K_m the Michaelis constant ($251 \text{ }\mu\text{M}$) and $[\text{S}]$ the substrate concentration. It follows that the more S is present, the slower thrombin is inactivated by AT. We also have to take into account that $[\text{S}]$, and consequently $K_m / (K_m + [\text{S}])$, is not constant be-

cause the substrate is consumed. To calculate, numerically, the course of [S] as it develops in time we cannot use time as a progress variable because the enzyme concentration changes in time. As a progress variable we therefore use the product of time and enzyme concentration ($[T] \cdot \text{time}$). This variable represents the “man-hours” of thrombin as we discussed in chapter 3. As the TG-curve approaches its end, this progress variable approaches the ETP. The course of product (hydrolyzed S) can be calculated stepwise via the formula

$$\Delta P_t = ([T]_t \cdot \Delta t) \cdot k_{\text{cat}} \cdot ([S] - P_t) / (K_m + [S] - P_t)$$

In which ΔP_t is the amount of substrate hydrolyzed during a time interval of Δt at the prevailing enzyme concentration $[T]_t$. P_t then is $\sum_1^n (\Delta P_t)$, where n is the number of the interval at time point t .

In figure 1 we calculate the remaining substrate as a function of the progress variable ($[T] \cdot \text{time}$) in a model in which E is fixed at 100 nM human thrombin, $[S]$ is 417 μM , Δt is 1 second, $K_m = 251 \mu\text{M}$ and $k_{\text{cat}} = 2.08 \text{ s}^{-1}$, i.e. under the conditions of a TG-experiment.

Figure 1. Progress of substrate consumption at fixed enzyme activity.

The residual amount of ZGRR-AMC and the progress-curve (thrombin*time) were numerically calculated in steps of 1 second in a mixture containing 100 nM human thrombin and 417 μM ZGGR-AMC. The gray curve shows the calculated residual substrate versus the progress variable thrombin*time and the thin black line through the gray curve is a 5th order polynomial fit. The fit constants were: 4.1693e-4 (y0); -7.7613e-8 (a); 2.4622e-12 (b); 2.3357e-16 (c); -2.6218e-21 (d); -8.1652e-25 (e).

This curve can be fitted to a polynomial of the 5th order (figure 1, thin black line in the gray curve)

$$S = S_0 + a.x + b.x^2 + c.x^3 + d.x^4 + e.x^5$$

In which S is the remaining [S], S_0 is the initial [S], x is the progress-variable [T]*time and a, b, c, d and e are constants (see figure 1). As such we obtain the relation between the remaining substrate S and the progress variable [T]*time.

For any given raw TG-data (fluorescent signal versus time) we can calculate the progress variable, [T]*time, as a function of time (see chapter 3) and hence, with the aid of the above formula the residual substrate can be calculated at any moment and hence the fraction of free thrombin at any moment.

This enables us to calculate step by step the course of [TAT] in time. We can calculate the increment of [TAT] over any time-interval during the course of thrombin generation. If at moment t interval n starts (with a Δt), than

$$\Delta TAT_n = \Delta t \cdot k_{dec} \cdot [T_t] \cdot K_m / (K_m + [S_t]) \cdot (AT_0 - TAT_t)$$

In which the subscript t indicates the value of the parameters at the time point t. The numerical integration is started at $t=0$ when no TAT is present. The resulting TAT-curve is shown in figure 2 (thin black curve). It gives the course of [TAT] in time.

Course of [α_2 M-T] against time (α_2 M-T-curve)

The progress-curve of the α_2 M-T complex during thrombin generation was already calculated in chapter 3 (figure 4, short dash thick black line). However, this curve is expressed as ‘man-hours’ thrombin, so we have to convert it into its α_2 M-T equivalent. The kinetic constants of human thrombin for ZGGR-AMC hydrolysis are: $K_m = 251 \mu\text{M}$ and $k_{cat} = 2.08 \text{ s}^{-1}$ while these constants of human α_2 M-T are: $K_m = 66.5 \mu\text{M}$ and $k_{cat} = 0.94 \text{ s}^{-1}$. The initial [S] is $417 \mu\text{M}$ and under these conditions the velocity of substrate-conversion of human thrombin is 1.604 times higher than those of human α_2 M-T. As a consequence we have to multiply the progress curve by 1.604 to get the ‘man-hours’ α_2 M-T responsible for the same amount of hydrolyzed substrate (fluorescent signal). The derivative of this curve gives [α_2 M-T] as a function of time.

Course of the α_1 -antitrypsin-thrombin complex in time.

The shape of the α_1 antitrypsin-T curve is of the same shape as the α_2 M-T curve, because both products are formed with a rate proportional to the thrombin concentration in plasma at each time point. The plasma concentration of this thrombin inhibitor is $22 \mu\text{M}$, so that, unlike the case of AT but like α_2 M (3.5

μM), we can neglect the decrease of the concentration through interaction with thrombin and use pseudo first order kinetics. The reaction constant of thrombin inactivation by α_1 -antitrypsin is about $250 (\text{M.s})^{-1}$ (see [4] figure 1). These parameters for $\alpha_2\text{M}$ are $3.5 \mu\text{M}$ and $1450 (\text{M.s})^{-1}$, respectively. This means that the total amount of thrombin bound to $\alpha_2\text{M} + \alpha_1\text{AT}$ is 2.1 times higher than the amount bound to $\alpha_2\text{M}$ alone. For that reason the $\alpha_2\text{M-T}$ -curve was multiplied with 2.1 giving the $\alpha_2\text{M-T} + \alpha_1$ -antitrypsin-T-curve. This curve is shown in figure 2 (thick black curve).

Figure 2. Thrombin concentration [T] against time

Short dash line: not yet inactivated [T] (TG-curve); Thin black curve: [T] bound to AT (TAT-curve); Thick black curve: [T] bound to $\alpha_2\text{M} + \alpha_1$ -antitrypsin ($\alpha_2\text{M-T} + \alpha_1$ -antitrypsin-T-curve); gray curve: total [T] (sum of the other three curves).

Calculation of FII-ase activity

The amount of prothrombin converted by FII-ase at each time-point during thrombin generation can now be calculated by taking the sum of the TG-curve, the TAT-curve and the $\alpha_2\text{M-T} + \alpha_1$ -antitrypsin-T-curve. This is shown in figure 2 (gray curve). It can be seen that the inactivation of thrombin by the plasmatic inhibitors begins immediately after thrombin formation starts. Initially thrombin formation is faster than thrombin inactivation, but later the second process becomes the larger one. This results in the rise and fall of thrombin in clotting plasma and a continuous increase of thrombin-inhibitor complexes until all thrombin is inactivated.

The course of prothrombin conversion velocity (FII-ase-curve) can now be calculated by taking the derivative of the total thrombin curve of figure 2. In figure 3 this is shown together with the derivative of the TAT-curve (TAT-forming velocity), the $\alpha_2\text{M-T} + \alpha_1\text{-antitrypsin-T}$ -curve ($\alpha_2\text{M-T} + \alpha_1\text{-antitrypsin-T}$ -forming velocity) and the TG-curve (thrombin-forming velocity). The sum of these last three curves gives again the FII-ase curve.

Figure 3. Thrombin forming velocity against time

Short dash black line: (not yet inactivated) thrombin forming velocity (dTG/dt); Thin black curve: TAT-forming velocity ($dTAT/dt$); Thick black curve: $\alpha_2\text{M-T} + \alpha_1\text{-antitrypsin-T}$ -forming velocity ($d(\alpha_2\text{M-T} + \alpha_1\text{-antitrypsin-T})/dt$); Continuous gray line: total thrombin forming velocity ($d(\text{total T})/dt$ or sum of the other three curves) = FII-ase curve; Short dash gray line: TG-curve ($[T]$ in nM).

Conclusion

A method has been developed to calculate the velocity of prothrombin conversion, independent of thrombin decay processes. It can be expected – but it remains to be demonstrated in practice – that this type of curve reflects the physiology of thrombin forming processes with more sensitivity than the thrombin generation curve itself.

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Chapter V

The necessity of continuous individual calibration in continuous fluorogenic measurement of the thrombin generation curve

Based on the article submitted to Thrombosis and Haemostasis
Assessment of Thrombin Generation with Fluorogenic Substrates. 1: Necessity of Continuous Individual Calibration.

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Summary

In fluorogenic thrombin generation (TG) experiments, the time course of thrombin is to be derived from the course of the concentration of the fluorescent product. Due to fluorescence quenching the signal from a fixed amount of product in plasma (2:3 diluted) is 70 ± 13 % of that in buffer. Calculating a calibration factor (CF) from the (initial) rate of fluorescence change (dF/dt) caused by a known amount of thrombin in buffer therefore causes significant underestimation of thrombin concentrations in plasma and adds >10% to experimental variability. Also, using a fixed CF does not account for the changes in CF that occur during the experiment due to substrate consumption and non-linearity of fluorescence with product concentration and thus distorts the form of the TG-curve. Continuous individual calibration (CIC), in which CF is determined in a parallel sample of the plasma under investigation and varies with the changing conditions during the experiment avoids these errors.

We determined the intra-individual coefficients of variation (CV) of the peak-height and ETP as obtained with CIC to those obtained with a fixed CF measured in buffer. With the fixed CF, CVs of these parameters varied between 18% and 49%; with CIC they lowered to 4–7%. We conclude that continuous comparison to a known thrombin activity in a parallel plasma sample is a necessary and sufficient condition for bringing the CV of TG-measurements into the range that is required for clinical application.

Introduction

Thrombin generation (TG) is undergoing a revival since it was recognized that it is a promising means of phenotyping the clotting system and that an over-all function test of the haemostatic-thrombotic system is a necessary complement to information on single factors and genes [1,2].

At this moment TG is being introduced in more and more clinical settings, notably in cases where traditional tests give no, uncertain or ambiguous results such as the monitoring of factor VIII-inhibitor bypassing therapy [3-5], the discrepancy between clotting factor levels and bleeding tendency in haemophilia [6] or in rare coagulation disorders [7], the monitoring of treatment by a mixture of different antithrombotics [8] or the anticoagulant effect of antiplatelet therapy [9]. Last but not least, thrombin generation appears to be a tool in the recognition of hypercoagulability. Thrombin generation is increased in patients at risk for venous thrombosis due to congenital inhibitor deficiencies [8, 10, 11] or acquired through the use of oral contraceptives [12, 13]. Thrombin generation is increased in the elderly [14, 15] and in patients with a tendency to recurrent venous thromboembolism [16, 17].

In all these fields the clinical usefulness of TG is being investigated and the above studies show promising results. Nevertheless the value of TG for clinical decision making remains to be demonstrated in larger studies. If it would be found to be of no clinical use in a given condition, then either that condition causes no change in TG or the method employed is not sensitive enough to detect existing changes. It therefore is essential that the experimental error is reduced to a minimum.

Among the techniques used to determine TG in a routine setting, those using fluorogenic substrates take a special place, because they allow to measure in non-defibrinated and platelet rich plasma [18, 19,3]. The problem then arises of how to relate the velocity of increase of the fluorescence intensity (dF/dt) to thrombin concentration.

It is a disadvantage of this technique that fluorescence intensity is not only dependent upon the concentration of the fluorophore (in our case AMC) but also upon the medium in which the fluorophore is present. The same amount of AMC causes a significant higher fluorescence in buffer than in plasma, because in plasma fluorescence is quenched. Further, the fluorescent product formed contributes to the quenching, the so called inner-filter effect. Therefore, a fixed thrombin activity in buffer will give an apparent initial reaction velocity that is higher than that in plasma. A second complication is that the relation between enzyme concentration and measured reaction velocity changes considerably during the experiment because of the innerfilter-effect and consumption of the substrate. Consequently a fixed enzyme activity causes a progressively lower increase of fluorescence. We therefore developed continuous individual calibration (CIC).

Upon the introduction of the fluorogenic TG essay, we calibrated by comparison to a known thrombin activity added to plasma in which antithrombin activity was destroyed by heating [18]. Later we used the α 2macroglobulin-thrombin complex (α 2M-T) which has persistent amidolytic activity that is insensitive to plasma antithrombins [19]. Practically this means that the activity of a fixed known amount of calibrator (α 2M-T) is measured in a parallel sample of the plasma under investigation and during the whole time course of the experiment. In this way at every level of fluorescence the appropriate calibration factor (CF) is available. This procedure thus compensates for the variations in quenching between plasmas and for the change in CF during the TG reaction. The drawback of CIC, evidently, is that it requires simultaneous measurement in two samples. With clinical application in mind, it therefore is a legitimate question to ask whether the precision brought by CIC is obligatory. This question is the more urgent since two different forms of the automated thrombin generation test are commercially available to the clinical laboratory, one of which (method A) uses a single sample and a fixed, buffer-based CF whereas the other (method B) uses CIC.

Materials and Methods

Chemicals

Z-Gly-Gly-Arg- aminomethylcoumadine (ZGGR-AMC) and aminomethylcoumadine (AMC) were obtained from Bachem (Switzerland). Phospholipids for the preparation of initiation reagent B (method B) were obtained from Avanti (USA). The chromogenic thrombin substrates were S2238 (Chromogenics, Mölndahl, Sweden) and DEMZGR-pNA (synthesised in house). All other chemicals were reagent grade and obtained commercially.

Solutions

The initiation reagents of method A (RC high and RC low) contain 71.6 pM tissue factor and a “high” or a “low” concentration of procoagulant phospholipids of unspecified composition and concentration. The initiation reagent B of method B consist of 30 pM relipidated recombinant human tissue factor, 24 μ M of a suspension of phosphatidyl serine (20 %) phosphatidyl ethanolamine (20 %) and phosphatidyl choline (60 %) in buffer, prepared as in ref 18.

The buffer for method A was from the provider and described as Hepes-NaCl-buffer containing 1% bovine serum albumin (BSA). For method B, two buffers are used. For dilution of reagents: 20 mM Hepes, 140 mM NaCl, 0.02% NaN₃, 5 mg/ml BSA at pH 7.35. For preparation of the fluorogenic substrate solution (Fluca): 20 mM Hepes, 0.02% NaN₃, 60 mg/ml BSA at pH 7.35.

The fluorogenic substrate solution for method A was from the provider and contained 1 mM ZGGR-AMC and 15 mM CaCl₂. The FluCa solution of method B contains ZGGR-AMC (2.5 mM) and CaCl₂ (100 mM) in buffer with 60 mg/ml BSA.

The calibrator solutions that came with the kit for method A (lots ST5500O and ST430PC) contained according to the label 865 and 768 nM thrombin. The calibrator solution for method B was obtained from Thrombinoscope (Maastricht, The Netherlands). After reconstitution of the lyophilized material with distilled water it consists of α 2M-thrombin with an activity that, according to the lable, is equal to that of 600 nM human thrombin in buffer.

Plasma:

Blood was obtained through antecubital venapuncture from consenting volunteers. Free flow or minimal suction is employed; vacuum containers are avoided. The blood is collected on 1:10 volume of sodium-citrate (0.13 M). The blood is centrifuged at 2900 g for 10 min at room temperature and the plasma is aspirated. This procedure is repeated once. The plasma is stored at -80 °C in 1 ml aliquots. Plasma samples for the determination of the intra-individual variation coefficient were taken, with informed consent, from volunteers in the placebo arm of a large phase 1 study. From each of six volunteers, twelve samples

were taken within 24 hours in a centre for pharmacological research. Plasma aliquots were prepared as described above and snap frozen in liquid nitrogen, stored at -80 °C until use and thawed at 37 °C for 15 minutes before analysis.

Chromogenic thrombin measurements

The calibrator activity on a slow chromogenic substrate in different plasmas was measured by adding to 80 μ l plasma 20 μ l of a 1200 nM solution of α 2macroglobulin-thrombin (Calibrator for method B equivalent to 600 nM thrombin) and 20 μ l DEMZZ (1.5 mM), so as to obtain a final concentration of 250 μ M substrate and the equivalent of 100 nM thrombin.

Thrombin generation measurement

Fluorescence intensities were measured in a 96-well plate fluorometer (Ascent Reader, Thermolabsystems, Helsinki, Finland) equipped with a 390/460 filter set (excitation/emission) and a dispenser was used for the addition of the substrate-calcium-solution at $t=0$. Round-bottom 96-well plates (Immulon 2HB) were used.

The method using buffer-calibration (method A) was that according to Varadi [4] commercialised as Technothrombin TGA, by Technoclone (Vienna, Austria). Calibration was carried out with the thrombin preparation provided at a series of concentrations (approximately 500, 250, 50 and 5 nM). According to the instructions the wells contained 40 μ l of thrombin dilution and 50 μ l ZGGR-AMC at 1 mM. It did not escape our attention that, if 10 μ L buffer would have been added as well, the substrate concentrations in the TG-experiment and the calibration experiment would have been identical and a 10% mistake in the reading of the thrombin concentrations from the calibrator curve would have been avoided; we faithfully followed the instructions, however. TG measurement wells contained 40 μ l plasma and 10 μ l initiation reagent A. The reaction was started by adding 50 μ l of the substrate- Ca^{++} solution. The end-concentrations thus were: plasma 2:5 diluted, substrate 500 μ M (TG-wells), tissue factor 7.16 pM, procoagulant phospholipids of unknown concentration and composition. The Ca^{++} -concentration after recalcification was 0.34 mM (7.5 mM added). The fluorescent traces were recorded as such and imported into the spreadsheet provided by the manufacturer for the calculation of the thrombin generation parameters.

The method using continuous individual calibration (method B) was that of Hemker [19] as commercialised by Thrombinoscope (Maastricht, The Netherlands) under the name Calibrated Automated Thrombinography (CAT). In this method each experiment consists of two sets of readings, one with a known fixed amount of thrombin activity i.e. the calibrator (CAL-wells) and the other in which thrombin generation occurs (TG-wells). By continuous comparison of the signal from the two wells the course of thrombin concentration is calculated in real time and displayed on the screen by a dedicated software program. TG-

wells contained 20 μ l initiation reagent B and 80 μ l plasma. CAL-wells were filled with 20 μ l calibrator and 80 μ l plasma. The reaction was started by adding, via the dispenser, 20 μ l of the FluCa solution to both the CAL- and TG-wells at $t=0$. The final concentrations for the TG-wells were: plasma 2:3 diluted, 416 μ M ZGGR-AMC, 5 pM recombinant tissue factor and 4 μ M procoagulant phospholipids. The Ca^{++} -concentration after recalcification was 4.8 mM (16.7 mM added). The same end-concentrations hold for the CAL-wells but then without tissue factor or procoagulant phospholipids but with the equivalent of 100 nM free human thrombin.

The raw fluorometer readings from method B (TG-wells) were also imported into a spreadsheet according to that used for method A for calculation of the parameters of the thrombogram using a fixed, buffer-based CF (see further method B2 in table 2).

