A Simple Method for the Determination of Plasma Extrinsic Coagulant Activity

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HUSSEIN, K.A. and MEDOUKH, N.T. A Simple Method for the Determination of Plasma Extrinsic Coagulant Activity. Tohoku J. exp. Med., 1986, 150 (2), 103-116 — A spectrophotometric study of plasma coagulation is presented with a method for the determination of plasma coagulant activity, via the extrinsic pathway. Dilute citrated plasma was coagulated by recalcification after the addition of a very small amount of thromboplastin. The maximum increase in absorbance per min during coagulation was used as a measure of coagulant activity. The method was applied to normal platelet-rich plasma samples, and the effect of changing the concentration of plasma, thromboplastin, fibrinogen, and heparin was studied. ——— plasma coagulant activity; extrinsic pathway; thromboplastin; fibrinogen; heparin

Tests commonly used for the determination of plasma coagulant activity depend mainly on measuring the reaction velocity, or measuring the yield of coagulation activity. The determination of the clotting time, as a measure of reaction velocity is based on the relationship between the clotting time and the coagulant activity, which is a complex relationship due to the sequential nature of the coagulation reactions (Bloom and Thomas 1981). A plot of the clotting time against coagulant activity yields a hyperbola on rectangular coordinates, where small differences in clotting time represent major differences in activity, when the clotting times are short, and represent minor differences in activity, when the clotting times are long. A standard curve must also be used with each test to convert the clotting times to units or percentages of maximum activity (Williams et al. 1977).

The determination of the product of coagulation activity usually adds one more stage to the test. For instance, thrombin formed during clotting is determined in the two stage prothrombin time by its ability to clot exogenous fibrinogen (Ware and Seegers 1949). The prothrombinase formed in the first stage of the thromboplastin generation test is determined in the second stage by its ability to clot substrate plasma (Brown 1984). Recently, thrombin generated during coagulation was determined by measuring the activity of thrombin eluted from the clot. This amount was found to be proportional to the total amount...
formed (Triantaphyliopoulos and Leech 1983).

In the presented work, the spectrophotometer was used to study the relationship between plasma coagulant activity and the maximum rate at which clot opacity develops during coagulation. The study gave rise to a method for the determination of plasma extrinsic coagulant activity initiated by a very weak stimulus, so as results can be used as a measure for the magnification power of the coagulation cascade.

**Materials and Methods**

**Tris buffer.** Tris buffer at pH 7.4 was prepared by adding 42 ml of 0.1 ml HCl to 50 ml of 0.1 M Tris, and diluting the solution to a total volume of 100 ml.

**Calcium chloride solution.** A stock 2 M solution was prepared by dissolving 29.4 g of CaCl₂ · 2H₂O in 100 ml of distilled water, and was stored at 4°C. A working solution containing 40 μmole/0.2 ml was prepared by diluting the stock solution 10 times using Tris buffer.

**Thromboplastin solution.** Rabbit brain thromboplastin preparations from “Sigma” were used. The contents of one vial were dissolved in 2 ml of deionized water at 37°C, as recommended by the manufacturer. The solution was used as a stock solution, and was diluted by Tris buffer to prepare different concentrations. Amounts of thromboplastin added in each experiment are referred to as a part of the stock solution.

**Fibrinogen solution.** Dried human fibrinogen “Kabi” was used. A fresh solution containing 60 mg in 10 ml Iris buffer was used in all experiments.

**Heparin solution.** Sodium salt of heparin “Calbiochem” containing 154.2 units/mg was used. Solutions were freshly prepared in Tris buffer.

**Human citrated plasma.** Fresh blood samples were collected in plastic tubes from blood donor volunteers at the Kuwait Blood Bank. The tubes contained one volume of sodium citrate solution (3.8%) and was filled to contain 9 volumes of blood. Erythrocytes were sedimented by centrifugation for 5 min at 100 × g, to prepare platelet-rich plasma. Platelet-poor plasma was prepared by centrifugation at 4°C for one hr at 18,000 × g. Platelet-poor plasma was used in one experiment only to demonstrate the effect of blood platelets at low concentrations of thromboplastin.

