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A modified, optimized kinetic photometric thrombin generation assay components to measure the potency of thrombin in plasma

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ABSTRACT

The purpose of the present study was to improve, optimize and modification, a kinetic photometric assay of Factor IIa activity with regard to blood sample types, calibration, analytic corrections, and activation reagents, in order to establish a rapid routine, less time consuming, and suitable method for the evaluation of large numbers of clinical samples. Our modified method was based on the continuous monitoring of the Factor IIa released from the reacting substance, in the presence of a specific chromogenic peptide substrate (S2238). The optimization of pH and of the concentrations of substrate, activator ($CaCl_2$), EDTA, and tissue factor was carried out as well as blood sample types (Platelet-rich and platelet-poor plasma samples) so as to ensure the best compromise between reliability, specificity, and sensitivity, However, the Factor IIa activity was compared in the plasma of different donors. In conclusion, our modified and optimized FIIa generation assay is a rapid and simple method for the diagnosis and monitoring of different clinical cases, at the same time it can be easily adapted to clinical chemistry analyzers.

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Introduction

Thrombin generation assays have been used since the 1950s as a powerful tool for investigating overall blood coagulability (1). As thrombin plays a fundamental role in the coagulation cascade, it is logical, that plasma thrombin generation (2), apart from several other important information, consists the most appropriate way to study the overall patients' haemostatic status. It also helps to monitor the patients' different status, more recently; it has been used for targeting potential extra-haemostatic effects and for identifying patients at risk for thromboembolism (3, 4).

These data could improve clinical diagnosis, anticoagulant monitoring, the epidemiology of thrombosis and or hemorrhagic risk, etc. Furthermore, it gives the opportunity to note the enzymatic activity of thrombin assists, by measuring the quantification of the composite effect of the multiple factors, so as to determine coagulation capacity and the environmental impact on these factors.

This piece of information makes extremely interesting the development of different methods for monitoring Factor IIa generation in vitro. Factor IIa generation assays are developed in an attempt to mimic and study both normal and pathological coagulation mechanisms. These assays are also used for preclinical screening and investigation of novel procoagulant and anticoagulant therapeutics. Several methods have already been developed in the clinical laboratory for measuring thrombin generation (5-8), such as chromogenic, and fluorogenic substrate assays, calibrated automatic thrombography, waveform analysis and also western blot techniques (5, 9, 10).

Various chromogenic substrate assays are present which are unfortunately not shown to give an effect on standard clotting tests, such as the prothrombin time (PT) and the partial thromboplastin time (aPTT). Additionally, with routine clotting tests, only the initial phase of thrombin generation is measured so the information provided by these tests is inadequate because it is known that after clot formation much of the thrombin action is still to come (11).

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Thrombin generation chromogenic assays may give the opportunity to evaluate not only the amount of thrombin generated, but also the length of time it remains active. Thus, the thrombotic or bleeding tendency of the patient can be monitored more safely and accurately. In the early 1970s, the development of synthetic chromogenic substrates, in clinical and research laboratories and their subsequent introduction in hemostasis testing, has opened new perspectives for the assay of many coagulation factors, fibrinolytic components, and inhibitors (12). Major advantages of the chromogenic substrates are their simplicity, rapidity and suitability for everyday use and a characteristic example of chromogenic substrate specific for thrombin is S-2238. However, The two major chromogenic techniques for the measurement of thrombin generation are the continuous and the sub-sampling method (4-10, 13, 14).

In the present work, we proposed an extremely simplified determination method of thrombin generation, which might be automatable. The most widely used chromogenic substrate (S-2238) was applied, several parameters involved in the reaction were examined and optimized and defibrination steps were omitted in order to understand the methods more rapidly and find the best suitable conditions for a routine assay. Moreover, western blot analysis for studying thrombin was for the first time developed in our laboratory, but it is not suggested for everyday application in the clinical laboratory because it is time-consuming.