Results and Discussion

The role of fluorescence quenching by plasma

In fig.1, left frame, two types of fluorescent traces are shown. Those obtained with a fixed amount of thrombin activity (200 nM of α 2MT, equivalent to 100 nM α thrombin) in buffer and in 2:3 and 2:5 diluted plasma and those caused by thrombin generation in plasma of these two dilutions. It is seen that the fixed activity gives a significantly higher velocity of fluorescence increase (dF/dt) in buffer than in plasma and that it is higher when plasma is more diluted. The calibration factor (CF) is calculated as $CF=100/(dF/dt)$ (dimension: nM.min per arbitrary unit of fluorescence). So CF calculated on basis of dF/dt in buffer is smaller than that on basis of dF/dt in plasma and thrombin concentrations in plasma will be underestimated if calculated with CF based on dF/dt in buffer (fig.1, right frame). When these plasmas are compared to a standard measured in the same plasma and in the same dilution as in the TG experiment this underestimation is avoided and significantly higher peaks are obtained (fig.1, right frame). Comparison of thrombin generation calculated with the appropriate CF in 2:3- and in 2:5 diluted plasma shows that dilution enhances the fluorescent signal but does not influence peak thrombin generation significantly (see also Chapter VIII).

In figure 2 it is seen that, when 100 nM thrombin activity is added to different plasma samples, the dF/dt is always considerably lower than that in buffer and varies significantly. The plasma samples used in figure 2 were obtained from the same individual in the course of 24 hour. This experiment was repeated in five more volunteers and table 1 shows the mean of five intra-individual averages and standard deviations.

Figure 1. Calibration against buffer and against an identical sample

Left frame: fluorescent traces.

Drawn line: Calibrator in buffer; ---: calibrator in plasma final dilution 2:5; Δ - Δ : calibrator in plasma final dilution 2:3; +-+: TG trace in plasma at final dilution 2:5; O-O: TG trace in plasma at final dilution 2:3.

Right frame: TG curves calculated from the data in the left frame.

Drawn line: plasma diluted 2:5 read against calibrator in buffer, Δ - Δ : plasma diluted 2:3 read against calibrator in buffer, +-+: TG trace in plasma diluted 2:5 read against calibrator in plasma diluted 2:5, O-O: TG trace in plasma diluted 2:3 read against calibrator in plasma diluted 2:3.

Figure 2. Intra-individual variation of reaction velocity in plasma compared to buffer

Fluorescent traces of 200 nM α 2M-T in buffer (top line) and in 12 plasma samples from the same individual, diluted 2:3 in buffer (other lines). The thick line represents the average of the 12 progress curves obtained in plasma. Upper horizontal line: End-level of the buffer trace; Middle horizontal line: average end-level of the plasma traces $\pm$ SD.

We discarded the data from one volunteer in which some samples showed evident haemolysis and that consequently showed even lower fluorescence and much higher variation than the others. It is seen that the velocities measured in plasma are less than $\sim 80\% \pm 10\%$ of that in buffer and that in the course of time they become still lower (see below). Consequently comparison to a fixed initial velocity measured in buffer underestimates thrombin concentrations by 10 – 50%, dependent upon the sample and the duration of the experiment. This variability necessarily adds to the variability of the concentration dependent parameters of the thrombogram that thus is increased by $>10\%$.

	Buffer	Plasma	Plasma
Dilution		2:3	2:5
N	24	5 x 12	5 x 12
t=0	100.0 $\pm$ 2.7	73.5 $\pm$ 12.5	82.0 $\pm$ 11.1
0-30 min	93.5 $\pm$ 1.4	64.6 $\pm$ 10.7	73.1 $\pm$ 9.2
t=30 min	76.3 $\pm$ 1.7	46.7 $\pm$ 7.5	55.4 $\pm$ 7.6

Table 1. Intra-individual variation of the velocities of fluorescence increase at fixed thrombin activity (200 nM α 2M-T)

All velocities are expressed as % of the initial velocity in buffer (average $\pm$ SD). Twelve PPP-samples from each of 5 volunteers. Means of the average values per volunteer.

We quantified the degree of quenching by measuring the signal caused by 70 μ M of AMC (the amount formed half way during a normal TG experiment) in these plasma samples (2:3 diluted) and in buffer. The fluorescent yield of each plasma was expressed as a percentage of that in buffer and the mean of the 5 intra-individual averages was found to be $70.4\% \pm 13.6\%$. This corresponds to the average value found for the initial velocity if expressed as a percentage of that in buffer (table 1, 2:3 diluted plasma). When we corrected each individual initial velocity for the effect of quenching, the average initial velocity increased to the level of that measured in buffer and the standard deviation was reduced ($104.4\% \pm 8.8\%$). That dF/dt at 100 nM thrombin activity is not the same in different reaction media (buffer/plasma) might in theory also be caused by the presence of modifiers of reaction velocity in the plasma-samples. We therefore determined the amidolytic activity (dOD/dt) of 200 nM of α 2M-thrombin on a chromogenic substrate (250 μ M DEMZ) in these plasma-samples (2:3 dilution) and expressed it as % of the value measured in buffer. The measured amidolytic

activity was somewhat smaller in plasma than in buffer (average $87.5 \% \pm 8.3\%$) but a correction procedure for each individual initial velocity (in 2:3 diluted plasma) on basis of the amidolytic activity in each plasma resulted in an average value which is still much lower than in buffer and in an increase of experimental variation ($84 \pm 17 \%$).

We conclude that variable quenching of fluorescence between plasma samples contributes significantly to the experimental variation in dF/dt at constant thrombin activity and therefore needs to be taken into account when dF/dt values are to be converted in thrombin concentrations. In line with this conclusion samples with observable haemolysis or icterus show a more pronounced underestimation and variability if calculated with a CF measured in buffer than “clean” samples do (results not shown).

Variance of the calibration factor during the experiment

In figs 1 and 2 and in table 1 it is seen that, at constant thrombin activity, the measured reaction velocity drops by about one third in the course of 30 min. This decrease is caused by substrate consumption in combination with the fact that the amount of fluorescent light emitted per μM AMC decreases as the AMC concentration increases (inner filter effect). Both effects are uniquely related to the amount of product formed and thereby to the measured fluorescence. Constant thrombin activity therefore will cause an increasingly lower dF/dt as the reaction proceeds.

Figure 3. Change of calibration factor with time and with the level of fluorescence
Left frame: CF as a function of time. Drawn black line: 100 nM $\alpha 2\text{M-T}$, dashed line: 200 nM $\alpha 2\text{M-T}$, dotted line: 400 nM $\alpha 2\text{M-T}$. Average of 12 experiments. Gray lines: the ± 2 SD values of the 200 nM $\alpha 2\text{M-T}$ trace.

Right frame: Same experiment as in the left frame, plot of CF against fluorescence intensity. Continuous line: 100 nM, dashed line: 200 nM, dotted line: 400 nM.

Consequently CF, i.e. $T/(dF/dt)$ (T = thrombin concentration), increases as the reaction proceeds and this increase is faster at higher calibrator concentrations (figure 3, left frame, CF plotted as a function of time). When plotted as a function of fluorescence, CF remains a unique function of the fluorescence level (figure 3, right frame).

Plasmas with a high thrombin generating capacity (ETP) will generate more fluorescence than plasmas with a low ETP. Therefore they will pass through a larger range of increasing CFs during the experiment than plasmas having a low thrombin generating capacity (figure 3, left frame). This makes that one cannot simply compensate for calibration errors by comparison to standard normal plasma. High activities would be underestimated in that way and low activities overestimated.

Substrate exhaustion

Due to substrate consumption the reaction rate at constant enzyme activity decreases during a TG experiment. In the limit, in plasmas with high thrombin generating capacity, substrate can drop to zero level. If this is not recognised it can be interpreted as zero thrombin activity. This is seen in figure 4 but can also be found in the literature [20].

Figure 4. Substrate exhaustion recognized and unrecognized

Normal plasma and normal plasma spiked with 1 μ M prothrombin and 2 μ M prothrombin were measured with method A (gray lines) and method B (black lines). In both methods 5 pM TF and 4 μ M PL was used as trigger. Left frame: fluorescent traces. Right frame: TG curves derived from the fluorescent traces of the left frame following the procedure of method A or B.

In the experiment of figure 4 we determined the fluorescent traces from a TG experiment in which normal plasma was spiked with prothrombin so as to obtain 1 and 2 μM in excess of the endogenous prothrombin (1.6 μM). Such high concentrations of prothrombin can be approached during factor VIII inhibitor bypassing therapy with prothrombin containing preparations [4]. The fluorescent traces were measured using as well method A as method B. In both methods 5 pM TF and 4 μM PL was used as trigger. The fluorescent traces were transformed into TG-curves following the recommended procedures of the manufacturers. Method A: with a fixed calibration constant obtained from activity measurements of thrombin in buffer and method B: by comparison to a continuously measured CF in a parallel plasma sample. From the fluorescent traces it is seen that at the highest prothrombin concentration fluorescence reaches an upper limit that must be ascribed to substrate exhaustion but that, in method A, is interpreted as zero thrombin activity. Zero amidolytic activity cannot be attained, however, because a considerable amount of $\alpha_2\text{M-T}$ complex has built up during the experiment. In a parallel experiment we found this to be equivalent to 50, 87 and 151 nM thrombin at the prothrombin concentrations used in the experiments of figure 4. In method B the software signals substrate exhaustion and the program stops calculating when it occurs (figure 4: right frame – higher curve).

Again it can be seen that buffer-calibration leads to underestimation. An additional disadvantage of calibration in buffer is that the steady end-level activity due to $\alpha_2\text{M-T}$ cannot be observed, so that its contribution to the amidolytic activity, as it develops during the experiment, cannot be calculated and hence no curve of the real thrombin concentrations can be obtained.

Experimental accuracy

From the above it is clear that CIC is required for accurate measurement of TG. The question remains whether this accuracy is of enough practical importance to justify the measurement in two samples simultaneously, as required for CIC. We therefore determined thrombin generation curves with the two commercially available methods. Method A uses a fixed CF, obtained from known thrombin activities measured in buffer, whereas method B uses CIC. Of method A both the “high” and the “low” phospholipid variant was tested. Method A1 uses RC high and method A2 uses RC low. Methods A and B differ not only in the manner of calibration but also in plasma dilution, tissue factor, phospholipid concentration and Ca^{++} concentration. In order to see what the influence is of the calibration method per se, we also calculated the parameters of the thrombogram from the raw data of method B using a fixed CF measured in buffer (method B2). As samples we used the same plasmas as those in table 1, i.e. 12 samples of each of six normal volunteers (and we again excluded the data from the one volunteer that showed haemolysis). For each volunteer we calculated

the average and the coefficient of variation of the twelve values and the results in table 2 are the mean of these five intra-individual averages and CVs.

	A1		A2		B1		B2	
	Average ± SD	CV (%)	Average ± SD	CV (%)	Average ± SD	CV (%)	Average ± SD	CV (%)
ETP (nM.min)	2332 ± 345	18	2385 ± 412	21	1976 ± 112	6	1389 ± 260	20
Peak (nM)	208 ± 50	29	171 ± 40	26	413 ± 14	4	244 ± 42	18
Slope (nM/min)	51 ± 20	49	37 ± 15	43	288 ± 19	7	179 ± 30	18
Lag-time (min)	22.6 ± 4.7	21	23.4 ± 4.7	18	2.8 ± 0.2	6	2.7 ± 0.2	7
TTP (min)	31.8 ± 5.6	18	31.7 ± 5.7	17	4.8 ± 0.3	5	4.5 ± 0.2	5

Table 2. Intra-individual coefficients of variation of the parameters of the thrombogram.

Influence of the method of calibration.

Method A1: fixed CF in buffer with RC high

Method A2: fixed CF in buffer with RC low

Method B1: CIC

Method B2: raw data of method B1 recalculated with a fixed CF in buffer

In order to be able to compare the results we expressed all thrombin concentrations as final concentrations in the wells as is done in method B1. This means that we recalculated the results obtained with the worksheet provided by the manufacturer of method A since the concentrations of thrombin obtained with this worksheet are concentrations in plasma. Obviously, the manufacturer of method A takes into account the dilution of plasma that is used and supposes that the concentration of thrombin formed in diluted plasma decreases proportionally with the dilution. As we already saw in figure 1 and as will be seen in chapter VIII, this is far from being true.

With method A, the distortion of the TG curve that is brought about by the use of a fixed CF makes that the steady end level of amidolytic activity from α 2M-thrombin formed during TG cannot be determined and consequently this activity cannot be subtracted to obtain the activity of free thrombin only. Therefore the ETP is arbitrarily, measured as the area under the curve until a fixed time point (60 min, as in ref.4) and comprises a significant amount of α 2M-T activity. The same is true for method B2.

From table 2 it is evident that method B1, the only one to use CIC, shows a much lower experimental variation than the other ones. From comparison with method B2, that is identical to B1 except for the method of calibration, it is seen that CIC reduces the CV of the concentration dependent parameters (ETP, peak,

slope) by >10%. As was to be expected, for the time dependent parameters no difference in CV was found. It is also seen that the values in B1 are higher than in B2, in line with the underestimation that is inherent to using a fixed CF determined in buffer under initial reaction conditions. Except ETP, all parameters determined with methods A1 and A2 show higher CVs than those in method B2, indicating that the method of calibration cannot be the sole cause of the high CVs found with methods A.

The absolute values of the different parameters cannot be compared directly between methods A and B because the reaction mixtures are different as to tissue factor and phospholipid content, Ca^{++} concentration and dilution. However, looking at the high values of the ETP in both methods A1 and A2 one might think that this is due to the fact that the contribution of the $\alpha 2\text{M}$ -thrombin component to the ETP is not subtracted (see above). This however counts also for method B2 which shows much lower ETP. In real, these high ETP-values are the result of the broad curves showing up with both methods A1 and A2 (look at the very low slope-values!). The time-course of a thrombogram in method A is about the double (or even more) of that of a thrombogram in method B. The reason(s) for these broad curves compared to method B probably have to be searched in the different reaction-conditions as e.g. the PL-concentration (not known) or calcium-concentration after recalcification (see methods and materials). Also the higher substrate-concentration and higher dilution of plasma that is used in method A can contribute to the higher ETP observed in this method (see respectively chapter VI and chapter VIII).

The intra-individual CV-values obtained with the different methods and shown in table 2 are very similar to those reported from the collaborative study carried out by the working group of the scientific and standardisation committee (SSC) of the international society of thrombosis and haemostasis (ISTH) (Chairman Elaine Gray, PhD) as reported at their meeting in Geneva in 2007 (oral communication).

These results clearly show that CIC is required for accurate measurement of TG-parameters. Nevertheless an important result has been obtained by Hron et al. [17] using method A. They showed that TG can be used to distinguish patients at low risk for recurrent VTE from those at high risk. In view of the fact that measurement of the aPTT also allows a certain stratification of such patients [21], a role of the clotting system seems certain. If we assume that the degree of quenching of fluorescence by plasma is not predictive of thrombotic risk, it follows that the use of CIC in thrombin generation analysis is very likely to allow an even better distinction between low and high risk groups.

We conclude that calibration by continuous comparison to a known thrombin activity in a parallel plasma sample is a necessary and sufficient condition to obtain the concentration dependent parameters of the thrombin generation curve with an experimental variation that is small enough to serve as a useful basis for clinical decision making.

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Chapter VI

Influence of the signaling substrate on the parameters of the thrombogram

Butenas S and K.G. Mann published an article in Journal of Thrombosis & Haemostasis entitled ‘Caution in the interpretation of continuous thrombin generation assays’ in which they express their concern about the influence of the signaling substrate on thrombin generation [1]. In this chapter we give a short summary of this article followed by our rebuttal (complete letter) as published in the same edition of Journal of Thrombosis & Haemostasis [2].

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Summary of ‘Caution in the interpretation of continuous thrombin generation assays.’[1]

S. Butenas and K.G. Mann

Thrombin plays a crucial role in its own formation. Adding an exogenous thrombin substrate to clotting plasma therefore inevitably influences these feedback mechanisms. During the initiation phase (lag-time) an artificial added substrate will compete with critical endogenous thrombin substrates present in plasma. As a consequence the initiation phase prolongs while the propagation phase is delayed. During the propagation phase the signaling substrate also interferes with the natural thrombin inhibitors present in plasma causing elevated values for total thrombin as well as higher peak thrombin concentrations. This is illustrated in figure 1 where thrombin generation is measured in the synthetic coagulation proteome in the absence and presence of 416 μM ZGGR-AMC. This coagulation proteome is a mixture of factors II, V, VIII, IX, X and XI, TFPI and AT-III all at mean physiologic concentrations. The addition of substrate prolongs the initiation phase from 120 s to 340 s and the peak thrombin concentration increases from 330 nM to 430 nM. Total thrombin generated is doubled (1210 and 2370 nM.min, respectively). (Note: here the author actually means the ETP, the man-hours of thrombin, not the concentration of thrombin).

Figure 1
Thrombin generation measured in the synthetic coagulation proteome with 5 pM TF and 4 μM PL in the absence ($\diamond$) and presence ($\blacklozenge$) of 416 μM ZGGR-AMC.

In conclusion, all parameters measured during the coagulation process are influenced by the presence of the synthetic substrate i.e. the initiation phase ($\sim$ clotting time), peak thrombin concentration and total thrombin generated during the reaction. This is of course inevitable and is the more important if coagulation factor deficiencies, such as haemophilia A, B and C are investigated since in these experiments low trigger-concentrations are used. This results in a prolonged initiation phase and an increased sensitivity to an exogenous substrate.

These considerations do not question the utility of continuous thrombin generation assays in clinical diagnosis. But one has to be careful with the quantitative interpretation of natural processes occurring during thrombin generation.

Caution in the interpretation of continuous thrombin generation assays: a rebuttal (H.C. Hemker and E. De Smedt) (full letter)

We share the concern of Butenas and Mann [1] about the influence of the signaling substrate in continuous measurement of thrombin generation. Any substrate necessarily binds to thrombin and, moreover, is liable to bind to other clotting proteases. It therefore always inhibits thrombin and is potentially an inhibitor of the clotting mechanism at other sites as well. Given the importance of thrombin-driven feedback reactions, inhibition of thrombin will, theoretically, always influence prothrombin conversion. Whether and under what conditions this is of practical importance remains to be answered for each single substrate. In the articles in which we introduced continuous thrombin generation measurement with chromogenic [2] or with fluorogenic [3] substrates, we have shown that prothrombin conversion is not significantly affected by the proposed substrates but thrombin decay is slowed down in a predictable manner because plasmatc antithrombins cannot interact with thrombin that is occupied by the substrate. It might suffice to refer to these articles but in view of its accruing clinical relevance we readdress the subject for the currently most-used substrate: Z-Gly-Gly-Arg-AMC.

Effects on the lag time

From table 1 it can be seen that Z-Gly-Gly-Arg-AMC indeed prolongs the initiation phase (lag time) of thrombin generation at low tissue factor (TF) concentrations, be it less in our experiments than in those of Butenas and Mann. We agree that this is likely to be due to inhibition of thrombin driven feedback reactions. The effect on the lag time in TF induced thrombin generation in plasma is weaker than that on the reconstituted ‘coagulosome’, which suggests that caution is in its place when extrapolating from reconstituted systems to the ‘isolated organ’ plasma. The information contained in the lag time is essentially the same as that in conventional clotting times. It is a key question whether the substrate interferes with the information that is unique for the thrombogram, i.e. that on the amount of prothrombin converted and on the time course of the thrombin production.