**Spectrophotometric assay.** Diluted plasma samples were coagulated by recalcification in 1 cm path length glass cuvettes. The increase in absorbance at 400 nm was recorded using a thermostated LKB Digital Ultraspectrophotometer “Cecil” supplied with a chart recorder.

Tris buffer and calcium chloride solutions were kept at 37°C in a temperature regulated water bath. Empty plastic tubes were kept warm in a rack immersed in the water bath, and empty cuvettes were kept warm in the spectrophotometer chamber. Plasma samples were kept at room temperature, in a small plastic tube.

Coagulation tests were done by adding plasma to the required solutions in a total volume of 3 ml of tris buffer. Solutions were transferred to a plastic tube in the following order: Tris buffer, thromboplastin, fibrinogen and heparin. The tube was rewarmed for 1/2 min and plasma was added (using an automatic pipette with disposable plastic tips) followed by 0.2 ml of calcium chloride solution. The solutions were mixed for 3 sec using a vortex mixer rotated at a low speed and transferred to the spectrophotometer cuvette. After exactly 18 sec from mixing the solutions the chart recorder was started and the absorbance adjusted to zero. The 18 sec were added to the preclotting time calculated from the chart record. Reproducibility of results can be checked by repeating a coagulation experiment using a constant concentration of plasma (0.3 ml/cuvette) and a constant amount of thromboplastin (0.6 μl/cuvette). Results are quite reproducible when plasma
samples are not warmed, or shaken, or unnecessarily transferred several times. These precautions are aimed to avoid activation of plasma surface active proteins. Adequate mixing of the coagulation mixture is important to get homogeneous clots. Homogeneous clots show the same absorbance when the cuvette containing the clot is reversed in the cuvette holder.

RESULTS

Chart tracing of the change in absorbance of diluted plasma during coagulation gave rise to “coagulation curves” as presented in Fig. 1. A tangent to the straight mid-part of the curve was drawn to enable the calculation of four values; the clot absorbance “CA”, the maximum rate of clot formation “MRCF”, the preclotting time “PCT”, and the time of clot formation “TCF”.

The “CA” is the absorbance caused by the opacity of the formed clot. The value was measured 5 min far from the tangent. When clots were left for longer times, the absorbance values increased very slowly to reach a constant value after 60 min, however, the increase was not more than 5%. This characteristic was the same for several plasma samples and was not significantly influenced by the fibrinogen concentration. Similar behavior was previously reported in the determination of fibrinogen by the turbidimetric method (Ellis and Stransky 1961).

The maximum increase in absorbance per min was calculated from the slope of the tangent. The value represents the maximum rate at which clot is formed (“MRCF”) when thrombin is generated at maximum rate.

The “PCT” represents the time period between mixing the coagulation mixture and the restoration of a constant level of absorbance. The “TCF” is the time period between mixing the coagulation mixture and the complete formation of the clot.

Fig. 1. Chart tracing of absorbance, during the coagulation of 0.3 ml plasma, by recalcification and 0.6 µl thromboplastin, in a total volume of 3 ml. Preclotting time “PCT” = 1.5 min, time of clot formation “TCF” = 1.6 min, clot absorbance “CA” = 0.49, maximum rate of clot formation “MRCF” = “CA” / “TCF” = 0.306.
mixture and the start of fibrin formation. The time was calculated by adding 18 sec to the time period shown by the coagulation curve. The 18 sec is the time spent between mixing the sample and starting the chart recorder.

The “TCF” represents the time during which opacity was increased, under the effect of maximum thrombin generation. When the “TCF” is added to the “PCT”, the sum can be considered to equal the clotting time commonly determined visually.