Materials and methods

Reagents

Substrate (S-2238): (Chemical Name: H-D-Phenylalanyl-L-pipecolyl-Larginine-p-nitroaniline dihydrochloride, MW: 625.6). The lyophilized powder was obtained from Chromogenix Instrumentation Laboratory Company - Lexington, MA 02421-3125 (USA). 25 mg of the lyophilized substrate were dissolved in 7 mL of distilled water to a concentration of 5.7 mmol/L , according to the manufactured instruction.

Activators (Tissue factor (TF)): Thromborel S (TF) was obtained from Dade Behring, Marbay, Germany. It has been preferred instead of contact activation, because it is a more physiological activator. The TF concentration always needs to be carefully controlled; too much will lead to excessive thrombin generation in the absence of FVIII, via the extrinsic pathway, and too little will give low thrombin generation even with normal concentrations of FVIII (14). In our assay, TF was used in dilution (½).

Buffer (A+B) (Total Volume 500 µl) was prepared by mixing 51µl of buffer A (0.05 M Tris-HCl, 0.1 M NaCl) with 379µl of buffer B (0.05M Tris-HCl, 0.1 M NaCl, and 20 mM EDTA). Tris-HCl has been chosen as the buffer medium because its pKa (8.1, 25 °C) makes it appropriate for measurements at pH 7.3-9.3 where most of the serine proteases were involved in coagulation shows their maximal activity.

Collection of Blood Samples

Acceptable samples for coagulation tests including plasma those are platelet-poor, platelet-rich and free from visible hemolysis. Blood was collected via atraumatic venipuncture into vacutainer tubes containing buffered Sodium Citrate (0.109 M, 9 parts of blood, and 1 part of citrate solution) as well as in vacutainers without buffered Sodium Citrate. To prepare plasma poor and plasma rich in platelets, the samples were centrifuged at 2,000 rpm for 20 min at room temperature and were either used immediately or kept at about 4 °C or frozen in –80 °C until further analysis. The samples were afterward used for modification and optimization of a kinetic photometric assay for the determination of thrombin activity as well as for studying the effect of FVIIa on thrombin generation.

Assay protocol

Thrombin generation in platelet-poor and platelet-rich non-defibrinated plasma was measured continuously at 37 °C. Routinely, to 30 µL of the sample were added 430 µL of buffer A+B followed by 10 µL of Thromborel S (Tissue Factor, 1/2) and 35µL of substrate S-2238 (0.37 mmole/L). Then thrombin generation was activated at zero time by adding 25µL (4.7 mmol/l) $CaCl_2$. The optical density was read at 405 nm every 30s up to 3 minutes. Thrombin generation curves were constructed with the optical density as a function of time. The linear interval of the reaction was determined using the kinetic program of the analyzer and the change in absorbance per minute

was calculated according to the manufacturer's instructions

Results and discussion

Optimization of assay components

The wide synthesis of chromogenic substrates and their subsequent introduction in hemostasis testing has opened new perspectives for the assay of many coagulation factors, fibrinolytic components, and inhibitors.

The method that we propose here has the purpose to optimize a rapid kinetic photometric thrombin activity assay for enabling the use of the thrombin generation method as a rapid and routine method in the clinical laboratory. Since it is well known that the reaction with a chromogenic substrate is sensitive to changes in pH, temperature, the composition of the buffer as well as that the reagents added to the analyzed sample influence the hydrolysis of the chromogenic substance by enzymes, it is necessary to establish optimized conditions before proceeding to the application of a chromogenic assay.

In our study, the optimization of the assay was based on the use of different pH and various concentrations of substrate S-2238, $CaCl_2$, EDTA. After some initial experiments the following assay conditions were chosen: pH 7.7, Substrate concentration (0.37 mmol/L), $CaCl_2$,(4.7 mmol/l) and EDTA (20 mmol/l).

Optimization of Substrate's concentration (S-2238)

Figure (1) shows the evaluation of the optimum conditions for the thrombin generation assay. Increasing the substrate's concentration increases the rate of reaction. However, enzyme saturation limits reaction rates. In our study for optimizing substrate's concentration thrombin generation was monitored in different concentrations of substrate (S-2238) as the following 0.05, 0.100, 0.13, 0.160, 0.210, 0.260, 0.320 ,0.370 and 0.420 mmol/l as shown in the Figure (1) and the optimal activity was achieved in 0.37mmol/L (Final concentration).