Effects on the amount of prothrombin converted

Plasma contains the natural antithrombin $\alpha 2M$, the ‘bait region’ of which is split by thrombin after which thrombin remains bound to the $\alpha 2M$ molecule and, although biologically inactive, retains its activity towards the signal substrate. The residual amidolytic activity, after thrombin generation is over, is due to this complex. The total amount of complex formed is a measure of the amount of thrombin molecules that have been available for reaction with $\alpha 2M$ (see further

[4]), whether they have been reversibly bound to the substrate or not. In figure 1A, we see that thrombin generation curves that are determined at different substrate concentrations (104–832 μM , 5 pM TF) eventually yield identical amounts of $\alpha 2\text{M-T}$ activity. Also, at 1 pM of TF, a concentration at which thrombin generation is dependent upon antihemophilic factors [5], the level is independent of the substrate concentration (table 1). Identical (low) end-levels of $\alpha 2\text{M-T}$ were also found when hemophilic plasmas were tested at different substrate concentrations (results not shown). We conclude that the presence of substrate did not alter the extent of prothrombin conversion.

Substrate (μM)	n	0	104	208	416	832
Lag-time 5 pM TF (s)	16		105 $\pm$ 6	104 $\pm$ 6	107 $\pm$ 8	127 $\pm$ 7
Lag-time 1 pM TF (s)	16		225 $\pm$ 13	226 $\pm$ 13	234 $\pm$ 11	253 $\pm$ 12
$\alpha 2\text{M-T}$ end-level 5 pM TF (nM)	8	92.2 $\pm$ 5.7	90.6 $\pm$ 6.5	93.9 $\pm$ 6.4	96.8 $\pm$ 5.9	94.8 $\pm$ 6.2
$\alpha 2\text{M-T}$ end-level 1 pM TF (nM)	8	74.7 $\pm$ 5.4	70.8 $\pm$ 6.3	79.8 $\pm$ 5.0	77.7 $\pm$ 4.9	77.2 $\pm$ 5.0
Inhib. by 10 nM TM, 5 pM TF (%)	8	68 $\pm$ 5	69 $\pm$ 4	67 $\pm$ 5	62 $\pm$ 4	45 $\pm$ 3
Inhib. by 4 nM TM, 1 pM TF (%)	8	81 $\pm$ 6	83 $\pm$ 7	78 $\pm$ 6	77 $\pm$ 5	63 $\pm$ 6

Table 1. Effect of substrate on feedback phenomena

The lag-time is defined as the moment that the thrombin concentration curve crosses the thrombin concentration value of 10 nM. Inhib.: the inhibition by TM (%) is defined as the $\alpha 2\text{M-T}$ end-level without TM (100 %) minus the $\alpha 2\text{M-T}$ end-level with TM (%).

As expected, the end-level of $\alpha 2\text{M-T}$ drops when a natural inhibitor of prothrombin activation is induced through addition of thrombomodulin. This decrease is more important when no substrate is present than it is at high substrate concentrations (table 1). So the thrombin-driven negative feedback through the activated protein C system is affected by the presence of substrate, be it that at the usual substrate concentration (416 μM) the effect is barely significant.

Effects on thrombin decay

Peaks of amidolytic activity increase significantly with the substrate concentration (figure 1A). This is not due to the higher reaction velocity at higher substrate concentration, because the reaction velocities are converted into enzyme concentrations by comparison with a fixed amount of thrombin activity measured at the relevant substrate concentration. The increase must be ascribed to the fact that thrombin is protected from antithrombins by the substrate. This is especially evident when the activity due to $\alpha 2\text{M-T}$ is subtracted (see [4] for the method employed) (figure 1B). In the reaction mixture, thrombin is partitioned

between the free form and that bound by substrate. Antithrombin acts on the free form only, i.e. on a fraction that is $K_m/(K_m + S)$ of the total enzyme. Multiplication of measured thrombin concentrations by $K_m/(K_m + S)$ should therefore compensate for the slowing down of thrombin decay by substrate. In accordance with this hypothesis, this multiplication produces curves that in good approximation are identical and independent of substrate concentration (figure 1C, see also [2]).

Figure 1. Influence of substrate concentration on thrombin activity. (A) Amidolytic activity, i.e. thrombin and $\alpha 2$ M-T activity; (B) thrombin activity only; (C) thrombin activity compensated for binding to substrate. Defibrinated pooled normal plasma. Reaction conditions as in [2]. Five pM tissue factor. Substrate concentrations from top to bottom: 832 μ M (black), 416 μ M (gray), 208 μ M (black), 104 μ M (gray).

Effects on the course of prothrombin conversion

In figure 1C, the effect of substrate binding on thrombin breakdown has been abolished. The resulting curves represent thrombin generation as if antithrombin

action would have been normal. The curves are so similar as to be indistinguishable, except for the effect on the lag phase. We conclude that not only the amount of prothrombin converted is not influenced by the substrate but also the time course of prothrombin conversion is hardly or not influenced.

In conclusion, to probe a system means disturbing it, so when proposing a new technique one has to investigate what this disturbance amounts to. This we did in our original publications and here we show additional evidence that the extent and the time course of prothrombin conversion are minimally disturbed by the presence of Z-Gly-Gly-Arg-AMC at 416 μM . Thrombin inactivation is slowed down to an exactly predictable extent. The necessary effect on thrombin-driven feedback, in plasma, does not cause an important increase in the lag time but does diminish the action of added thrombomodulin by about 10%.

These conclusions pertain to Z-Gly-Gly-Arg-AMC at the usual concentration, i.e. at around twice its K_m . They should not be extrapolated to other substrates or to higher concentrations. They certainly should not be interpreted as *laissez passer* for any other substrate. Thrombin generation experiments with high-affinity substrates, e.g. those performed in [6] are very likely to reflect a coagulation mechanism in which feedback mechanisms are severely disturbed.

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Chapter VII

During thrombin generation there is a shift from chemical to diffusional control

In 2005 we published an article in Journal of Thrombosis & Haemostasis about the shift from chemical to diffusional control in thrombin-generation during coagulation [1]. As reaction on the above article Orfeo T. and K.G. Mann wrote a comment in which they question our conclusions and give their point of view about the use of mathematical and biological models [2]; on which we published a rebuttal [3].

In this chapter we render this discussion about diffusion. We present our first article as it was published [1] after which the comments of Orfeo T. and K.G. Mann are reproduced, each one followed by a summary of our refutation. For the sake of completeness the rebuttal itself [3] is also added.

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During coagulation thrombin generation shifts from chemical to diffusional control.

H. C. Hemker, E. De Smedt, P. W. Hemker

Haemostasis and thrombosis hinge on the formation of thrombin. The underlying reaction mechanism is a web of proenzyme-enzyme conversions, controlled by positive and negative feedback reactions [1]. Current paradigm has it that thrombin formation is the result of chemical interactions between the components of this web and therefore is uniquely determined by the initial concentrations and reaction constants of its components [2,3].

Essential steps of the thrombin forming process occur at membranes that contain negatively charged phospholipids [1]. In an *in vitro* model system such procoagulant phospholipids are normally added in the form of relatively small vesicles that remain in suspension, so that the binding sites for the clotting factors are available in free solution. *In vivo* the binding takes place on membrane surfaces that present in a wound or ruptured plaque [4]. Factor VII(a) binds to tissue factor incorporated in cell membranes and factors II, X(a), V(a), IX(a) and VII(a) on adhering and aggregating activated platelets and on membrane remnants. Experiments in the presence of platelets and tissue factor bearing cells suggest that, as soon as such structures are present, physical transport through diffusion might play a role in determining thrombin generation velocities [5,6,7]. In coagulation experiments in plasma *in vitro*, procoagulant phospholipids are added to PPP in the form of vesicles; in PRP they are provided by activated platelets [8]. At the moment that a clot forms ~98 % of thrombin is still to be generated [9, 10]. The total amount of thrombin formed is an important determinant of the quality of haemostasis or the extent of thrombosis [11] and this thrombin is almost all formed within the fibrin mesh of the clot. In that mesh the plasma is stagnant and the procoagulant surfaces of blood platelets and membrane fragments stick to the fibrin fibres [12]. Prothrombin conversion and other activation reactions thus are likely to be located on the insoluble fibrin mesh rather than in free and homogeneous solution and it is conceivable that not only chemical reaction rates but also diffusional transport to these surfaces determines the rate of thrombin formation.

Whether diffusion or chemical interaction is rate limiting can be decided on basis of temperature dependence. Diffusion velocity is proportional to absolute temperature, i.e. will show a ~14% increase between 10 and 50 °C, whereas biochemical reactions will roughly double their pace if the temperature is raised by 10 °C (see e.g. [13]). Here we show that the temperature dependency of thrombin generation in clotting plasma is such that we must assume that, when a clot has formed, diffusion starts to co-determine the rate of thrombin formation.

Thrombin inactivation, on the contrary, is caused by stoichiometric reaction with specific plasma proteins, the most important of which is AT. In contrast to the activation reactions, inactivation takes place in free solution and therefore is less likely to be diffusion limited.

If the velocities of both activation and inactivation are determined by chemical reaction rates, a rise in temperature will enhance both reactions to roughly the same extent and the change in thrombin activity with temperature will be small. If diffusional transport would govern activation but thrombin inactivation would be a chemical reaction, then activation would increase ~3% by raising the temperature from 25 to 35 °C, but inactivation would double so that a 50% decrease of the free thrombin formed would result. If diffusion plays a limited role that is more important in the activation reaction than in the inactivation, a limited decrease of thrombin activity would be seen with rising temperatures.

We tested thrombin generation in plasma at four temperatures: 25, 30, 35 and 40°C under three conditions. A: in defibrinated plasma with added procoagulant phospholipids; B: in normal plasma with the same amount of added phospholipids and C: in platelet rich plasma (figure 1). Defibrination with Arvin is known to precipitate fibrin but not otherwise to affect the clotting system [14].

To 80 µl of citrated plasma we added 20 µl of a mixture of 30 pM recombinant tissue factor and 24 µM phospholipid vesicles (20% dioleoyl phosphatidyl serine, 20% dioleoyl phosphatidyl ethanolamine and 52% dioleoyl phosphatidyl choline and 8% cholesterol). Thrombin generation was started by adding 20 µl CaCl₂ containing 2.5 mM of the thrombin substrate Z-Gly-Gly-Arg-AMC. The fluorescent product AMC was detected by its emission at 460 nm upon excitation at 390 nm [15]. When fibrinogen is present, the clot forms at the moment that also the fluorogenic thrombin substrate starts to be converted, the bulk of thrombin being formed within the clot.

In figure 1 it is seen that, with increasing temperature, the lag-time of thrombin formation shortens under all circumstances. This is to be expected because during the lag-time all reactions take place in free solution and will follow chemical rate laws, i.e. are accelerated significantly by a rise in temperature.

In figure 1B and 1C it is seen that, as soon as fibrin is present less thrombin is formed at higher temperatures than at lower ones. This indicates that thrombin forming reactions are less accelerated by a rise in temperature than thrombin inactivating reactions are, which can be explained by assuming that the role of diffusion in the generation of thrombin is more important than in its decay.

Figure 1. Fluorescence development from a thrombin specific substrate in plasma

A: Defibrinated platelet poor plasma with added phospholipids; B: non-defibrinated platelet poor plasma with added phospholipids; C: platelet rich plasma. In all experiments the fluorescence was measured in 120 μ L of citrated plasma to which were added at zero-time (final concentrations) 5 pM TF, 16.7 mM CaCl₂ and 0.42 mM ZGGR-AMC. In each frame, from right to left the temperatures were: 25 °C (black), 30 °C (gray), 35 °C (black) and 40 °C (gray).

This effect of diffusion starts to play a rate determining role in the generation of thrombin around the moment of clot formation. So, whereas the initiation phase is a purely chemical process, the contribution of physical restraints to thrombin generation during the propagation phase is not to be neglected. In mixtures without fibrinogen (e.g. [2]) or in mathematical simulation (e.g. [3]), thrombin generation velocity is governed by chemical conversion rates only and diffusion is not taken into account. In models where the prothrombin converting enzyme is adsorbed at a macroscopic surface, thrombin generation is diffusion limited at all but extreme concentrations of the reactants [16]. Because of the transition of chemical- to diffusional limited reaction velocity around the moment of clotting neither of these approaches can be quantitatively extrapolated to the in vivo si-

tuation. It follows that thrombin generation in the body is best modelled by measuring it directly in a sample of isolated organ i.e. in clotting platelet rich plasma with addition of the relevant components of the vessel wall (tissue factor, thrombomodulin) and/or white cells.

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Citations of the comment of Orfeo T and KG Mann and our refutation in short

Orfeo and Mann: The authors have attempted to extend the ETP methodology (?) to examine whether a fundamental change in reactant availability (delivery or type of rate limiting steps) is influenced by fibrin formation in clotting plasma. Pivotal to their interpretation of temperature dependence of thrombin generation is the simplifying assumption that the enthalpies of activation defining the entire ensemble of binding and catalytic events are relatively uniform. This assumption appears rash.

Hemker, de Smedt and Hemker: Present common knowledge agrees precisely that the dependence upon temperature (which is what enthalpy means in this context) of the ensemble of chemical reactions is more important than that of the ensemble of diffusional transport. Probably Orfeo and Mann want to suggest that we need to know the details on each and every reaction within the ensemble. This obviously is impossible. It also is unnecessary, were it only because we compare the temperature dependence of two situations that differ in the presence of fibrin only. The chemical mechanism of thrombin generation is not altered by the presence of fibrin, so the large majority of thermodynamics of the reactions will be identical in both conditions. Unless Orfeo et al. postulate that fibrin has an influence on the enthalpies of reactions in which it does not participate. Physical conditions change dramatically however: the medium becomes unstirred and inhomogeneous on the μ -meter scale which necessitates diffusional transport to and from phospholipid surfaces.

Orfeo and Mann: A number of initial assumptions in this letter are incorrect. The lag phase of the reaction is a consequence of the generation of catalysts required to provide the factor Xa, factor IXa and thrombin which participate in intrinsic factor Xase and prothrombinase generation on platelet surfaces. All these reactions involve complexes localized on membrane surfaces, including the triggering complex VIIa-TF, which activates factor X and factor IX on the surface of exogenously introduced vesicles but requires diffusional transfer of these reactants to activated platelet surfaces (or synthetic phospholipid vesicles) for the efficient progression of the reaction. None of these proteolytic or binding reactions take place in solution and all involve diffusional transfer of proteins on and off membranes and diffusional events constrained to surfaces.

Hemker, de Smedt and Hemker: An interesting remark is made by Orfeo and Mann on the role of diffusion in thrombin generation on vesicle surfaces before fibrin forms. Indeed lateral diffusion of molecules adsorbed at the phospholipid-solute interface plays a role in all reactions that are located at such an interface, whether in suspension or caught on a fibrin web. As our laboratory was the first

to demonstrate lateral diffusion we are unlikely to underestimate its role. We also recall, however, that lateral diffusion only can be made rate limiting under extreme conditions, i.e. at macroscopic surfaces and very low prothrombinase concentrations. Therefore its influence is not observed with vesicles in suspension and the interactions between vesicles and clotting factors and between clotting factors on a vesicle surface can be described by normal chemical collision theory. This type of diffusion therefore can, and is, justifiably left out of the equations both in Dr Mann's chemical and computational models as well as in ours.

Most of our readers must be familiar with the pictures of a fibrin web onto which phospholipid platelets and/or phospholipid particles adhere and we know of no other mechanism for reactants to be transported to and from such immobilised surfaces in a stagnant fluid than diffusion through the liquid phase. To the understanding reader it must be evident that we meant this type of diffusional transport in our letter and we see no flaw in our reasoning that such diffusional transport will start to count at the moment of clotting.

Orfeo and Mann: "A brief examination of the data provided highlights the interpretive challenge that using a diluted, citrated plasma system to elucidate events at the level of thermodynamics and physical chemistry presents. The authors provide time course data displaying the generation of a fluorescent signal derived from hydrolysis of a fluorogenic substrate by thrombin produced during a series of tissue factor initiated reactions in recalcified, citrated plasma conducted at different temperatures."

"We have estimated the maximum slope (dF/dT) for each of the presented time courses and present the temperature dependence of these slopes for the cases of defibrinated and non-defibrinated plasma (figure 1, solid lines). Note that at each temperature the maximum thrombin level achieved is greater in the presence of fibrinogen/fibrin. However, in principle, to convert substrate hydrolysis rate data measured at different temperatures to thrombin concentrations, standard curves must be constructed at each temperature. The data presented by Hemker *et al.* appear to be 'raw'; that is uncorrected for the expected temperature dependence of thrombin activity on the substrate. This makes interpretation of the data either by visual inspection or secondary manipulation problematic. To address this issue we have measured the temperature dependence between 25 and 40°C of thrombin hydrolysis of the synthetic substrate Spectrozyme TH (American Diagnostica Inc, Greenwich, CT, USA) and have used that value (approximately 40% change in rate per 5°C change in temperature) to correct maximum slope values extracted from the presented time courses so that they reflect only the amount of thrombin present. These corrected data (dashed lines) are also presented in figure 1. Two observations can be made here: at any given temperature, the maximum level of thrombin achieved is greater in the presence of fibrinogen/fibrin; and maximum thrombin levels in the two reaction systems ($\pm$ fibrinogen/fibrin) actually decrease uniformly and in parallel with increasing

temperature. These trends are not consistent with the hypothesis that the onset of fibrin formation represents a transitional point between two qualitatively different reaction milieus.

Figure 1. Secondary analyses of fluorescence vs. time plots resulting from the clotting of plasma in the presence of a thrombin specific substrate.

Maximum slopes (dF/dT) of each progress curves at 25, 30, 35 and 40°C were estimated for the reactions in defibrinated plasma (Hemker et al. [1], figure 1, left frame) and non-defibrinated plasma (Hemker et al. [1], figure 1, middle frame). Solid lines represent uncorrected dF/dT values, dotted lines represent dF/dT values corrected for the effect of temperature on fluorogenic substrate hydrolysis, using our estimate of the temperature dependence of thrombin hydrolysis of the synthetic substrate Spectrozyme TH. ♦ defibrinated plasma; ▲ plasma with fibrinogen.

Hemker, de Smedt and Hemker: We preferred to show the raw data because we know that a conclusion is more transparent and convincing if it does not depend on data handling. The finding as such appeared to us of sufficiently general interest to attempt a presentation that is maximally clear to the non specialist. From our graphs anybody can see that in the absence of fibrin the sum of effects on all the reactions playing (including that of thrombin converting substrate) equals out, so that as much thrombin product is formed at higher temperatures as at lower ones. In the presence of fibrin, however, less product is formed at higher temperatures, i.e. the balance between thrombin activation and thrombin inactivation shifts in favour of the latter. This conclusion can be arrived at with the naked eye and does not require any special treatment of the data. However, standard curves were measured at each temperature but they were not presented or used for data handling because it might be confusing and was not necessary to demonstrate the phenomena.

Orfeo and Mann, from our data, derive that the peak velocity of product formation does not show the same temperature dependence as the total amount of substrate converted. They omit to say how this could influence our conclusions. Little experience with plasmatic thrombin generation curves suffices to learn that there is no strict relation between thrombin peak-height and total thrombin activity. Because the relative contribution of diffusion and chemistry shifts during the process, we have to take into account the integral time range of the process. Therefore it is the total amount of thrombin that should be discussed and not the peak values only. This again underlines why it is sometimes better to have a look at the raw data in order not to confuse ourselves. Concerning the correction made for the temperature dependence of thrombin activity on the substrate, we did not see the relevance of doing this since this effect is the same in both conditions (with and without fibrinogen).