A constant concentration of plasma (0.3 ml/cuvette) was coagulated by recalcification with increasing concentrations of thromboplastin (Fig. 2). Samples with zero thromboplastin coagulated by contact with the cuvette glass surface, via the intrinsic pathway. The “MRCF” was increased by increasing thromboplastin, with platelet-rich and platelet-poor plasmas. At low concentrations of thromboplastin, platelet-rich plasma gave higher “MRCF” values showing the effect of blood platelets. At the concentration of 4 µl of thromboplastin/cuvette the “MRCF” of both plasmas became equal showing that the effect of blood platelets was completely substituted by the thromboplastin preparation phospholipids. The “CA” of platelet-rich plasma showed a slight decrease as the coagulant activity was increased by thromboplastin. The “PCT” showed a rapid drop reflecting the effect of phospholipids, and the “TCF” showed a less marked gradual decrease.

Fig. 3 shows the results of increasing the concentration of plasma as the only variable factor. Plasma was coagulated by recalcification using a constant concentration of thromboplastin (0.6 µl/cuvette). The increase in plasma concentration caused a linear increase in the “MRCF” and the “CA” and a gradual decrease in the “PCT”, however, the “TCF” was almost constant.

Repeating the last experiment with different plasma samples gave rise to
similar results, however, values were different. Fig. 4 shows the “MRCF” and the “CA” values of three plasma samples with different fibrinogen content.

When the “MRCF” values in Fig. 4 were plotted against their corresponding “CA” values, a common curve was obtained (Fig. 5). The curve shows that the “MRCF” of different plasma samples, at the variable plasma concentrations used, increased linearly by the increase in “CA”.

This relationship was further tested by using 20 different plasma samples at a constant plasma concentration (0.3 ml/cuvette) and with a constant thromboplastin concentration (0.6 μl/cuvette). Results of these experiments are shown in
The effect of increasing plasma concentration on the "MRCF" and the "CA" values was also studied after raising the coagulant activity of plasma, by increas-

Fig. 5. A plot of "MRCF" against "CA", of samples shown in Fig. 4 (closed circles), and 20 samples coagulated at a constant plasma and thromboplastin concentration (0.3 ml plasma and 0.6 \( \mu \)l thromboplastin/cuvette open circles).

<table>
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<th>PCT</th>
<th>TCF</th>
<th>MRCF</th>
<th>CA</th>
<th>PCT</th>
<th>TCF</th>
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Table 1. Coagulation curve values

Coagulation curve values of samples presented in Fig. 5 by open circles. "TCF" average, 1.55–1.65; mean, 1.605; s.d., 0.0339.

Fig. 5. and Table 1.

The effect of increasing plasma concentration on the "MRCF" and the "CA" values was also studied after raising the coagulant activity of plasma, by increas-
ing the amount of added thromboplastin, and after decreasing the coagulant activity by the addition of heparin. The concentration of thromboplastin was increased to 1.2, 1.5 and 1.8 μl of thromboplastin/cuvette. Heparin was added (with 0.6 μl of thromboplastin) at concentrations of 0.06 and 0.12 units/cuvette. Fig. 6 shows the results of these experiments.

The effect of increasing the concentration of fibrinogen on the “MRCF” and “CA” values was first studied by adding fibrinogen solution to variable concentrations of plasma, coagulated by recalcification and a constant amount of thromboplastin (0.6 μl/cuvette). To each plasma concentration fibrinogen was increased in a series of experiments (Fig. 7). In each experiment the added fibrinogen was increased by 0.1 ml of fibrinogen solution (200 mg %, in Tris buffer). Absorbance values higher than 1.3 were avoided to ensure precise opacity readings.

The concentration of fibrinogen was also increased using a constant concentration of plasma (0.3 ml/cuvette) and four levels of thromboplastin concentrations; 0.6, 1.2, 1.5 and 1.8 μl/cuvette or with two concentrations of heparin (0.06 and 0.12 units/cuvette, with 0.6 μl of thromboplastin). Results are shown in Fig. 8.

The effect of increasing the concentration of fibrinogen on the “PCT” and the “TCF” was studied using a constant plasma concentration (0.3 ml plasma/cuvette) at variable conditions of coagulant activity; using 0.6 μl of thromboplastin.