Optimization of tissue factor (Thromborel S)

We also studied the influence of thromborel S (TF) on thrombin generation essay, The concentration of TF is not given by the manufacturer and for this reason, TF is expressed as final dilution and volume, In the present study, we used different thromborel S volume as the following, (0, 2,5, 5, 7.5, 10, 11 and 12) µl as shown in the Figure (2), the optimal activity was achieved by using 10 µl.

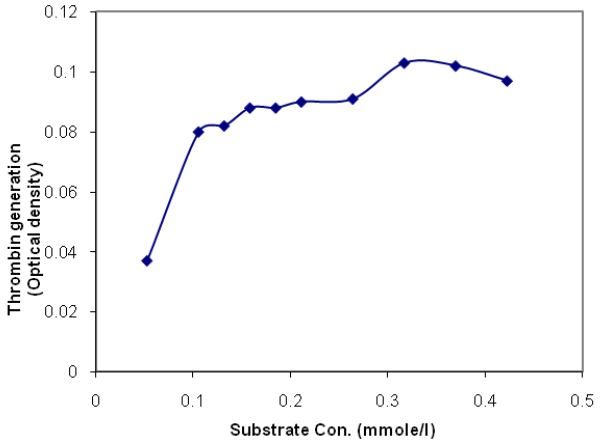

Figure 1. The effect of substrate con. on thrombin production

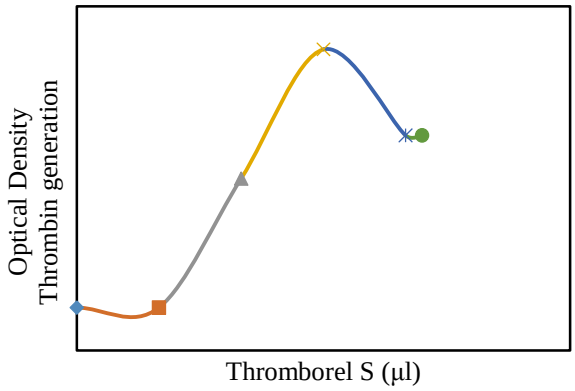

Figure 2. The influence of thromborel S on thrombin generation essay

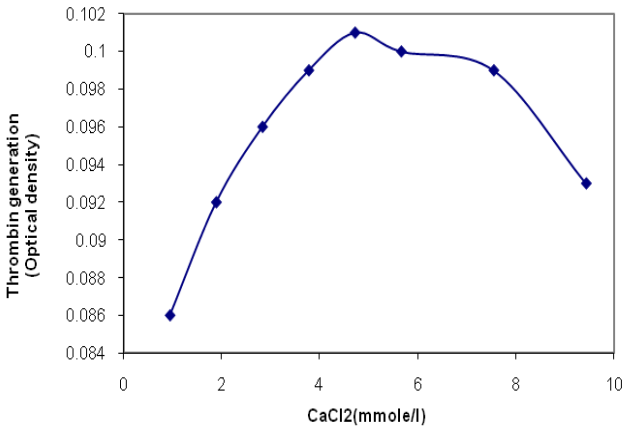

Figure 3. The effect of $CaCl_2$ Con. on the thrombin generation essay

Optimization of activator ($CaCl_2$)

In our study, thrombin generation was triggered in the presence of $CaCl_2$. During the optimization of the procedure different con. of $CaCl_2$ were used us the following (0.94, 1.89, 2.8, 3.80, 4.7, 5.70, 7.5 and 9.4 mmol/l and the optimal activity was noted in the 4.7mmol/l (Final concentration) see Figure (3)

Optimization of pH

In addition to substrate's concentration, $CaCl_2$ and thromborel S, which are strongly influenced thrombin generation, pH also had a strong effect on the thrombin generation curve. Usually, the enzyme activity is measured at the pH optimum for the proteolytic activity of the enzyme. Thrombin has been shown to be active in the pH range of 5-10. In our study, we used different pH, (7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 8.0, 8.1 and 8.2) as shown in the Figures (4 and 5) and the optimal pH of enzyme in our assay conditions was noted in pH 7.7.