Further confusion is caused by the remark that more thrombin is generated in the presence of fibrin than in its absence. This is perfectly true and has been observed by us before. It is caused by the changes that fibrin brings about in the pattern of interaction between thrombin and antithrombins. Antithrombin is not able to inactivate fibrin-bound thrombin meaning that fibrin as such does not increase thrombin inactivation but in contrary reduces it. Nevertheless, despite this, the balance between formation and inactivation shifts in the presence of fibrinogen in the sense of the latter at higher temperatures. This point thus strengthens our argument rather than invalidating it.

We conclude that the remarks of Orfeo and Mann on our observations are correct but do not purport to our interpretation and conclusions.

Mathematical and biological models of blood coagulation. A rebuttal. (Full letter)

H. C. Hemker, E. De Smedt, P. W. Hemker.

The comment of Orfeo and Mann [1] on the letter in which we showed that thrombin generation in a clot is not dependent on chemical reaction rates only but on diffusional phenomena as well [2] raises some interesting points that we are glad to discuss further.

We preferred to show the raw data because we know that a conclusion is more transparent and convincing if it does not depend on data handling. The finding as such appeared to us of sufficiently general interest to attempt a presentation that would be most clear to the non-specialist. From our graphs [2] it is readily apparent that in the absence of fibrin the sum of effects on all of the participating reactions (including hydrolysis of the fluorogenic substrate by thrombin) balances out, such that as much thrombin product is formed at higher temperatures as at lower ones. In the presence of fibrin, however, less thrombin is formed at higher temperatures, i.e. the balance between thrombin activation and thrombin inactivation shifts in favour of the latter. This conclusion can be arrived at by inspection and does not require any special treatment of the data.

We are well acquainted with the ways in which fluorescence data can be converted into thrombin concentrations, as these techniques were developed in our laboratory [3]. In further investigations on the role of diffusion in thrombin generation we will certainly use such approaches to study the phenomenon in more quantitative detail, but for the demonstration of its existence this is not necessary and may be confusing.

Orfeo and Mann themselves furnish an excellent example of such confusion. From our data they derive that the maximal velocity of substrate hydrolysis does not show the same temperature dependence as the total amount of fluorogenic substrate converted. They omit to say how this could influence our conclusions. The maximal velocity of product formation indicates the peak thrombin activity. Experience with plasmatic thrombin generation curves demonstrates that there is no strict relationship between thrombin peak height and total thrombin activity (i.e. the endogenous thrombin potential [4]). Because the relative contribution of diffusion and chemistry shifts during the process, we have to take into account the integral time range of the process. Therefore it is the total amount of thrombin that should be discussed and not the peak values only.

Further confusion is caused by the remark that more thrombin is generated in the presence of fibrin than in its absence. This is perfectly true and has been observed by us before [3]. It is caused by the changes that fibrin brings about in the pattern of interaction between thrombin and antithrombins. It shows that fibrin as such does not increase thrombin inactivation but nevertheless shifts the

balance between formation and inactivation toward the latter at higher temperatures. This point thus strengthens our argument rather than invalidating it.

We conclude that the remarks of Orfeo and Mann on our observations are correct but are not relevant to our interpretation and conclusions.

An interesting remark is made by Orfeo and Mann on the role of diffusion in thrombin generation on vesicle surfaces before fibrin forms. Indeed lateral diffusion of molecules adsorbed at the phospholipid-solute interface plays a role in all reactions that are located at such an interface, whether in suspension or caught in a fibrin web. As our laboratory was the first to demonstrate lateral diffusion [5], we do not underestimate its role. We also recall, however, that lateral diffusion can only be rate limiting under extreme conditions, i.e. at macroscopic surfaces and very low prothrombinase concentrations. Therefore its influence is not observed in reactions between clotting factors in solution on vesicles in suspension and hence these interactions can be described by normal chemical collision theory. This type of diffusion therefore can, and is, justifiably left out of the equations both in Dr Mann's chemical and computational models (as cited in [1]) as well as in ours. (In technical terms: the interaction between clotting factors and the surface of a free phospholipid vesicle in aqueous medium is limited by the diffusion controlled maximal rate ($10^9 \text{ M}^{-1}\text{s}^{-1}$) like any chemical reaction while the interaction between molecules at the phospholipid surface is determined by the much higher lateral diffusion rate ($10^{16} \text{ M}^{-1}\text{s}^{-1}$). This latter step therefore never becomes rate limiting.)

Most of our readers must be familiar with the pictures of a fibrin web onto which platelets and/or phospholipid particles adhere and we know of no other mechanism for reactants to be transported to and from such immobilised surfaces in a stagnant fluid than diffusion through the liquid phase. To the understanding reader it must be evident that we meant this type of diffusional transport in our letter and we see no flaw in our reasoning that such diffusional transport will start to contribute at the moment of clotting.

As to our interpretation, Orfeo and Mann state "pivotal to [our] interpretation of the temperature dependence of thrombin generation is the simplifying assumption that the enthalpies of activation defining the entire ensemble of binding and catalytic events are relatively uniform". Present common knowledge agrees precisely with the dependence upon temperature (which is what enthalpy means in this context) of the ensemble of chemical reactions being more important than that of the ensemble of diffusional transport. Perhaps Orfeo and Mann wanted to suggest that we need to know the temperature dependence of all individual reactions within the ensemble. This is obviously impossible. It is also unnecessary, because we compared the temperature dependence of two situations that differ only in the presence of fibrin. Unless Orfeo and Mann postulate that fibrin has an influence on the enthalpies of reactions in which it does not partake, the large majority of the thermodynamics of the reactions in presence and absence of fibrin will be identical because the chemical mechanism of thrombin generation is not altered by the presence of fibrin. Physical conditions change dramati-

cally however: the medium becomes unstirred and inhomogeneous on the μ -meter scale which necessitates diffusional transport to and from phospholipid surfaces.

The general impression that the comment of Orfeo and Mann left with us represented a certain resistance to the idea that there may be more to the coagulation process than chemistry alone. We regret the absence of a more constructive view, such as the proposition of an alternative hypothesis to explain our observations.

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Until now Orfeo T. and Mann K.G. refrained from answering to our rebuttal.

Chapter VIII

The effect of plasma dilution on thrombin generation

Dilution of plasma reduces the participation of the intrinsic pathway and the Jossen-loop during thrombin generation and shifts it towards the direct extrinsic pathway. Dilution induces a second wave of prothrombinase-activity due to suppression of protein S dependent inhibition.

In preparation. Will be submitted to Thrombosis and Haemostasis as the third manuscript of a series of manuscripts with working title:

Assessment of Thrombin Generation with Fluorogenic Substrates. 3. The effect of plasma dilution on thrombin generation.

E. De Smedt, R. Wagenvoort, H.C. Hemker

Introduction

Several methods exist to determine thrombin generation that use different dilutions of plasma or whole blood. In thrombelastography (TEG) [1] almost undiluted whole blood is used because the natural thrombin substrate fibrinogen is used as an indicator. As fibrinogen is exhausted long before thrombin generation is over, this cannot serve to measure the entire thrombin generation curve. So adding an artificial substrate is obligatory and the blood / plasma inevitably has to be diluted. Also calcium, trigger and/or phospholipids have to be added. In the 'thrombin generation time' [2] whole blood is diluted 1/3, meaning that plasma is diluted $\sim 1/5$. Other methods determine thrombin generation in platelet poor plasma like there are the CAT-method (plasma diluted 2/3) [3] and the Technoclone-method (plasma diluted 2/5) [4]. This poses the question how thrombin generation is influenced by dilution of plasma. Also from a practical point of view it is interesting to investigate this question since it can possibly reduce the amount of blood or plasma required for measuring thrombin generation.

In this article we use the CAT-method to investigate the effect of dilution on thrombin generation in platelet poor plasma. In a first stage platelet poor plasma was diluted at constant concentrations of calcium and citrate. In a second stage we kept prothrombin and antithrombin constant as well, so as to investigate the effect of dilution on prothrombinase formation. We will see that as a rule of thumb, dilution fosters procoagulant mechanisms and defavorises inhibitory mechanisms. Furthermore a shift is observed from the intrinsic system and Jossen-loop towards the direct extrinsic system.

Materials and methods

Materials

Platelet poor plasma

Blood is obtained through antecubital venipuncture. Free flow or minimal suction is employed; vacuum containers are avoided. The blood is collected in plastic tubes of 10 ml containing 1 ml sodium-citrate (0.13 M) put on room temperature 1h in advance. While collecting the blood each tube is tilted slowly three times so as to mix the content well. The blood is centrifuged at 4000 rpm for 10 min at room temperature and the plasma is centrifuged a second time (*idem*). Finally all plasma is collected and ultra-centrifuged at 18000 rpm for 1h at 4°C giving platelet poor plasma. In order to avoid contamination with procoagulant microparticles from aging platelets, PPP is prepared as quick as possi-

ble. A pool of platelet poor plasma is collected from healthy subjects and is used as reference plasma (NPPP).

Haemophilia plasma (informed and with consent) was obtained from Yessim Dargaud.

Proteins

Human prothrombin was prepared according to [5], human antithrombin according to [6].

TFPI was a kind gift of Dr. Theo Lindhout, PS was a kind gift of Dr. Tilman Hackeng. Recombinant human TM and APC were a kind gift of Asahi (Japan).

Buffers

BSA5 contains 20 mM Hepes, 140 mM NaCl, 0.02% NaN₃ and 5 mg/ml bovine serum albumine (BSA) at pH 7.35. BSA60 contains 20 mM Hepes, 0.02 % NaN₃ and 60 mg/ml BSA at pH 7.35.

Reagents

The phospholipids used are a mixture of 60% Phosphatidyl choline, 20% phosphatidyl serine and 20% phosphatidyl ethanolamine. The stocks are obtained by Avanti (USA).

Trigger and substrate are commercially available: lyophilized innovin from Dade-Behring and Z-Gly-Gly-Arg-AMC from Bachem, Switzerland.

Methods

TG-curves

All thrombin generation curves were obtained using the CAT-method in which plasma is diluted 4/6: 80 µl plasma + 20 µl reagents + 20 µl FluCa (substrate + calcium) [3]. In our investigation we used also higher dilutions of plasma obtained with the same buffer as for preparation of FluCa (20 mM Hepes, 60 mg/ml BSA, pH 7.35). To this buffer is added 21.7 mM citrate and 2.4 mM calcium so as to keep their concentration at a constant level independent of the dilution of plasma. In a further stage also 1.26 µM human prothrombin and 2.15 µM human antithrombin was added. As a result citrate and calcium are always kept constant and prothrombin and antithrombin if desired.

Determination of h-prothrombin and h-antithrombin in plasma

Human prothrombin in plasma was determined by activating it with an excess of staphylocoagulase. Then the thrombin-concentration was measured with the chromogenic substrate S2238.

Human antithrombin in plasma was determined by adding a fixed amount of thrombin and heparin to a dilution series of plasma. The remaining thrombin-concentration after 10' at 37 °C was measured with S2238 in each dilution and these values were plotted against the amount of plasma in each dilution (µl plasma). The slope of this linear curve gives the concentration of thrombin that

has been inactivated per μl plasma. This corresponds with the concentration of antithrombin present in this μl plasma.

Determination of prothrombinase curves

FII-ase curves were calculated following the procedure described in chapter 4.

Results

The concentration of TF available determines the pathway of coagulation [7-10]. As a rule of thumb, the lower the trigger concentration the more complex the mechanism of thrombin generation is. Under our standard conditions (2:3 diluted plasma), 5 pM TF triggers mainly the extrinsic pathway and thrombin generation is just slightly dependent upon factors VIII and IX. If less trigger is used, the contribution of factors VIII and IX via the Jossen-loop increases significantly and at 1 pM TF the Jossen-loop is the main pathway during thrombin generation. At concentrations lower than 1 pM TF also factor XI starts to play a role (intrinsic system). Without TF, contact activation triggers the intrinsic pathway while the extrinsic pathway and Jossen-loop do not contribute anymore. This is illustrated in figure 1.

Figure 1. Different pathways of thrombin generation

TG is measured in NPPP (NP) and a severe haemophiliac (Haem) (4:6 dilution). The first number gives the trigger concentration (pM TF), the second number the PL-concentration (μM). When no TF was added, the plasma was activated with kaolin to obtain reproducible lag-times.

We used three different concentrations of TF so as to trigger respectively mainly the extrinsic system (5 pM TF), mostly the Jossen-loop (1 pM TF) or only the intrinsic system (no TF). If no trigger is added, one depends upon the minimal contact activation provided by the plastic 96-well plates and lag-times are long and variable. So to obtain reproducible lag-times we activated the plasma with kaolin before. Under these conditions thrombin generation was measured in NPPP and a severe haemophilia patient ($<5\%$ VIII). Figure 1

shows that thrombin generation becomes increasingly dependent of factor VIII the less trigger is used.

Figure 2. TG in diluted NPPP at constant concentration of calcium and citrate.

TG is measured in the presence of
 5 pM TF, 4 μ M PL (figure A)
 1 pM TF, 4 μ M PL (figure B)
 no TF (+ kaolin) with 1 μ M PL (figure C)

So, thrombin generation was measured using no TF, 1 pM TF and 5 pM TF in a dilution series of NPPP at constant concentration of calcium and citrate (figure 2). The evolution upon dilution of the three most important parameters of the thrombogram (lag-time, peak-height and ETP) is presented in figure 3.

Surprisingly, the ETP increases in dilutions up to 1:6, drops thereafter and attains the level of the 4:6 dilution until the 1:24 dilution. The peak-height shows a qualitatively similar pattern but starts decreasing earlier, after 2:6 dilution. The lag-time has a tendency to shorten if TF is present and only prolongs significantly in the absence of TF. The high values of ETP, despite low peak-heights, are mainly caused by the broadening of the thrombin generation curves upon dilution. This phenomenon must be due to slower inactivation of thrombin or to prolonged formation of thrombin or to both.

Figure 3. Evolution of the parameters of the TG of figure 2.

TG was measured in diluted NPPP at constant concentration of calcium and citrate in three different conditions (see figure 2). Evolution of lag-time (A) peak-height (B) and ETP (C) is presented against the dilution of plasma (plotted on a logarithmic scale) for each of the three conditions: 5 pM TF, 4 μ M PL (blue), 1 pM TF, 4 μ M PL (green) and no TF (+ kaolin) with 1 μ M PL (red).

Figure 4. The effect of dilution on thrombin decay

A: thrombin generation curves of figure 2A plotted as Log thrombin-concentration against time. A trendline was calculated through the decay-phase so as to obtain the down-slopes. Two dilutions (1:6 and 1:24) are not shown to not overload the figure. The colors fit with the colors used in figure 2A.
B: down-slopes of the semi-logarithmic decay of the thrombin generation curves of figure 2A (see trendlines of figure 4A).

To estimate the effect on thrombin decay we plotted the thrombin generation curves of figure 2A as logarithm of thrombin-concentration against time. It appeared that almost the entire down-slope became a straight line, suggesting semi-logarithmic decay (figure 4A). The obtained down-slopes were proportional to the plasma dilution (figure 4B). Also in the other conditions the same evolution in down-slopes was observed (results not shown). We concluded that dilution of antithrombin is the main cause of the minor response of ETP to dilution

To single out the effect of dilution on prothrombinase activity we kept the concentrations of prothrombin and antithrombin constant and equal to that of 4:6 diluted plasma and repeated the experiments (figures 5 and 6).

Figure 5. TG in diluted NPPP at constant concentration of calcium, citrate, prothrombin and antithrombin.

TG is measured in the presence of
 5 pM TF, 4 μ M PL (figure A)
 1 pM TF, 4 μ M PL (figure B)
 no TF (+ kaolin) with 1 μ M PL (figure C)

Figure 6. Evolution of the parameters of the TG of figure 5

Thrombin generation was measured in diluted NPPP at constant concentration of calcium, citrate, prothrombin and antithrombin in three different conditions (see figure 5). Evolution of lag-time (A) peak-height (B) and ETP (C) is presented against the dilution of plasma (plotted on a logarithmic scale) for each of the three conditions: 5 pM TF, 4 μM PL (blue), 1 pM TF, 4 μM PL (green) and no TF (+ kaolin) with 1 μM PL (red).

We observed that:

- A. Thrombin generation hardly decreases in TF triggered plasmas even up to 1:96 dilution. The ETP in 1:96 diluted plasma is even higher than in 4:6 diluted plasma. In sharp contrast, contact activated plasma loses all activity if diluted more than 24 times.
- B. In TF triggered plasma, despite the constant concentration of prothrombin and antithrombin the down-slopes are again less steep, the higher the dilution of plasma, not however in contact activated plasma.

We will first focus on observation A and later on we will discuss observation B.

Observation A

The difference in behavior of the intrinsic and the extrinsic system suggests that dilution of an inhibitor of extrinsic thrombin generation plays a role, TFPI being an obvious candidate. We therefore repeated the experiments keeping TFPI at a concentration equal to that in 4:6 diluted plasma (figure 7). This causes throm-

bin generation to decrease with plasma dilution. Like in the intrinsic system almost no thrombin generation is seen anymore in the high dilutions. We therefore hold dilution of TFPI responsible for the persistent activity of the extrinsic pathway and the Jossen-Loop at high dilutions in figures 5A and 5B. This also explains the shortening lag-time in the low dilutions (figures 3A and 6A).

Figure 7. Constant concentration of TFPI

TG in diluted NPPP at constant concentration of calcium, citrate, prothrombin, antithrombin and TFPI (2 nM). TG is measured in the presence of 1 pM TF and 4 μ M PL.

The intrinsic system however, in which TFPI does not play a role, is seriously diminished in 1:6 diluted plasma and is completely inactive if plasma is diluted more than 24 times (figure 5C). The absence of thrombin generation at 1:24 dilution in figure 5C/6C needs not to be in contradiction to the persistence of low thrombin generation in figure 2C/3C because in the latter case antithrombin is diluted and in the former not. The inactivity of the intrinsic system at high dilutions poses the question of the role of factors VIII and IX at low TF concentration. Indeed, although we know that the Jossen-loop is the main pathway at 1 pM TF under our standard conditions (figure 1 – 4:6 diluted plasma), we don't know if and how much the Jossen-loop contributes to thrombin generation upon dilution. To investigate this further we measured two samples of a haemophilia patient, the one drawn before and the other drawn after infusion with FVIII (<5% and 80% FVIII) triggered with 1 pM TF and keeping calcium, citrate, prothrombin and antithrombin constant (figure 8). The plasma with FVIII (dotted lines in figure 8C) shows the same evolution in lag-time, peak-height and ETP as NPPP (figure 5B). The plasma without FVIII (full lines in figure 8C), however, shows very low peak-height and ETP in the low dilutions but approaches the values of the sample after infusion upon dilution. The two samples of this patient can easily be distinguished in 4:6 diluted plasma but it becomes increasingly harder to distinguish them the more the plasma is diluted. At dilutions > 6x the samples become identical.

Figure 8. TG in diluted haemophiliac plasma at constant concentration of prothrombin and antithrombin

Measured at constant concentration of calcium, citrate, prothrombin and antithrombin in the presence of 1 pM TF and 4 μ M PL. Figure A: TG before infusion with FVIII (<5% FVIII). Figure B: TG after infusion with FVIII (80% FVIII). Figure C: evolution of lag-time (blue), peak-height (green) and ETP (red) against the dilution of plasma (plotted on a logarithmic scale) for the haemophiliac before infusion with FVIII (full line) and for the haemophiliac after infusion with FVIII (dotted line).

This indicates that in highly diluted plasma the Jossen-loop does not function anymore, just as observed for the intrinsic system. In agreement with this decreasing contribution of the Jossen-loop upon dilution, is the observation that also when prothrombin and antithrombin are *not* kept constant, the difference between plasma with and without factor VIII disappears upon dilution (figure 9).

Figure 9. Thrombin generation in diluted haemophilic plasma

Measured at constant concentration of calcium and citrate in the presence of 1 pM TF and 4 μ M PL. Figure A: TG before infusion with FVIII (<5% FVIII). Figure B: TG after infusion with FVIII (80% FVIII). Figure C: evolution of lag-time (blue), peak-height (green) and ETP (red) against the dilution of plasma (plotted on a logarithmic scale) for the haemophilic before infusion with FVIII (full line) and for the haemophilic after infusion with FVIII (dotted line).

Observation B

We concluded before (figure 4) that dilution of antithrombin was the main cause for the minor response of ETP to dilution (broadening curves). However, since in figure 5 the concentration of antithrombin and prothrombin is the same in each dilution and since the break-down velocity of thrombin during thrombin generation is a second order reaction given by $v = k_{dec} \cdot [IIa] \cdot [AT]$ in which k_{dec} is a constant, the reason for the protracting down-slopes has to be found in the production-phase i.e. the conversion of prothrombin into thrombin by prothrombinase. Also, at dilutions > 12x a change in the shape of the thrombin

generation curves is observed (most clear at 1 pM TF) suggesting that there is a second wave of prothrombinase activity. We concluded that probably an inhibitor of prothrombinase is affected by dilution, maybe activated protein C [11, 12].

To investigate the role of the APC-system, the experiment of figure 5B (1 pM TF, constant concentration of prothrombin and antithrombin) was repeated with 3 nM TM or 3 nM APC added. The inhibition caused by adding TM or APC decreased with increasing dilution (figures 10A and 10B).

Figure 10. Influence of TM and APC on TG and FII-ase activity in diluted plasma

TG measured in diluted NPPP at constant conc. of calcium, citrate, prothrombin and antithrombin in the presence of 4 μ M PL, 1 pM TF $\pm$ TM (figure A) and in the presence of 4 μ M PL, 1 pM TF $\pm$ APC (figure B). Only the lowest and highest dilution are shown in order to not overload the figures. In figures C and D the prothrombinase-curves of figures A and B are presented .

This reduction is not unexpected since both PC and PS are diluted. Despite the low concentration of PS, APC (activated by TM or added exogenously) reduces clearly the broadening of the thrombin-generation peak in 1:96 diluted plasma. Therefore prothrombinase-activities were calculated from the thrombin generation-curves presented in figures 10A and 10B following the procedure as de-

scribed in chapter 4. These prothrombinase-curves are presented in figures 10C and 10D. As expected, a second wave of prothrombinase-activity is observed in 1:96 diluted plasma. This second peak is partly reduced if PC is activated by TM and completely if APC is added exogenously.

This poses the question why this extra activity of prothrombinase is not seen in low dilutions (e.g. 4:6) while the APC-system is not activated, or added exogenously. To further investigate the role of dilution of protein S, we repeated dilution 1:96 keeping PS at a concentration equal to that in 4:6 diluted plasma and measured thrombin generation with 5 pM TF, 1 pM TF, 1 pM TF + 3 nM TM and 1 pM TF + 3 nM APC (figure 11).

Figure 11. Influence of PS on thrombin generation in diluted plasma

TG measured in diluted NPPP at constant conc. of calcium, citrate, prothrombin and antithrombin in the presence of 4 μ M PL, 5 pM TF (figure A); 4 μ M PL, 1 pM TF (figure B); 4 μ M PL, 1 pM TF + TM (figure C) and 4 μ M PL, 1 pM TF + APC (figure D). In each condition thrombin generation was also measured while PS was held at a concentration equal to that in 4:6 diluted plasma.

We concluded that PS has the same effect as APC: it abolishes the broadening of the thrombin generation curves. Again prothrombinase-activity was calculated from the thrombin generation peaks (figure 12) and indeed, the second prothrombinase-wave showing-up in 1:96 diluted plasma disappears if PS is *not* diluted. Even more, PS inhibits the whole prothrombinase-curves and prolongs the lag-times (figures 11 and 12). We can only conclude that PS, just as APC and independent of APC, has an inhibitory effect on prothrombinase. The inverse is true as well: APC is able to inhibit prothrombinase if PS is diluted 96 times (figure 11D/12D). However, if APC and PS are allowed to cooperate, their inhibitory effect on prothrombinase is much stronger and thrombin generation is completely inhibited.

Figure 12. Prothrombinase-curves of figure 11

TG measured in diluted NPPP at constant concentration of calcium, citrate, prothrombin and antithrombin in the presence of 4 μ M PL, 5 pM TF (figure A); 4 μ M PL, 1 pM TF (figure B); 4 μ M PL, 1 pM TF + TM (figure C) and 4 μ M PL, 1 pM TF + APC (figure D). In each condition thrombin generation was also measured while PS was held at a concentration equal to that in 4:6 diluted plasma.

Conclusions

The more the plasma is diluted the less the Josso-loop and the intrinsic system participate in thrombin generation. The direct extrinsic pathway, in contrary, is unchained by the dilution of TFPI and this compensates amply for the loss of contribution of the Josso-loop in the 1 pM TF condition. As a result, the relative participation of the Josso-loop in prothrombinase formation decreases significantly and becomes negligible if plasma is diluted more than six times as illustrated in figure 9. This figure shows that it becomes very hard to distinguish two samples of a haemophiliac (<5% and 80% FVIII) if thrombin generation is measured in six times diluted plasma. It is essential to be aware of these shifts when results obtained with methods using higher dilutions of plasma are to be interpreted. This is the more important since methods exist in which whole blood is diluted three times (i.e. plasma ~ 5 times) [2].

The experimental condition that is nearest to normal plasma is still 2:3 diluted plasma. Therefore the question remains how the contribution of the different pathways to prothrombinase formation in undiluted plasma would be (in-vivo condition). In the light of the experiments presented in this article we expect that the contribution of the Josso-loop to prothrombinase formation in the in-vivo situation may be more important than hitherto thought.

The broadening of the thrombin generation curves upon dilution results from a second wave of prothrombinase activity which is caused by dilution of PS. If PS is not diluted or if the APC-system is activated or added exogenously, this second wave is suppressed. However, PS is able to inhibit prothrombinase-activity right from the beginning and independent of APC (figure 12). This is confirmed by the prolonging lag-times if PS is not diluted (figure 11). It supports the idea of an independent down regulation of prothrombinase activity by protein S [13, 14]. Hackeng et al. observed this APC-independent anticoagulant effect of PS particularly at low TF-concentrations and described an interplay between TFPI and PS in the inhibition of TF-activity [15]. Our data, the contrary, suggest that PS may not need TFPI to down-regulate thrombin-generation in an APC-independent way since also TFPI is diluted 96 times in the experiments shown in figure 11. We also observe inhibition by PS at high TF-concentration (figure 11A). Our data further suggest that APC is able to inhibit prothrombinase-activity independent of PS since PS is diluted 96 times in figure 11D. So, both are able to inhibit prothrombinase on their own (ETP $\pm$ 50% inhibition), but their effect is much higher (ETP $\pm$ 95% inhibition) if they are allowed to cooperate (figure 11D).

As a rule of thumb it can be postulated that dilution affects more the negative feedback mechanisms of coagulation (TFPI and protein S) than the pro-coagulant actors of the system. As a result the highest ETP is obtained if plasma

is diluted about six times (figure 2). Further a shift is observed from the intrinsic system and Josso-loop towards the direct extrinsic system.

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Chapter IX

Protocols for measuring thrombin generation

- I. Timed subsampling onto a fibrinogen solution
- II. Timed subsampling onto a chromogenic substrate
- III. Continuous chromogenic measurement of TG
- IV. Continuous fluorogenic measurement of TG (CAT)

Preface

In this chapter a short protocol is given of the techniques used for measuring thrombin generation from the early fifties until now. Of the CAT-method a more extensive description is given together with possible applications. As such an overview is given of measuring thrombin generation during half a century. Each of these methods has its benefits and disadvantages and each of them can still be required to solve a specific research problem.

Timed subsampling onto a fibrinogen solution is the oldest method and may seem outdated. However, it is the only method able to measure α -thrombin alone without meizo-thrombin and α 2M-thrombin since only α -thrombin is able to convert fibrinogen into fibrin. This technique can be applied as well in PPP, PRP as in whole blood. It requires extraordinary skill however and no automated or semi-automated form is available.

Timed subsampling onto a chromogenic substrate measures α -thrombin as well as meizo-thrombin and α 2M-thrombin. It can be carried out in PPP and in PRP but only the free activity, in the fluid phase, is measured. This technique allows the researcher to add drugs or other components (e.g. a clot) to the system while TG is going on. It is also possible to induce shear-stress by stirring. This has been shown to be important in patients with von Willebrand's disease. Thrombin generation is significantly impaired in patients with von Willebrand's disease. Using the subsampling method the defect is much more pronounced in PRP than in PPP [1] while in the CAT-method this could not be demonstrated [2]. This is due to the fact that the role of vWF in the thrombin-induced procoagulant response of platelets requires at least a minimal shear, as shown by Briede et al. [3].

A variation on this technique is subsampling onto an AT-heparin mixture which makes it possible to measure only α 2M-thrombin and meizo-thrombin.

Continuous chromogenic measurement of TG detects also α -thrombin, meizo-thrombin and α 2M-thrombin. It can only be carried out in defibrinated PPP but this technique makes it possible to measure thrombin generation curves at high throughput. Furthermore, there is no need for continuous individual calibration (in opposite with the next method) since there is no inner-filter-effect or significant substrate-depletion because substrates with favourable kinetic profile and solubility are available. Also the color of plasma has no influence on this type of measurements. The method is at the basis of a commercial thrombin generation measurement setup.

Continuous fluorogenic measurement of TG detects α -thrombin, meizothrombin and α_2 M-thrombin and is just as the previous technique able to measure thrombin generation curves at high throughput. The big advantage of this method is that TG can be measured in the presence of fibrin and platelets and that not only the free thrombin is measured but also the clot-bound thrombin. Continuous individual calibration is an absolute requirement because of inner-filter-effect, substrate depletion and the influence of the color of plasma (see chapter V).

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Timed subsampling onto a fibrinogen solution [1, 2]

General procedure:

In the early fifties, thrombin generation was obtained by timed subsampling of aliquots of clotting whole blood or recalcified citrated plasma onto a fibrinogen solution. Clotting times were measured for each time-point and converted into thrombin units via a calibration curve established with an international thrombin standard. As such a thrombin generation curve was constructed.

Preparation and handling materials:

Here is described how the materials were obtained at that time. Nowadays, if one would perform this experiment, other materials might be preferred.

Fibrinogen Solution

Plasma was treated with $\text{Al}(\text{OH})_3$ to remove prothrombin and the fibrinogen was separated from this prothrombin-free plasma by two successive precipitations with ammonium-sulfate (pH 6.6) and dialysis against citrate saline. The fibrinogen solutions used had a concentration of 2.5 g/l in 0.15 M NaCl.

Thrombin calibration curve

A curve was constructed relating fibrinogen clotting times to concentrations of thrombin (repeated for each batch of fibrinogen). The international thrombin standard used was defined as followed: one thrombin unit is the amount of thrombin that converts one ml of fibrinogen at 2.5 g/l in 15 seconds. A series of 10 thrombin-dilutions was made (0.5 – 20 U/ml) and 100 μl of each dilution was added to a tube at 37°C containing 300 μl of fibrinogen solution. The clotting time in each tube was recorded and a simple curve of clotting times plotted against concentration of thrombin was used for determining the thrombin content of an unknown solution (U/ml).

Protocol:

Immediately after venipuncture, 2 ml of blood is added to a siliconised glass centrifuge tube containing 200 μl of saline. This tube is shaken gently to mix the content well and set at 37°C. The purpose of the saline is to control the volume of reagents added in other experiments. After 1 minute 100 μl of the blood is added to the first of a row of tubes, also at 37°C, each containing 300 μl of fibrinogen solution. This is repeated further at one-minute intervals as soon as possible and the clotting times of these blood-fibrinogen mixtures are recorded. From these clotting times the thrombin concentration in the whole blood at each time of sampling is assessed via the thrombin calibration curve. By plotting thrombin concentration against time, a thrombin generation curve is obtained.

The fibrin formed in the centrifuge tube during this sampling process is pressed to one side of the tube and removed by mean of a wooden swab-stick to which it adheres conveniently. In this way fluid samples can continue to be taken without interruption.

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Timed subsampling onto a chromogenic substrate [1, 2]

General procedure:

Thrombin itself is measured directly with the aid of a chromogenic substrate (S2238). The thrombogram is obtained via determination of the thrombin content in small subsamples taken at short intervals from clotting blood or plasma. Usually defibrinated plasma is used but it is also possible to measure thrombin generation in normal PPP or PRP. In this case the fibrin clot formed in the reaction mixture during the sampling process is wound on a small spatula and discarded. In this way fluid samples can continue to be taken without interruption. The sampling time is automatic recorded and also semi-automatic measurement of the thrombin activities is possible which allows executing experiments in parallel.

Preparation and handling materials:

1) Platelet poor plasma:

The blood is obtained through antecubital venipuncture. Free flow or minimal suction is employed; vacuum containers are avoided. The blood is collected in plastic tubes of 10 ml containing 1 ml sodium-citrate (0.13 M) put on room temperature 0.5 h in advance. While collecting the blood each tube is tilted slowly three times so as to mix the content well without causing contact-activation. Presenting air bubbles are pricked through. The blood is centrifuged at 2900 g for 10 min at room temperature and the plasma is aspirated with a pipette and centrifuged a second time (idem). In order to avoid contamination with procoagulant microparticles from aging platelets, PPP is prepared as quick as possible.

Defibrinated PPP:

Ancrod is added to PPP so as to reach a final concentration of 1 U/ml. The plasma is mixed well (vortex) and kept at 37 °C for 10 minutes. Then it is put on ice for 10 minutes. To remove the clot one has to turn very slowly with a spoon against the wall of the tube and take out the clot. Because Ancrod does not cause haemolysis, it can also be added to whole blood, rendering defibrinated plasma directly upon centrifugation.

Ancrod, the fibrinogen clotting enzyme of the Malayan Pit Viper, was the commercial preparation Arvin (Knoll AG, Ludwigshafen, Germany).

Buffers:

Buffer A: 50 mM Tris.HCl, 100 mM NaCl, 0.5 g/l bovine serum albumin, pH 7.35

Buffer B: 50 mM Tris.HCl, 100 mM NaCl, 0.5 g/l BSA, 20 mM EDTA, pH 7.9

Substrate:

The chromogenic substrate used for thrombin is S2238 (H-D-Phe-Pip-Arg-pNA.2HCl).

Trigger

As a trigger recombinant tissue factor (rTF) is used. Recombinant tissue factor not containing polybrene or Ca^{++} was a kind gift from Dade Behring (Marburg, Germany).

Phospholipids vesicles:

20% phosphatidylserine and 80% phosphatidylcholine

Protocol:

Set-up:

To 240 μl defibrinated plasma is added 40 μl of buffer A and 20 μl phospholipids-solution and this mixture is incubated at 37 °C for 5 minutes. Buffer A may contain any substance of which the influence on thrombin generation has to be investigated. Coagulation is triggered at zero time with 60 μl trigger-solution containing CaCl_2 and rTF (for the extrinsic pathway). For the intrinsic pathway defibrinated plasma is incubated with 40 μl of buffer A in which 0.25 mg/ml kaolin is suspended. After 4 min, 20 μl phospholipids-solution is added and 5 min after the start of the incubation thrombin generation was triggered with 60 μl trigger-solution containing CaCl_2 . If necessary the volumes indicated may be proportionally altered.

After addition of the trigger, 10 μl of the reaction mixture is sampled at intervals of about 10-15 seconds in disposable plastic microcuvettes (at 37 °C) with 490 μl buffer B containing 200 μM of S2238. After sampling the cuvette is immediately and rigorously mixed on a Vortex mixer. The EDTA in buffer B neutralizes the Ca^{2+} present in the cuvette what makes coagulation to stop. The already formed thrombin reacts with S2238 and after about 2 minutes the reaction in the cuvette is stopped by the addition of 300 μl citric acid (1 M). The cuvette is again thoroughly mixed and can be kept at room temperature until reading. The optical density (OD) is read at 405 nm in a suitable spectrophotometer (LKB-Ultrospec) or Hemker-photometer. The generation of p-Nitroaniline is linear in time up to an OD of 0.9. If necessary incubation times longer or shorter than two minutes can be used so as to obtain a suitable sensitivity and not to exceed 0.9 OD.

The subsampling and the stopping pipettes are time recording pipettes (Gilson, France). It is essential to keep the plunger of the pipette down after subsampling and to wipe the pipette tip carefully in order to avoid contamination of the incubation mixtures with EDTA from the buffer in the cuvette. Plastic tubes can be

used instead of cuvettes but glass tubes have to be avoided because they adsorb thrombin and give unpredictable results.

If TG is measured in PRP, 240 μ l of plasma is diluted with 60 μ l buffer A and incubated at 37°C for 5 min. Coagulation is triggered with 60 μ l CaCl_2 (100 mM) containing rTF equivalent to a final concentration of 7.5 pg/ml.

Material-list:

Defibrinated PPP, buffer A and B, substrate, rTF, CaCl_2 , phospholipids (20% PhS, 80% PhC)

Preparations:

Trigger solution for the extrinsic system contains rTF (1/240) in 100 mM CaCl_2 (final concentration 16.7 mM). Clotting time around 30 s.

Trigger solution for the intrinsic system contains 100 mM CaCl_2 .

Phospholipids-solution contains 20 μ M PhS/PhC.

Data-handling:

The spectrophotometer as well as the time recording pipette is connected to a personal computer that calculates the increase of OD in time (OD/min) from the measured OD values and the times of subsampling and stopping. Thrombin amidolytic activities are calculated by comparing the OD/min values of the samples to those of a standard calibration curve prepared using known amounts of human α -thrombin (active site titration). Also a correction has to be made for the α 2M-T complex. This is done by a simple algorithm based on the fact that the α 2M-T formation velocity is proportional to the concentration of free thrombin. This algorithm is used in a computer program that calculates free thrombin curves from the amidolytic activity curves.

Protocol for determining the decay constant of thrombin in plasma:

In the same way, using the sub-sampling technique, the decay constant of thrombin in plasma can be determined. Instead of triggering thrombin generation, a known amount of thrombin is added to defibrinated PPP and by subsampling the rate of reaction with AT (in plasma) is measured.