The effect of FVIIa on Thrombin generation

In addition to the previously mentioned parameters, the effect of recombinant activated *FVII* on thrombin generation was studied in the presence of 10 µL of Thromborel S.

By contrast, we found that the thrombin generation in plasma-rich platelets was significantly increased in the presence of rVIIa as compared to that observed without the presence of rVIIa Figure (6).

Thrombin production kinetics in different samples

In our modified and optimized protocol for the determination of thrombin production, the maximal amount of generated thrombin in non-clotted plasma samples can be measured easily in a few minutes. The continuous determination of thrombin production in response to the activation time is shown in Figure (7) for different platelet-rich in plasma samples from healthy donors. It clearly shows that thrombin production kinetics may vary from one person to another inter-individual variation), however, It is clearly indicated in the curve, that thrombin generation after activation of the reaction is rapidly increased and reaches a peak value (Tmax); the amount of produced thrombin decreased gradually thereafter.

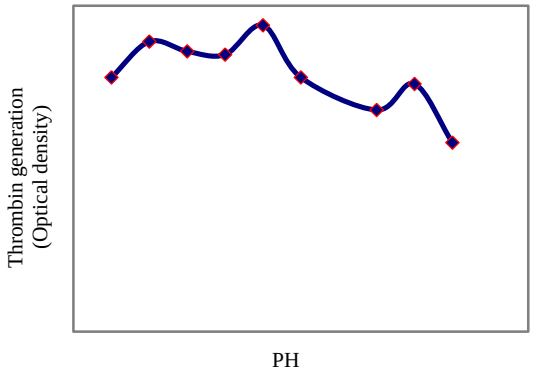

Figure 4. The effect of pH. on the thrombin generation essay

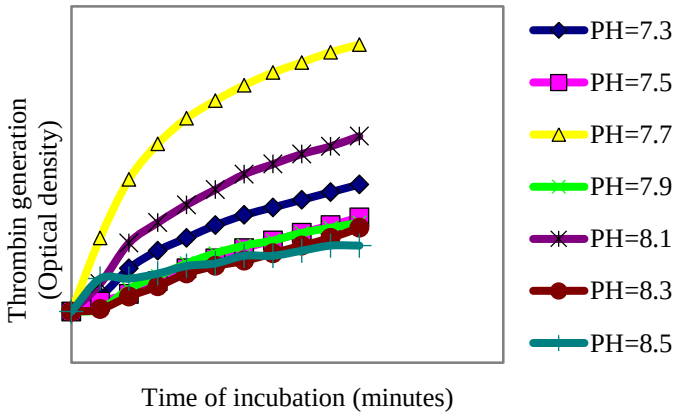

Figure 5. Determination of discontinuous thrombin production for increasing time intervals (0-10 min, measuring every 1 minute) in seven different pH

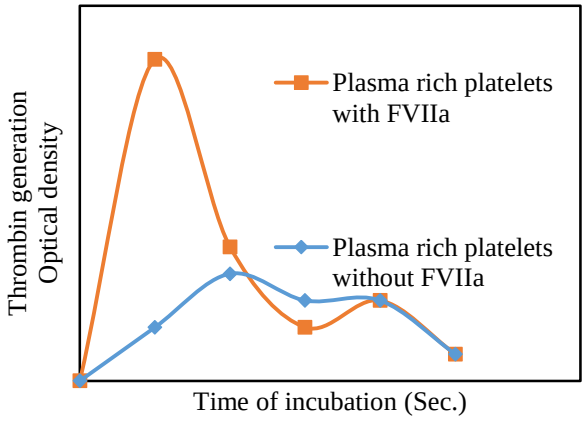

Figure 6. The influence of recombinant FVIIa on thrombin production essay in plasma rich platelet