Method:

At zero time 60 μ l of h-T (1500 nM) is added to 240 μ l defibrinated PPP and 60 μ l buffer A, at 37°C. Sub-sampling is started immediately at suitable intervals (5-10 s) by adding aliquots of 10 μ l into cuvettes with 490 μ l Buffer B containing 200 μ M S2238. After sampling the cuvette is quickly and rigorously mixed on a Vortex mixer and after about 2.5 minutes the reaction in the cuvette is stopped by the addition of 300 μ l citric acid (1 M). The cuvette is thoroughly mixed and kept at room temperature until reading. The optical density (OD) is

read at 405 nm and amidolytic activities are calculated (see before: data handling).

The amidolytic activities (C_t) were fitted to the three parameter curve $C_t = C_\infty + (C_0 - C_\infty) \cdot e^{-k_{\text{dec}} \cdot t}$, where C_0 is the initial amidolytic activity and C_∞ is the residual, steady end-level activity due to the $\alpha_2\text{M-T}$ complex.

The decay constant (k_{dec}) is the sum of the AT dependent part (k_1) and the $\alpha_2\text{M}$ dependent part (k_2). The ratio $R = k_{\text{dec}}/k_2$ is equal to C_0/C_∞ , i.e. the ratio of the amount of thrombin added and the amount of $\alpha_2\text{M-T}$ at the end of the experiment. This ratio can be determined because of the persistent amidolytic activity of the $\alpha_2\text{M-T}$ complex.

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Continuous chromogenic measurement of TG [1, 2]

General procedure:

A slow reacting chromogenic substrate (SQ68) is added directly to clotting plasma. The reaction mixture consists of four parts of defibrinated plasma, one part of trigger solution and one part of start solution. The reactions are carried out in a laboratory automaton as e.g. the Cobas Bio and Cobas Fara centrifugal analysers (F. Hoffmann-La Roche, Basel, Switzerland) capable of measuring the optical density at 405 nm at 30 s intervals during 12 to 15 min. This method is restricted to defibrinated platelet poor plasma since clot-formation would disturb the OD-measurement. Defibrination of large series of samples is the rate limiting step of the procedure.

Preparation and handling materials:

Platelet poor plasma:

Idem see 'Timed subsampling onto a chromogenic substrate'.

Defibrinated PPP:

Idem see 'Timed subsampling onto a chromogenic substrate'.

Buffers:

Hepes-buffer: 20 mM Hepes-NaOH, 150 mM NaCl, 0.5 g/l bovine serum albumin, pH 7.35

Substrate:

Malonyl- α -aminoisobutyryl-arginine para-nitroanilide methyl ester hydrochloride (SQ68) was synthesized by Serbio Laboratories, France.

Trigger

As a trigger recombinant tissue factor and Actin FS[®] is used. Recombinant tissue factor not containing polybrene or Ca⁺⁺ was a kind gift from Dade Behring (Marburg, Germany). Actin FS[®] was a kind gift of Dade Productions (Düdingen, Switzerland), a suspension of ellagic acid and soy bean phosphatides.

Protocol:

Set-up:

In the Cobas machine 80 μ l of defibrinated plasma with 20 μ l of rTF without Ca²⁺ are incubated for 30 seconds at 37 °C, which is sufficient for temperature equilibration. Thrombin generation is started by adding 20 μ l of a pre-warmed start solution containing 100 mM CaCl₂ and 3 mM substrate. After the start of the reaction the optical density at 405 nm is recorded at intervals of maximally

30 s for at least 12 min. Instead of 80 μ l plasma it is also possible to add 75 μ l plasma and 5 μ l of a substance to be tested.

Material-list:

Defibrinated PPP, Tris-buffer, Hepes-buffer, substrate, rTF, Actin FS[®], CaCl₂

Preparations:

Trigger solution for the extrinsic system contains about 300 pM rTF in Hepes-buffer.

Trigger solution for the intrinsic system contains 1: 2.5 diluted Actin FS[®] in Hepes-buffer.

Start solution contains 100 mM CaCl₂ and 3 mM substrate in Hepes-buffer.

Data handling:

Data handling is undertaken on a personal computer connected to the centrifugal analysers. A series of OD readings and the times of the readings are transformed to give the OD as a function of time and from these data the ETP can be calculated off-line. A correction has to be made for the α 2M-T complex as seen in the subsampling method with a chromogenic substrate. This is done by a simple algorithm based on the fact that the α 2M-T formation velocity is proportional to the concentration of free thrombin. This algorithm is used in a computer program that calculates free thrombin curves from the amidolytic activity curves.

References

1. Hemker, H.C., et al., *Continuous registration of thrombin generation in plasma, its use for the determination of the thrombin potential*. Thromb Haemost, 1993. 70(4): p. 617-24.
2. Wielders, S., et al., *The routine determination of the endogenous thrombin potential, first results in different forms of hyper- and hypocoagulability*. Thromb Haemost, 1997. 77(4): p. 629-36.

Continuous fluorogenic measurement of TG: CAT-method

General procedure:

Principle

Thrombin generation is monitored by a fluorogenic substrate (ZGGR-AMC) added to the plasma at time zero together with the trigger (calcium). The thrombogram is measured in a 96-well plate fluorometer (Ascent Reader, Thermolab-systems, Helsinki, Finland) equipped with a 390/460 filter set (excitation/emission) and a dispenser used for the addition of the substrate-calcium-solution. Transparent round-bottom 96-well plates (Immulon 2HB) have to be used. If flat-bottom plates are used the optical unit has to be changed so as to read from beneath. Each experiment consists of two sets of readings, one with a known fixed amount of thrombin activity (calibrator, CAL), the other in which thrombin generation (TG) occurs. By continuous comparison of the signal from the two wells the course of thrombin concentration is calculated in real time and displayed on the screen by a dedicated software program (Thrombinoscope BV, Maastricht, The Netherlands). For explanation about the use of the software see the manual of Thrombinoscope BV.

Set-up of an experiment

Each plasma-sample requires its own calibrator-measurement (see chapter II and IV). The recommended set-up of an experiment is in quadruplicate. This means 4 CAL-wells and 4 TG-wells per plasma-sample. This is called a group. As such 12 groups can be measured in one experiment. If the same plasma is measured under different conditions (e.g. another drug tested) different groups can share the same calibrator.

Before measuring thrombin generation each well is filled with 20 μ l calibrator (CAL-wells) or reagents (TG-wells) and 80 μ l plasma. The reaction is started with 20 μ l of a solution, containing calcium and substrate, added via the dispenser ($t=0$). This solution of calcium and substrate is called FluCa. The final volume in each well is 120 μ l. The reagents (RG) consist of a buffer-solution containing trigger, procoagulant phospholipids and/or any reagent one likes to test. As trigger recombinant tissue factor or innovin is used (commercially available). FluCa is a buffer-solution containing substrate (2.5 mM) and Ca^{2+} (100 mM). After the plate is filled with calibrator, reagents and plasma, the plate is put in the fluorometer at 37°C for minimally 5'. Meanwhile the dispenser of the fluorometer is flushed with a warm Ca^{2+} -solution (100 mM), emptied and flushed again with FluCa. The reaction is started by dispensing 20 μ l FluCa into the wells. The software program registers this as zero-time, shakes the plate for 10 s and starts reading. The interval between two readings of the fluorescence signal and the total time that TG is measured can be chosen.

Preparation and handling materials:

1) *Platelet poor plasma:*

Idem see 'Timed subsampling onto a chromogenic substrate'.

Also a pool of platelet poor plasma from healthy subjects is made (NPPP). This is used as reference plasma. All the plasma of these subjects is collected and the pool is ultra-centrifuged at 2900 g for 1h at 4 °C. PPP and NPPP are stored in the freezer at -80 °C. Just before each experiment the plasma is thawed, held on ice and a few minutes in advance the plasma is put at room temperature.

2) *Platelet rich plasma:*

The blood is collected identically as for PPP. Eventually CTI is added together with the sodium-citrate to avoid contact-activation (0.8 μ M final concentration). The blood is centrifuged at 265 g for 10 min at room temperature (>20 °C) and the plasma is aspirated with a pipette. The result is PRP.

PRP does not always contain the same amount of platelets. For a standard PRP measurement it is advised to adjust the platelets at 150 000 pt/ μ l. To do so, a few ml of the PRP is kept at 37 °C, 200 μ l is kept separately to measure the platelets and the rest of the PRP is centrifuged at room temperature (>20 °C) at 2900 g for 10 minutes. The result is PPP and does not contain any platelets anymore.

During this centrifugation step the platelets of the PRP are measured in the Coulter-counter (~ 300 000 tot 500 000 pt/ μ l) which is turned on and primed 0.5 h in advance. Avoid air bubbles during the measurement. Next, the few ml PRP at 37 °C is adjusted with PPP to obtain 150 000 pt/ μ l. The PRP has to be tilted slowly a few times before pipetting. The adjusted PRP is kept at 37 °C.

PRP has to be handled gently (do not vortex!) and kept above 20 °C to avoid activation of the platelets. The experiment should be started within 1 h after venipuncture. The PRP can not be frozen because this would cause the platelets to breakdown.

3) *Calibrator*

The calibrator is provided by Thrombinoscope (Maastricht, The Netherlands) and contains lyophilized α 2M-T equal to an activity of 600 nM h-T after reconstitution. It is stored at 4 °C and is dissolved in 1 ml milli-Q and kept on ice.

4) *Buffers*

Two buffers are used: 'BSA5' for preparing dilutions of the reagents and 'BSA60' for the FluCa. BSA5 contains 20 mM Hepes, 140 mM NaCl, 0.02% NaN_3 and 5 mg/ml BSA at pH 7.35. BSA60 contains 20 mM Hepes, 0.02 % NaN_3 and 60 mg/ml BSA at pH 7.35. To dissolve BSA it is brought on top of the buffer without stirring to allow it to dissolve slowly. This can take more than an hour. Next the buffer is filtered using a corning filter system 255 ml

with 0.2 μm PES membrane. The buffers are stored in the freezer at $-20\text{ }^{\circ}\text{C}$. They are kept at room temperature during an experiment.

5) *Phospholipids (PL)*

The phospholipids used are a mixture of 60% Phosphatidyl choline (PhC), 20% phosphatidyl serine (PhS) and 20% phosphatidyl ethanolamine (PhE). The stocks are obtained at Avanti (USA). The phospholipids are stored in the freezer at $-80\text{ }^{\circ}\text{C}$. They are kept at room temperature during an experiment.

Example of a 2 ml preparation of 1 mM PhS-PhE-PhC (20-20-60):

Stocks: - PhS (10 mg/ml; 2.5 ml; MW: 810.03; CHCl_3) $\rightarrow$ 12.34 mM

- PhE (25 mg/ml; 4 ml; MW: 744.04; CHCl_3) $\rightarrow$ 33.60 mM

- PhC (25 mg/ml; 4 ml; MW: 786.15; CHCl_3) $\rightarrow$ 31.80 mM

32 μl PhS + 12 μl PhE + 38 μl PhC is pipetted in a glass tube. Nitrogen-gas is blown in the tube until all CHCl_3 has disappeared and two ml buffer is added (140 mM NaCl, 20 mM Hepes, pH 7.35). The resulting solution is a trouble suspension and has to be sonicated for 10 min with a MSE Mark II 150-watt ultrasonic disintegrator, set at 9 microns peak to peak amplitude until the solution is clear. Alternatively the trouble PL-suspension can also be extruded through a 400 nm filter until the solution is clear.

6) *Trigger*

As a trigger both recombinant tissue factor and innovin (Inn) can be used. Recombinant tissue factor not containing polybrene or Ca^{++} was a kind gift from Dade Behring (Marburg, Germany) and was used previously in our lab. Lyophilized innovin is commercially available (Dade-Behring) and is used nowadays in our lab. It has to be dissolved in 10 ml milli-Q and this solution has the same activity as a TF-solution of 6 nM. Dissolved Inn is stored in the freezer at $-80\text{ }^{\circ}\text{C}$ and is kept on ice during an experiment.

7) *Substrate*

The substrate (Z-Gly-Gly-Arg-AMC) is commercially available (Bachem, Switzerland). A solution is made of 100 mM substrate in DMSO and stored in the freezer at $-80\text{ }^{\circ}\text{C}$.

8) *FluCa*

About 10 minutes before measuring thrombin generation the FluCa solution is prepared. This is a mixture of buffer (BSA60), substrate and CaCl_2 so as to obtain a concentration of 2.5 mM substrate and 100 mM CaCl_2 . This mixture is immediately vigorously mixed and the resulting clear solution is kept at $37\text{ }^{\circ}\text{C}$.

9) *TM, APC, CTI, kaolin, Arvin, heparin, hirudin, melagatran, penta-saccharide*

These reagents are commercially available and are stored in the freezer at -80 °C. Kaolin is stored in the fridge. They are thawed and kept on ice during an experiment. Kaolin is kept at 37 °C.

Protocol for PPP

Thrombin generation is measured in PPP at a final concentration of 4 µM PL and 1/1200 Inn (5 pM rTF) or 1/12000 Inn (0.5 pM rTF).

Set-up:

2 groups (same calibrator)

- 4 wells with calibrator

- 4 wells with 4 µM PL and 1/1200 Inn (RG A)

- 4 wells with 4 µM PL and 1/12000 Inn (RG B)

Material-list:

PPP; PL (1 mM); Inn; calibrator; substrate (100 mM); buffers (BSA5, BSA60); CaCl₂ (1 M)

Preparations:

RG A contains 1/200 Inn and 24 µM PL in BSA5.

RG B contains 1/2000 Inn and 24 µM PL in BSA5.

TG is measured during 40 min at 15 s time-intervals.

Protocol for PRP

Thrombin generation is measured in PRP at a final concentration of 1/12000 Inn (without PL). The PRP first has to be adjusted at 150 000 platelets/µl.

Set-up:

1 group

- 4 wells with calibrator

- 4 wells with 1/12000 Inn (RG A)

Material-list:

PRP; Inn; calibrator; substrate (100 mM); buffers (BSA5, BSA60); CaCl₂ (1 M)

Preparations:

RG A contains 1/2000 Inn in BSA5.

TG is measured during 60 min at 15 s time-intervals.

Applications:

1) Check quality of platelet poor plasma

TG is measured in PPP without Inn (only PL) and without PL (only Inn). So the reaction mixture contains 4 μM PL or 1/1200 Inn.

Set-up:

- 2 groups (same calibrator)
- 4 wells with calibrator
- 4 wells with 4 μM PL (RG A)
- 4 wells with 1/1200 Inn (RG B)

Material-list:

PPP; PL (1 mM); Inn; calibrator; substrate (100 mM); buffers (BSA5, BSA60); CaCl_2 (1 M)

Preparations:

RG A contains 24 μM PL in BSA5.
RG B contains 1/200 Inn in BSA5.

TG is measured during 60' at 15'' time-intervals.

2) Measure the intrinsic system in PPP

TG is measured in PPP activated with kaolin at a final concentration of 1 μM PL (no trigger).

Set-up:

- 2 groups (same calibrator)
- 4 wells with calibrator
- 4 wells with 4 μM PL and 1/1200 Inn (RG A)
- 4 wells with 1 μM PL (kaolin-activated PPP) (RG B)

➔ The wells with kaolin-activated plasma have to be filled with 90 μl plasma (instead of 80 μl) and 10 μl RG-solution (instead of 20 μl) so as to compensate for the extra dilution of the plasma during activation. For the same reason the calibrator needs not to be measured separately in kaolin-activated plasma since the plasmas are diluted equally in the well.

Material-list:

PPP; PL (1 mM); Inn; kaolin (0.5 mg/ml); CTI (83 μM); calibrator; substrate (100 mM); buffers (BSA5, BSA60); CaCl_2 (1 M)

Preparations:

RG A contains 1/200 Inn and 24 μM PL in BSA5.

RG B contains 12 μM PL in BSA5.

Kaolin-activated plasma:

Kaolin has to be kept at 37°C and needs to be mixed well (vortex) before use. 80 μl PPP is activated with 10 μl kaolin (0.5 mg/ml) and the plasma is kept at 37 °C for 7 minutes. The time is important and has to be followed exactly because de-activation of clotting-factors by kaolin can happen if the time is preceded. The plasma-tube has to be tilted a few times during these 7 minutes so as to avoid sedimentation of the kaolin. After this activation step the plasma is centrifuged for 5 minutes at 10 000 g. A little pellet of kaolin can be seen at the bottom of the tube and the plasma is separated from this pellet. All these steps have to be done as fast as possible.

TG is measured during 60 min at 15 s time-intervals.

Remark: if PPP is treated with CTI the contact-activation by kaolin is inhibited. The standard concentration of CTI used to inhibit contact-activation is 0.72 μM .

3) Check the APC-system of plasma in PPP and PRP

TG is measured in PPP with TM to activate the APC-system of the plasma or with exogenous added APC to check its influence on thrombin generation.

Set-up:

3 groups (same calibrator)

- 4 wells with calibrator

- 4 wells with 4 μM PL and 1/1200 Inn (RG A)

- 4 wells with 4 μM PL, 1/1200 I and 6 nM TM (RG B)

- 4 wells with 4 μM PL, 1/1200 I and 6 nM APC (RG C)

Material-list:

PPP; PL (1 mM); Inn; TM (1 μM); APC (600 nM); calibrator; substrate (100 mM); buffers (BSA5, BSA60); CaCl_2 (1 M)

Preparations:

RG A contains 1/200 Inn and 24 μM PL in BSA5.

RG B contains 1/200 Inn, 24 μM PL and 36 nM TM in BSA5.

RG C contains 1/200 Inn, 24 μM PL and 36 nM APC in BSA5.

TG is measured during 40 min at 15 s time-intervals.

Remark: The effect of the APC-system can also be measured in PRP but in this case only 1/12000 Innovin is added and no PL.

4) Check the influence of fibrinogen on TG in PPP

TG is measured in PPP and in PPP defibrinated with Arvin.

Set-up:

2 groups with normal PPP (same calibrator)

- 4 wells with calibrator
- 4 wells with 4 μM PL and 1/1200 Inn (RG A)
- 4 wells with 4 μM PL and 1/12000 Inn (RG B)

2 groups with PPP without fibrinogen (same calibrator)

- 4 wells with calibrator
- 4 wells with 4 μM PL and 1/1200 Inn (RG A)
- 4 wells with 4 μM PL and 1/12000 Inn (RG B)

Material-list:

PPP; PL (1 mM); Inn; Arvin or Ancrod (50 U/ml); calibrator; substrate (100 mM); buffers (BSA5, BSA60); CaCl_2 (1 M)

Preparations:

RG A contains 1/200 Inn and 24 μM PL in BSA5.

RG B contains 1/2000 Inn and 24 μM PL in BSA5.

PPP without fibrinogen:

Arvin is added to PPP so as to reach a final concentration of 1 U/ml. The plasma is mixed well (vortex) and kept at 37 °C for 10 minutes. Then it is put on ice for 10 minutes. To remove the clot one has to turn very slowly with a spoon against the wall of the tube and take out the clot.

TG is measured during 60 min at 15 s time-intervals.

Remark: This is only possible in PPP because in PRP platelets would be removed as well together with the clot.

5) The effect of several anti-coagulantia on thrombin generation in PPP and PRP

TG is measured in PPP with several anti-coagulantia as there are unfractionated heparin (UFH), Melagatran (M), Hirudin (H) and Penta-saccharide (Penta-S)

Set-up:

10 groups (same calibrator)

- 4 wells with calibrator
- 4 wells with 4 μM PL and 1/1200 Inn (RG A)
- 4 wells with 4 μM PL, 1/1200 Inn and 0.04 U/ml UFH (RG B)
- 4 wells with 4 μM PL, 1/1200 Inn and 0.3 μM M (RG C)

- 4 wells with 4 μ M PL, 1/1200 Inn and 0.4 μ g/ml H (RG D)
- 4 wells with 4 μ M PL, 1/1200 Inn and 5 μ g/ml Penta-S (RG E)
- 4 wells with 4 μ M PL and 1/12000 Inn (RG F)
- 4 wells with 4 μ M PL, 1/12000 Inn and 0.04 U/ml UFH (RG G)
- 4 wells with 4 μ M PL, 1/12000 Inn and 0.3 μ M M (RG H)
- 4 wells with 4 μ M PL, 1/12000 Inn and 0.4 μ g/ml H (RG I)
- 4 wells with 4 μ M PL, 1/12000 Inn and 5 μ g/ml Penta-S (RG J)

Material-list:

PPP; PL (1 mM); Inn; UFH (1 U/ml); Hirudin (100 μ g/ml); Melagatran (30 μ M); Penta-saccharide (100 μ g/ml); calibrator; substrate (100 mM); buffers (BSA5, BSA60); CaCl_2 (1 M)

Preparations:

RG A contains 1/200 Inn and 24 μ M PL in BSA5.
RG B contains 1/200 Inn, 24 μ M PL and 0.24 U/ml UFH in BSA5.
RG C contains 1/200 Inn, 24 μ M PL and 1.8 μ M M in BSA5.
RG D contains 1/200 Inn, 24 μ M PL and 2.4 μ g/ml H in BSA5.
RG E contains 1/200 Inn, 24 μ M PL and 30 μ g/ml Penat-S in BSA5.
RG F contains 1/2000 Inn and 24 μ M PL in BSA5.
RG G contains 1/2000 Inn, 24 μ M PL and 0.24 U/ml UFH in BSA5.
RG H contains 1/2000 Inn, 24 μ M PL and 1.8 μ M M in BSA5.
RG I contains 1/2000 Inn, 24 μ M PL and 2.4 μ g/ml H in BSA5.
RG J contains 1/2000 Inn, 24 μ M PL and 30 μ g/ml Penta-S in BSA5.

TG is measured during 80 min at 15 s time-intervals.

Remark: The effect of these anti-coagulantia on thrombin generation can also be measured in PRP but in this case only 1/12000 Innovin is added and no PL.

6) Multiple-dose studies on new drugs

See standard-protocol of PPP.

Important is here that each plasma-sample is measured with its own calibrator.

7) Determine pharmaco-dynamics of new drugs

See standard-protocol of PPP.

Important is here that each plasma-sample is measured with its own calibrator.

Chapter X

General discussion and conclusions

Thrombin generation as an overall-function test in clinical labs

Thrombosis is the cause behind common diseases as stroke and myocardial infarction and thereby has become the second cause of death and disease in the western world [1]. Of the three components that, according to Virchow, foster thrombosis, i.e. the vessel wall, blood flow and coagulability, the latter one remains the most elusive. The mechanism of blood coagulation may be known in astonishing detail but, despite decades of investigation in the field, clinicians do not have a universal test at their disposal to determine this “coagulability”. Several clotting-time tests are available, each one giving information about another detail of the complicated web of blood coagulation reactions. None of these is an adequate overall function test like e.g. the electro-cardiogram for heart rhythm problems. This thesis contributes to the question whether thrombin generation is a suitable candidate for this role.

How is it possible that a test that is known for over half a century only now postulates for this function ? Three main raisons can be pointed out.

In the first place the test was “out of fashion” and pushed into oblivion by the advent of modern biochemical and molecular biology techniques. In the second place it was labor intensive and unsuitable for application in the modern, largely automated, clinical laboratory and in the third place – maybe the most important - it did not fit in the traditional view of primary and secondary haemostasis.

During the last few years things are changing. In research laboratories it becomes more and more recognized that thrombin plays a pivotal role not only in secondary haemostasis and venous thrombosis but also in arterial thrombosis and primary haemostasis, right from the beginning when the platelet-reactions start. Therefore the thrombin generating capacity of blood is more and more being accepted as a – if not the - central element in the physiological function of haemostasis.

Thrombin generation was slowly being brought back into fashion by the work of Béguin and Hemker, that in a series of articles that started in 1986 showed how this technique could be used for the unraveling of the mode of action of heparins and other antithrombotics and for the recognition of the receptors that are responsible for the platelet procoagulant reaction.

As a consequence several research laboratories are exploring manners to follow-up thrombin generation in the fluid organ blood. The ideal way to assess the function of the thrombin generating system in a patient would be measuring the concentration of thrombin as a function of time and space in a platelet aggregate at the site of a lesion. This is technically impossible at this moment but a good approximation became feasible with the arrival of Calibrated Automated Thrombography. With this method it is possible to measure thrombin generation in platelet rich plasma or platelet poor plasma in the presence of elements of the

vessel wall, i.e. tissue factor and thrombomodulin. This technique is the issue of this thesis.

Calibrated Automated Thrombography (CAT)

The CAT-method, presented in **chapter II**, makes it possible to measure thrombin generation at high throughput and with an acceptable experimental error (<5%) which makes it a promising tool for clinical routine-labs. Von Willebrand's disease can be detected [2] and the effect of platelet-inhibitors (e.g. aspirin, clopidogrel) can easily be followed [3, 4]. It is also possible to obtain useful partial information from observations in PPP e.g. detect deficiencies in clotting factors (except factor XIII) or observe the effect of anticoagulants (Antivitamin K, heparin (-likes), direct thrombin- or FXa-inhibitors). This is the more important since there is no single clotting-time test that reacts similarly to these different types of anticoagulants. Also the function of the protein C system can be probed both in PPP and in PRP. Addition of TM allows to detect the function of the entire system, but, as thrombin is required for TM to express its activity, TM only affects the second half of the thrombin generation curve (peak-height, ETP) but not the lag-time which makes its effects not detectable with clotting-time tests.

Particularly interesting is the lupus anticoagulant. This antibody causes an increase of the clotting time and thus is deemed "anticoagulant" but it also brings about a thrombotic tendency. This is called the 'LAC paradox'. The LAC-paradox disappears as soon as one resorts to thrombin generation where it is seen that indeed the lag-time (=clotting time) is prolonged but also that thrombin generation is not inhibited by activation of the protein C system [5]. This underlines that there exists no strict relation-ship between the length of the initiation phase (~clotting-time) and the amount of thrombin to be formed (95% of thrombin is formed after clotting). Therefore measuring a thrombogram produces much more information than a clotting time.

Calculation of the TG curve starting from raw data

In **chapter III** it is shown that two fit-procedures are needed for the conversion of the raw data into a TG curve. The first one is needed to convert the raw data into 'ideal data' i.e. data that would have been obtained if substrate consumption and inner filter effect would not occur. The second one is carried out on the 'ideal' progress curve and is needed to obtain the course of free thrombin from the course of amidolytic activity, the latter being the sum of free thrombin and $\alpha 2\text{M-T}$. Another advantage of fitting the progress-curve is that we avoid large

experimental scatter that arises from working with the first derivative of experimental data.

Calculation of prothrombinase-activity

Thrombin is the pivotal enzyme in the coagulation cascade and FII-ase activity is the final step in the formation of thrombin. The FII-ase activity is dependent on the components of the FII-ase complex: factor Xa, factor Va and the phospholipids, which in their turn are dependent upon the function of the other clotting factors and regulatory systems and the platelets. The FII-ase activity therefore reflects the activity of the clotting system but not that of thrombin breakdown. In **chapter IV** it is shown how the FII-ase activity can be calculated starting from the thrombin generation curve, if the initial concentration of AT is known. It can be expected – but it remains to be demonstrated in practice – that this type of curve reflects the physiology of thrombin forming processes with more sensitivity than the thrombin generation curve itself.

Necessity of continuous individual calibration

In order not to miss abnormalities in thrombin generation it is of the utmost importance that the experimental error is reduced to a minimum. In the CAT-method the splitting of a fluorogenic substrate is monitored by comparing it to a constant known thrombin activity in a parallel, non-clotting sample. As such a calibration factor (CF) is assessed during the complete experiment under the same conditions as that prevailing in the thrombin generation experiments. This is necessary to obtain clinically acceptable intra-individual coefficients of variation as is shown in **chapter V**. There we compared the intra-individual experimental error obtained with two commercial available methods. One is the CAT-method [6] using continuous individual plasma calibration, the other one the Technoclone-method [7] using a fixed CF based on initial thrombin activity measured in buffer. It is shown that a fixed CF in buffer introduces serious underestimation since the yield of fluorescent signal depends strongly on the color of plasma. This underestimation is not constant but increases during TG due to substrate consumption and to absorption of fluorescent light by the forming fluorescent product (inner filter effect). This introduces large coefficients of variation which can be reduced to clinically meaningful levels by using continuous individual plasma calibration.

Influence of signaling substrate on thrombin generation

Measuring a system always means disturbing it. During thrombin generation a signaling substrate is added which binds to thrombin and therefore also inhibits thrombin. Since thrombin plays a pivotal role in its own formation via several feedback mechanisms, inhibition of thrombin will, theoretically, influence prothrombin conversion. We investigated already what this disturbance amounts to at the time that we proposed the CAT-method (chapter 2). We showed that under our conditions the proposed substrate did not affect prothrombin conversion significantly and that thrombin decay is slowed down in a predictable manner because the artificial substrate added inhibits the inactivation of thrombin by plasmatic antithrombins. Since this subject is clinically extremely relevant and since Butenas S and K.G. Mann expressed their concern about this issue we showed additional evidence as presented in **chapter VI**. We concluded that not only the amount of prothrombin converted but also the time course of prothrombin conversion are minimally affected by the presence of ZGGR-AMC at 416 μM . The inactivation of thrombin by plasmatic antithrombins is slowed down in an exactly predictable extent. Thrombin-driven feedback mechanisms are affected although this does not cause an important increase in the lag-time. They do cause, however, a reduction in the inhibition of added TM by about 10%. It is important to stress that these conclusions are restricted to the presented substrate at the usual concentration (416 μM).

In order to restrict the discussion to the soluble system, as did Butenas and Mann, we used defibrinated plasma in these experiments. The effect of the presence of fibrin(-ogen) is further discussed in chapter VII.

Shift from chemical to diffusional control

With the arrival of the CAT-method it became possible to measure thrombin generation in the presence and in the absence of fibrinogen. This was done in platelet poor plasma at different temperatures as shown in **chapter VII**. Out of these experiments we concluded that thrombin generation shifts from chemical to diffusional control during coagulation. Until now it was granted for true that thrombin generation was determined by the initial concentrations and reaction constants of the participating components. Nevertheless, as soon as a fibrin-web forms, phospholipid platelets and/or phospholipid particles adhere onto this web and the reactants can only be transported to and from such immobilized surfaces in a stagnant fluid by diffusion through the liquid phase. Such diffusional transport will start to count at the moment of clotting. This means that diffusion starts to co-determine the rate of thrombin formation from the moment clotting takes place. In mixtures without fibrinogen or in mathematical simulation, thrombin generation is uniquely determined by the initial concentrations and

reaction constants of the components and diffusion is not taken into account. In models where prothrombinase is adsorbed at a macroscopic surface diffusion is rate-limiting at all but extreme concentrations of the reactants. None of these approaches is true since there is a shift from chemical to diffusional control during coagulation around the moment of clotting. As a consequence the best approach for assessing thrombin generation in the body is measuring it directly in a sample of the isolated organ i.e. in clotting PRP with addition of the relevant components of the vessel wall (TF, TM).

The effect of plasma dilution on thrombin generation

Measuring a system not only means disturbing it (chapter 4) it also means diluting it since a signaling substrate, calcium, trigger and/or phospholipids need to be added. In **chapter VIII** it is investigated what the influence is of dilution on thrombin generation in platelet poor plasma using the CAT-method. It is shown that the ETP increases upon 1:6 dilution of plasma and drops thereafter. This is caused by dilution of tissue factor pathway inhibitor (TFPI) and protein S. Dilution of these inhibitory mechanisms affects thrombin generation more than dilution of procoagulant mechanisms. Dilution suppresses protein S dependent inhibition and this induces a second wave of FII-ase activity which results in a prolonged decay-phase. Dilution of TFPI unchains the direct extrinsic pathway. Both phenomena result in higher (or prolonged) thrombin-activity. At the same time the intrinsic pathway and Jossen-loop are significantly reduced by dilution. The intrinsic pathway loses all activity at dilutions > 24x while the contribution of the Jossen-loop at low trigger-concentration becomes almost zero in 1:6 diluted plasma. As a consequence one has to be very careful with the interpretation of results obtained in highly diluted plasma. This is especially the case in conditions where the Jossen loop is involved as e.g. in phenotyping haemophiliacs.

Relevance for science and society

Thrombin generation is undergoing a revival since it is recognized that it is a promising means of phenotyping the clotting system. Since about half of us all will die from thrombotic disease, the medical importance of a valid overall function-test of the haemostatic-thrombotic system is hard to overestimate. In the clinical laboratories there is a great need for such a test to be able to classify patients with increased risk on thrombosis or bleeding and to follow-up and optimize anti-thrombotic or anti-bleeding therapy. Measuring thrombin generation seems promising to fulfill this need. It looks as if it is a suitable candidate for being the electro-cardiogram of the haemostatic-thrombotic system. This com-

parison, however, should not be carried beyond its limits. The thrombin forming capacity of the blood is a typical “on demand” function, whereas the ECG monitors a necessarily continuous function. Continuously ongoing thrombin generation does occur and may be increased in diseased states [8, 9]. It is detected via by-products of the coagulation process in the circulation (TAT complexes, prothrombin fragment 1-2, fibrin degradation products). Such products can be seen as “smoke-detectors” signaling an ongoing fire. The thrombin generating potential is to be seen as an indicator of the “fire-risk” i.e. the potential thrombin-forming capacity of the non triggered fluid organ blood. Another difference is that not the entire person has to be available to measure the thrombogram, a few ml of blood is sufficient, be it that the nearer the test is to physiological conditions, the nearer the patient should be to the laboratory. Thrombin generation in PPP can be conveniently carried out on frozen and thawed samples that thus can be stored and shipped. Thrombin generation in PRP needs to be done within the hour after venapuncture.

In this thesis we presented the CAT-method as a technique to measure thrombin generation. The CAT-method was originally designed as an automated routine-method to perform quick semi-automated thrombin generation experiments in the research laboratory with essentially more possibilities than previous chromogenic techniques or subsampling. However, the CAT-method is not only a technical innovation; it is also a new and better concept to probe the mechanisms behind haemostasis and thrombosis since it investigates an essentially larger portion of the physiological mechanism than previous tests. Because of the use of fluorescence as a detection mechanism turbid media can be studied. Therefore the role of the platelets, the role of fibrin, the role of fibrin-platelet interaction and the role of diffusion-controlled mechanisms are reflected as well. In principle it is also possible to add white blood cells and to carry out the experiments in wells lined with cultured endothelium. So, until the advent of a whole blood method or measurement of thrombin in a wound in situ, the CAT-method appears to be the most comprehensive way for measuring thrombin generation.

A number of investigations were presented in this thesis that are required before clinical studies can be undertaken to establish the actual value of measuring thrombin generation as a clinical test. Studies on clinical applications are being undertaken in several laboratories around the world and in various fields e.g.

A: Phenotyping of patients at high risk for thrombosis (hyper-coagulability) [10] or bleeding (hypo-coagulability) [11, 12]: high or low thrombin generation is a risk factor as such, independent of its cause.

B: Control and optimize anti-thrombotic therapy: reduction of the thrombin generation capacity is probably the essence of anti-thrombotic therapy, so measuring thrombin generation is possibly a universal test for the control of anticoagulant therapy.

C: Control and optimize anti-bleeding therapy: reinforce thrombin generation capacity is probably the essence of anti-bleeding therapy, so the effects of both factor VIII-inhibitor bypassing therapy (FEIBA) and Novoseven can probably be monitored by measuring thrombin generation [7, 13].

D: Finding and testing of new anti-thrombotics: on basis of the available evidence one may safely assume that a candidate drug that inhibits thrombin generation by about 50% will have an anti-thrombotic effect and not cause bleeding. The CAT-method provides a mean to monitor the efficacy of new drugs in human experiments and clinical trials.

In short: The data available at this moment – and increasing from day to day – are promising. Future research will certainly also delineate its limitations but it does not seem overdone to expect that thrombin generation will be a lasting and valuable addition to the possibilities of the clinical haemostatic laboratory.

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Nederlandse samenvatting

Trombine generatie als een algemene functie-test in klinische laboratoria

Trombose is de tweede grootste oorzaak van dood en ziekte (beroerte, embolie) in de westerse wereld. Volgens Virchow zijn drie zaken van belang bij het ontstaan van trombose nl. de vaatwand, de bloedstroming en de stolbaarheid van het bloed waarvan de laatste het moeilijkst te bepalen is. We mogen dan wel heel veel weten van het mechanisme achter de bloedstolling na tientallen jaren aan onderzoek, toch beschikken klinici nog steeds niet over een universele test om de stolbaarheid van bloed te bepalen. Er bestaan nochtans verschillende testen die gebaseerd zijn op stollingstijden maar elke test geeft informatie over een ander detail van het complexe web dat bloedstolling is. Geen enkele van deze testen kan dienen als een algemene functionerings-test zoals het elektrocardiogram voor hart-ritme stoornissen. De vraag kan dan ook gesteld worden of het meten van trombine generatie geen mogelijke kandidaat is voor zo een test en dit proefschrift wil bijdragen tot het antwoord op deze vraag.

Er zijn drie belangrijke redenen waarom het meten van trombine generatie, wat toch al een halve eeuw gekend is, nooit eerder naar voren is geschoven om deze rol te vervullen. Ten eerste duwde de vooruitgang in de moderne biochemische en moleculair biologische technieken de techniek wat in de vergetelheid. Ten tweede was het meten van trombine generatie heel erg arbeids-intensief en dus niet geschikt om te implementeren in de erg geautomatiseerde klinische laboratoria. Ten derde en misschien is dat wel de belangrijkste reden, paste deze techniek niet in het heersende denkbeeld van 'primaire hemostase' en 'secundaire hemostase'.

De laatste jaren wordt het echter meer en meer erkend dat trombine niet alleen een belangrijke rol speelt in 'secundaire hemostase' en veneuze trombose maar ook in 'primaire hemostase' en arteriële trombose. En dit vanaf het allereerste begin als de plaatjes-reacties starten. Als gevolg hiervan wordt de trombine vormende capaciteit van bloed meer en meer aanvaard als een – of zelfs het – centrale element in de fysiologie van de hemostase. Verder brachten Béguin en Hemker deze techniek langzaam opnieuw in de belangstelling met een serie artikelen, gestart in 1986, waarin werd aangetoond hoe deze techniek kan worden gebruikt om de werking van heparines en andere anti-stollingsmiddelen te verklaren en om de receptoren te bepalen die verantwoordelijk zijn voor de plaatjes-reacties.