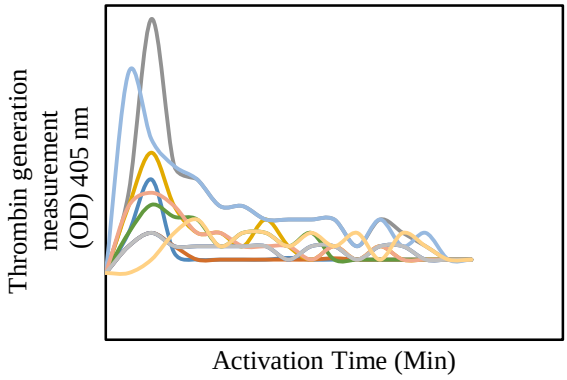

Figure 7. Values represent the discontinuous determination of platelet-dependent thrombin generation measurement for increasing time intervals (0-15 min, measuring every 30 s) in 10 different healthy individuals

Our modified and optimized method for determining thrombin production was also applied for calculating thrombin activity in 100 plasma samples of healthy individuals and 100 patients suffering from different diseases such as cardiovascular disease and diabetes mellitus (data not shown). The present study was shown that a hypercoagulable state can be diagnosed using our modified method, however, the modified method was also able to test the efficiency of oral anticoagulants in patients.

It is clear that a study for thrombin generation via the intrinsic pathway of coagulation is essential for further understanding of the normal clotting procedure and its various defects, because the ability of blood to form clots without artificial assistance, probably determines its haemostatic efficiency. Although the early thrombin generation is very important for hemostasis, excessive production of thrombin, could become a risk factor for thrombotic episodes. For this reason, the profile of thrombin production could be of great importance.

Several scientific groups have developed assays for the estimation of thrombin generating and have combined the measurement of the vitamin K-dependent coagulation factors based on synthetic chromogenic substrates. Various prothrombin activating principles have formed the basis of these methods.

Thrombin generation assays have been widely used in an attempt to understand if and how thrombin generation can be used as an index of coagulability under pathologic conditions, such as diabetes and hyperlipidemia (15, 16). By means of thrombin generation assays, the roles of coagulation factors and platelets in similar and other disorders could be clarified, assisting in their better treatment. Furthermore, the effect of everyday lifestyle and habits on coagulation, such as cigarette smoking can also be studied by assays (17). The above examples indicate how much necessary it is to find rapid and accurate methods for the measurement of thrombin generation, so as to understand better and also quantify how both externals and internal factors influence thrombogenesis.

Although measuring the hemostatic function of the blood is important both in bleeding and thrombotic disorders, there is no universal test of this function available to the clinician. However, clotting times of different types do not indicate a thrombotic tendency of unknown origin or mild hemostatic disorders. An ideal laboratory test should de efficient enough to determine the clinical implication of a biochemical coagulation diagnosis and also determine the effects of both procoagulant and anticoagulant medication. Any uncertainty in calculating thrombin generation may have disastrous results and lead to sub-therapeutic or overly aggressive dosing in medication based on information derived from laboratory tests. Therefore, it is really important to choose the right method so as to appropriately evaluate the coagulant status of a patient. Currently, however, there is no single haemostasis clinical laboratory test able to reflect the overall in vivo biology and this reality makes necessary the continuous search for new methods and optimization of the already known ones.
In the method described here, chromogenic assay's parameters showed an important interindividual variability, which has also been observed in other studies (18, 19)

Concerning the effect of rFVII on thrombin generation, it is already known that the activated factor VII (FVIIa)/ tissue factor enzyme complex functions as the initiator of the coagulation cascade in vivo. FVIIa is of particular interest because it has been found to induce haemostasis in various bleeding disorders. Even trace amounts of FVII are sufficient for initiating blood coagulation in vitro. Our results showed a wide variety of the amounts of thrombin generated in plasmas from different patients, in accordance with previous results (20), which suggests that the threshold of FVII required to initiate haemostasis in vivo depends on one or more plasmatic or cellular factors.

Our assay, without requiring any ultra-sophisticated equipment, combines the efficiency and good characteristics of commonly used chromogenic substrate assays with the advantage of being adaptable to automation and showing greater reproducibility and speed. That is, the reason why we omitted the reptilase treatment. It was an attempt to simplify a widespread method of measuring thrombin generation because one of our purposes was to shorten the reaction time.

Acknowledgments
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Interest conflict
None.

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