Ondertussen zijn verschillende laboratoria op zoek naar manieren om de trombine generatie in het orgaan bloed te meten. De ideale manier om het functioneren van het trombine generatie-systeem in een patient op te volgen zou er in bestaan de trombine concentratie te meten in functie van tijd en ruimte in het plaatjes-aggregaat in de wonde. Tot op heden is dit technisch niet mogelijk maar een goede benadering is wel beschikbaar onder de vorm van de CAT-methode ofwel 'Calibrated Automated Thrombography'. Deze methode maakt het mogelijk trombine generatie op te volgen in zowel plaatjes-arm als plaatjes-rijk plasma en dit in de aanwezigheid van elementen van de vaatwand zoals tissue-factor en trombomoduline. Deze techniek is het onderwerp van dit proefschrift.

Calibrated Automated Thrombography (CAT)

De CAT-methode, voorgesteld in **hoofdstuk II**, maakt het mogelijk om trombine generatie te meten in 48 monsters tegelijk met een aanvaardbare experimentele fout van minder dan 5%. Bijgevolg is het een beloftevolle techniek voor klinische routine-laboratoria. De ziekte van Von Willebrand kan opgespoord worden en het effect van plaatjes-remmers zoals aspirine en clopidogrel kan worden gevolgd. Het is ook mogelijk om nuttige informatie te verkrijgen uit metingen in PPP zoals bvb. het opsporen van tekorten aan stollingsfactoren (behalve FXIII) of het vaststellen van het effect van anti-stollingsmiddelen (anti-vitamine K, heparines, directe trombine- of FXa-remmers). Dit is des te belangrijker aangezien er geen enkele stollingstijd-test bestaat die gelijkaardig reageert op al deze anti-stollingsmiddelen. Ook de functie van het proteïne C-systeem kan worden getest zowel in PPP als in PRP. Als TM wordt toegevoegd kan het hele systeem worden getest maar aangezien TM trombine nodig heeft om actief te zijn, is het effect van TM alleen maar te zien in de tweede helft van de trombine generatie-curve (piek-hoogte, ETP) en niet in de lag-time. Vandaar ook dat het effect niet te zien is in stollingstijd-testen.

Bijzonder interessant is het 'lupus anticoagulant'. Dit antilichaam veroorzaakt een toename van de stollingstijd en wordt dus beschouwd als een anti-stollingsmiddel maar het vertoont ook een stollingsbevorderende werking. Dit wordt de 'LAC-paradox' genoemd. De LAC-paradox verdwijnt als men zich baseert op trombine generatie waarbij men kan zien dat de lag-time of stollings-tijd inderdaad toeneemt maar ook dat de trombine generatie niet geremd wordt als het proteïne C-systeem wordt geactiveerd. Dit onderstreept dat er geen strikt verband bestaat tussen stollingstijd en de hoeveelheid trombine die gevormd wordt (95% van de trombine wordt gevormd na de stolling). Bijgevolg geeft het meten van een trombogram veel meer informatie dan een stollingstijd.

Berekening van de TG-curve vertrekkende van de ruwe data

Zoals uitgelegd in **hoofdstuk III** zijn er twee fit-procedures nodig om de ruwe data om te zetten in een TG-curve. De eerste fit-procedure is nodig om de ruwe data om te zetten in 'ideale data' d.w.z. data die we zouden verkrijgen indien er geen substraat-uitputting of inner-filter effect zou aanwezig zijn. De tweede fit-procedure wordt uitgevoerd op de ideale voortgangs-curve ('progress-curve') en is nodig om de vrije trombine te berekenen op basis van de totale amidolytische activiteit. De amidolytische activiteit is immers het resultaat van zowel vrij trombine als $\alpha 2\text{M-T}$. Een bijkomend voordeel van het fitten van de voortgangs-curve is dat men een grote spreiding van de meetpunten vermijdt die men zou krijgen indien men met de eerste afgeleide van de ruwe data zou werken.

Berekening van de protrombinase-curve

Trombine is het centrale enzym in het complexe web van de bloedstolling en FII-ase activiteit is de laatste stap in de vorming van trombine. De FII-ase activiteit is afhankelijk van de componenten van het FII-ase complex: FXa, FVa en de fosfolipiden die op hun beurt weer afhankelijk zijn van het functioneren van de andere stollings-factoren en regulatie-mechanismen en de plaatjes. Bijgevolg geeft de FII-ase activiteit een beeld van het hele stollings-systeem zonder de trombine afbraak. In **hoofdstuk IV** wordt uitgelegd hoe de FII-ase activiteit kan worden berekend vertrekkende van de TG-curve, als de beginconcentratie van AT bekend is. Het valt te verwachten – maar dat moet nog bewezen worden in de praktijk – dat de FII-ase curve een veel beter beeld geeft van de fysiologie van trombine vormende processen dan de TG-curve zelf.

De noodzakelijkheid van continue individuele calibratie

Het is van het allergrootste belang dat de experimentele fout beperkt wordt tot het minimum om afwijkingen in trombine generatie te kunnen detecteren. In de CAT-methode wordt de splitsing van het fluorescerende substraat opgevolgd en vergeleken met die van een bekende constante hoeveelheid trombine in een parallel niet-stollend monster. Op die manier wordt de calibratie-factor (CF) gedurende de hele meting bepaald onder dezelfde condities als dewelke heersen in de TG-experimenten. Dit is noodzakelijk om klinisch aanvaardbare intra-individuele variatie-coëfficiënten te bekomen zoals wordt aangetoond in **hoofdstuk V**. We vergeleken de intra-individuele experimentele fout verkregen met twee commerciële methodes. Enerzijds de CAT-methode die continue individu-

ele calibratie gebruikt en anderzijds de Technoclone-methode die een vaste CF gebruikt die verkregen is door de beginsnelheid te meten van een bekende trombine concentratie in buffer. Zoals men kan zien zorgt het gebruik van een vaste CF bepaald in buffer voor een ernstige onderschatting omdat de opbrengst van het fluorescerende signaal erg afhankelijk is van de kleur van het plasma. Deze onderschatting is niet constant maar neemt toe tijdens de meting van de TG-curve omwille van substraat-uitputting en inner-filter effect. Dit zorgt voor grote variatie-coëfficiënten die teruggebracht kunnen worden tot een klinisch aanvaardbaar niveau door gebruik te maken van continue individuele calibratie.

De invloed van het substraat op de TG

Een systeem meten betekent altijd dat het systeem verstoord wordt. Tijdens het meten van trombine generatie wordt er een substraat toegevoegd dat aan trombine bindt en bijgevolg trombine ook remt. Aangezien trombine een belangrijke rol speelt in zijn eigen vorming via allerlei feedback-mechanismen valt theoretisch te verwachten dat deze remming van trombine ook de trombine vorming beïnvloedt. Hoe groot dit effect wel is, werd reeds onderzocht toen we de CAT-methode voorstelden (hoofdstuk II). In dit artikel toonden we reeds aan dat het substraat onder onze omstandigheden geen significante invloed heeft op de omzetting van protrombine en dat de trombine afbraak op een voorspelbare manier wordt vertraagd omdat het toegevoegde substraat de afbraak van trombine door remmers aanwezig in het plasma vertraagt. Omdat dit onderwerp klinisch uiterst relevant is en omdat Butenas S. en K.G. Mann hun bezorgdheid te kennen gaven over dit onderwerp werd dit opnieuw onderzocht en deze extra bewijzen worden getoond in **hoofdstuk VI**. Uit deze experimenten konden we besluiten dat niet alleen de hoeveelheid protrombine die wordt omgezet maar ook de tijd waarin dit gebeurt minimaal worden beïnvloed door het aanwezige substraat (416 μM ZGGR-AMC). De inactivering van trombine door de remmers aanwezig in het plasma wordt op een perfect voorspelbare manier vertraagd. De feedback mechanismen die afhankelijk zijn van trombine worden weliswaar beïnvloed maar dit zorgt niet in een toename van de lag-time. Het zorgt wel voor een afname van de remming veroorzaakt door extern toegevoegde TM met ongeveer 10%. Het is echter erg belangrijk om te benadrukken dat deze conclusies enkel betrekking hebben op het bestudeerde substraat (ZGGR-AMC) bij de gebruikte concentratie (416 μM).

Omdat we de discussie wouden beperken tot een systeem in oplossing, zoals Butenas en Mann ook doen, hebben we in deze experimenten gebruik gemaakt van gedefibreerd plasma. Het effect van fibrine (fibrinogeen) wordt verder nog besproken in hoofdstuk VII.

Een verschuiving van chemisch gecontroleerde naar diffusie gecontroleerde trombine generatie

Met de komst van de CAT-methode werd het mogelijk om trombine generatie te meten zowel mét als zonder fibrinogeen. We hebben dit gedaan in plaatjes arm plasma en dit bij verschillende temperaturen zoals te zien is in **hoofdstuk VII**. Uit deze experimenten konden we besluiten dat er een verschuiving optreedt van chemisch gecontroleerde trombine generatie naar diffusie gecontroleerde trombine generatie gedurende de bloedstolling. Tot op heden werd voor waar aangenomen dat trombine generatie werd bepaald door de beginconcentraties en reactie-constanten van de betrokken reactiecomponenten. Nochtans, van zodra er een fibrine-netwerk wordt gevormd, hechten phospholipiden-plaatjes en/of phospholipiden-deeltjes zich aan dit netwerk en de reactiecomponenten kunnen alleen naar en van zulke geïmmobiliseerde netwerken gevoerd worden in een stilstaande vloeistof via diffusie door de vloeibare fase. Dit transport via diffusie wordt van belang op het moment dat de stolling begint of met andere woorden diffusie begint de snelheid van trombine vorming mee te bepalen vanaf het moment dat stolling plaatsvindt. In omstandigheden zonder fibrinogeen of in wiskundige simulaties wordt trombine generatie enkel en alleen bepaald door de beginsnelheden en reactie-constanten van de reactiecomponenten. Diffusie wordt niet in rekening gebracht. In een model waarin prothrombinase geadsorbeerd is aan een macroscopisch oppervlak is diffusie wél snelheidsbepalend maar dan onder alle omstandigheden tenzij de reactiecomponenten in extreme concentraties aanwezig zijn. Geen van beide benaderingen is juist omdat er een verschuiving optreedt van chemische controle naar controle door diffusie rond het ogenblik waarop het bloed stolt. Bijgevolg is de beste benadering om de trombine generatie in het menselijk lichaam te bepalen, het direct te meten in een monster van het geïsoleerde orgaan d.w.z. in stollend PRP met toevoeging van de relevante reactie-componenten van de bloedwand (TF, TM).

Het effect van verdunning van plasma op de trombine generatie

Een systeem meten betekent niet alleen dat het systeem verstoord wordt (hoofdstuk 4), het wordt ook onvermijdelijk verdund aangezien er substraat, calcium, een reactiestarter en/of phospholipiden moeten worden aan toegevoegd. In **hoofdstuk VIII** hebben we met behulp van de CAT-methode onderzocht wat de invloed is van verdunning op de trombine generatie in PPP. We stelden vast dat de ETP eerst toeneemt tot aan de plasma-verdunning 1:6 en dan pas afneemt. Dit wordt veroorzaakt door de verdunning van 'tissue factor pathway inhibitor' (TFPI) en proteïne S. Verdunning van deze remmers beïnvloedt de trombine

generatie meer dan de verdunning van stollings-bevorderende mechanismen. Verdunning onderdrukt proteïne S afhankelijke remming en dit veroorzaakt een tweede golf van FII-ase-activiteit wat op zijn beurt resulteert in een verlengde afbraak-fase. Verdunning van TFPI ontketent het directe extrinsieke reactiemechanisme. Beide fenomenen monden uiteindelijk uit in een hogere of verlengde trombine-activiteit. Tegelijkertijd worden het intrinsieke reactiemechanisme en de 'Josso-Loop' beduidend geremd door verdunning. Het intrinsieke reactiemechanisme verliest alle activiteit bij verdunningen $> 24\times$ terwijl de bijdrage van de Josso-Loop bijna volledig verdwijnt als het plasma 1:6 verdund is. Men moet bijgevolg erg voorzichtig zijn met het interpreteren van resultaten die verkregen zijn in sterk verdund plasma. Dit is vooral belangrijk in omstandigheden waarbij de Josso-Loop betrokken is zoals bijvoorbeeld bij het classificeren van hemofilië-patienten.

Belang voor wetenschap en maatschappij

Trombine generatie is aan een heropleving bezig sinds algemeen erkend wordt dat het een beloftevolle techniek is om het stollings-systeem te typeren. Het medisch belang van een algemene functionerings-test van het stollings-systeem kan nauwelijks overschat worden aangezien ongeveer de helft van ons zal sterven aan stollings-ziekten. In klinische laboratoria is er een grote behoefte voor zulk een test die het mogelijk maakt om patiënten te classificeren met een verhoogd risico op trombose of bloeding en om anti-trombose of anti-bloedings therapie op te volgen en te optimaliseren. Het meten van trombine generatie lijkt erg beloftevol om aan deze behoefte te voldoen. Het lijkt er op dat het een geschikte kandidaat is om de rol van 'elektro-cardiogram van het stollings-systeem' op zich te nemen. Deze vergelijking mag men echter niet te letterlijk nemen. De trombine-vormings-capaciteit van bloed is een typische 'on demand' functie terwijl het ECG een noodzakelijkerwijs continue functie opvolgt. Continue trombine generatie op een laag niveau bestaat in vivo en kan daarnaast in verhoogde mate voorkomen in bepaalde ziekten. Het wordt opgespoord via bijproducten van de bloedstolling in de bloedsomloop (TAT-complexen, protrombine-fragment 1-2, producten van fibrine-afbraak). Dergelijke producten kunnen beschouwd worden als rook-melders die een brandend vuur signaleren. De ETP of het potentieel om trombine te genereren moet worden bekeken als een indicator van het risico op vuur oftewel de potentiële trombine-vormende capaciteit van het niet-stollende vloeibare orgaan bloed. Nog een verschil is dat niet de hele persoon aanwezig hoeft te zijn om trombine generatie te meten, een paar ml bloed is voldoende. Het is wel zo dat hoe meer men de fysiologische omstandigheden wil nabootsen, hoe meer de patient beschikbaar moet zijn voor het laboratorium. Trombine generatie in PPP kan perfect gemeten worden in bevroren en vervolgens ontdooide plasma-monsters die dus gemakkelijk kunnen be-

waard en verzonden worden. Trombine generatie in PRP moet binnen het uur na de bloed-afname worden gemeten.

De CAT-methode, zoals we ze in dit proefschrift hebben voorgesteld, was oorspronkelijk ontworpen als een geautomatiseerde routine-methode om snel semi-geautomatiseerde trombine generatie experimenten uit te kunnen voeren in onderzoeks-laboratoria met meer mogelijkheden dan de eerder ontwikkelde chromogene techniek of de subsampling-methode. De CAT-methode is echter niet alleen een technische innovatie; het is ook een nieuw en beter concept om de mechanismen achter hemostase en trombose te onderzoeken omdat het een veel groter gedeelte van het fysiologisch mechanisme onderzoekt dan de voorgaande testen. Ook troebele media kunnen bestudeerd worden omdat een fluorescerend substraat wordt gebruikt als detectie-middel. Als gevolg hiervan worden ook de bijdrage van de plaatjes, fibrine, fibrine-plaatjes interacties, en diffusie gecontroleerde mechanismen mee in rekening gebracht. Het is in principe ook mogelijk om witte bloedcellen toe te voegen en om experimenten uit te voeren in wells die bedekt zijn met endothelium-weefsel. Zolang het niet mogelijk is om te meten in volbloed of om trombine te meten in de wond zelf lijkt de CAT-methode bijgevolg de techniek die het dichtst aanleunt bij de fysiologische omstandigheden.

In dit proefschrift werden reeds een heleboel onderzoeken gepresenteerd die nodig zijn vooraleer klinische studies kunnen worden op touw gezet om te bepalen wat de echte waarde is van trombine generatie metingen als klinische test. In verschillende laboratoria over heel de wereld en in verschillende onderzoeks-domeinen worden studies over klinische toepassingen uitgevoerd zoals bvb.

A. Het classificeren van patiënten met een verhoogd risico op trombose (hyperstolbaarheid) of bloeding (hypo-stolbaarheid): hoge of lage trombine generatie is een risico-factor op zich, ongeacht de oorzaak.

B. Het opvolgen en optimaliseren van anti-trombose therapie: het verlagen van de trombine generatie capaciteit is waarschijnlijk de essentie van anti-trombose therapie, dus het meten van de trombine generatie is mogelijk een universele test om anti-stollings therapie op te volgen.

C. Het opvolgen en optimaliseren van anti-bloedings therapie: het terug verhoogen van de trombine generatie capaciteit is waarschijnlijk de essentie van anti-bloedings therapie, dus de effecten van zowel 'factor VIII-inhibitor bypassing therapy (FEIBA)' en Novoseven kunnen waarschijnlijk worden opgevolgd door de trombine generatie te meten.

D. Het vinden en testen van nieuwe anti-stollingsmiddelen: op basis van de beschikbare gegevens kan men gerust aannemen dat een kandidaat medicijn dat de trombine generatie met ongeveer 50% remt een anti-stollend effect zal hebben en dat het geen bloeding zal veroorzaken. Met de CAT-methode hebben we dus een goed middel voor handen om het effect van nieuwe medicijnen te onderzoeken in experimenten met menselijk plasma en in klinische onderzoeken.

Samengevat, de beschikbare gegevens tot nu toe – die trouwens nog toenemen van dag tot dag – zijn veelbelovend. Toekomstig onderzoek zal ongetwijfeld ook de beperkingen van deze methode blootleggen maar het lijkt niet overdreven om te verwachten dat het meten van trombine generatie een blijvende en waardevolle toevoeging zal blijken aan het instrumentarium van ieder klinisch laboratorium.

Dankwoord

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Curriculum vitae

Erik De Smedt is geboren op 6 januari 1976 in Mechelen, België. In 1988 begon hij aan het algemeen secundair onderwijs aan het Sint-Theresia-college te Kapelle-op-den-Bos en in 1994 behaalde hij zijn diploma in de richting Latijn-Wetenschappen. Hij studeerde in 1997 af als Regent Wetenschappen-Aardrijkskunde (leraar secundair onderwijs) aan de Katholieke Hogeschool Mechelen waarna hij een nieuwe opleiding startte voor laborant (analist) aan de Katholieke Hogeschool Leuven, departement Rega-school. In 2000 studeerde hij met onderscheiding af (diploma A1 Scheikunde, optie milieuzorg). Vervolgens ging hij werken bij Tibotec NV in Mechelen, een farmaceutisch bedrijf dat o.a. aidsremmers ontwikkelt (ondertussen opgekocht door Johnson & Johnson). Na een jaar, in 2001, verhuisde hij naar Meeuwen (Belgisch Limburg) en ging aan de slag bij Synapse BV in Maastricht. Vrij snel werd besloten tot een promotie-onderzoek waarvoor nog een extra opleiding diende gevolgd te worden aan de Hogeschool Heerlen ter verkrijging van het HBO-diploma Chemie optie Biochemie. Dit werd met succes (cum laude) afgerond in november 2003. Het promotie-onderzoek werd verricht binnen de onderzoeksgroep CARIM aan de Universiteit Maastricht onder de leiding van Dr. Rob Wagenvoord (copromotor) en Prof. Dr. H.C. Hemker en Prof. Dr. H. Ten Cate (promotoren) hetgeen uiteindelijk heeft geleid tot dit proefschrift.