Fig. 6. The effect of increasing the concentration of plasma on the “MRCF” and “CA” values. Plasma was coagulated by recalcification after the addition of thromboplastin indicated, or after the addition of heparin (and 0.8 μl thromboplastin).
plastin/cuvette (Fig. 9), 1.2 μl of thromboplastin/cuvette (Fig. 10), and 0.24 units of heparin with 0.6 μl of thromboplastin/cuvette (Fig. 11). Results of these experiments showed that increasing the concentration of fibrinogen caused a gradual decrease in the “PCT” accompanied by a gradual increase in the “TCF”.

The group of experiments in which a constant plasma concentration was used (0.3 ml/cuvette) are shown in Fig. 12. The middle part of curve A represents samples coagulated with 0.6 μl of thromboplastin/cuvette, where the “MRCF” values increase by the increase in plasma fibrinogen (represented by the “CA” values). Curves B and B’ represent plasma samples coagulated with higher concentrations of thromboplastin, and curves C and C’ represent those coagulated with 0.6 μl thromboplastin and increasing concentrations of heparin. The curve D represents the increase in fibrinogen concentration in samples coagulated with 0.6 μl thromboplastin.

**DISCUSSION**

Platelet-rich citrated plasma samples were coagulated by recalcification after the addition of a very small amount of thromboplastin. The amount of throbo-
Plasma Extrinsic Coagulant Activity

The added thromboplastin initiated the coagulation of plasma diluted 10 times via the extrinsic pathway. The change in absorbance during coagulation was recorded as coagulation curves, which gave rise to four values; "PCT", "TCF", "MRCF" and "CA" (Fig. 1).

Although the amount of thromboplastin used was very small, it was quite sufficient to prevent interferences from the intrinsic pathway, activated by plasma contact with the cuvette glass surface. This was achieved by the phospholipids

Plasma was coagulated by recalcification with the amounts of thromboplastin indicated, or with heparin and 0.6 μl of thromboplastin, in a total volume of 3 ml.

Fig. 8. The effect of adding fibrinogen to a constant plasma concentration (0.3 ml/cuvette) on the “MRCF” and “CA” values. Fibrinogen was increased as in Fig. 7. Plasma was coagulated by recalcification with the amounts of thromboplastin indicated, or with heparin and 0.6 μl of thromboplastin, in a total volume of 3 ml.
contained in the thromboplastin preparation, which decreased the "PCT" to less than half its value when no thromboplastin was added (Fig. 2).

The method present for the determination of plasma coagulant activity depends on measuring the effect of thrombin formed during coagulation rather than measuring the clotting time. However, the "PCT" and the "TCF" were
Plasma Extrinsic Coagulant Activity

The effect of increasing heparin with 0.3 ml plasma and 0.6 μl thromboplastin/cuvette (solid lines); and the effect of adding fibrinogen to 0.3 ml plasma with 0.24 units of heparin and 0.6 μl thromboplastin/cuvette (dashed lines).

Fig. 12. Plasma samples at a constant concentration (0.3 ml/cuvette) were coagulated by recalcification and 0.6 μl thromboplastin (curve A), or with higher concentrations of thromboplastin (curve B and B'), or with 0.6 μl thromboplastin and increasing concentrations of fibrinogen (curve D).

determined to compare changes in the reaction velocity with changes in the amount of formed thrombin, as a product of coagulation activity.

The “CA” value represents the degree of turbidity which develops during coagulation. This value was previously reported to show a linear correlation with
fibrinogen concentration (Ellis and Stansky 1961). However, at high concentrations of fibrinogen the straight line relationship gave rise to a curve. Results showed that the relationship was also affected by the velocity of fibrin formation; an increase in coagulant activity caused a small decrease in the “CA” value (Fig. 2), and a small increase in this value was noted when the coagulant activity was decreased (Fig. 11).

The “MRCF” represents the maximum rate at which turbidity develops during coagulation. This rate was recently determined photometrically as the clotting velocity (Becker et al. 1984), and was used as a measure of fibrinogen concentration, in plasma samples coagulated by the snake venom batroxobin. The method is based on the observation of Hemker (Hemker et al. 1979) who found that the clotting time of plasma, coagulated using excess thrombin, is not only a function of fibrinogen concentration, but also a function of clotting velocity. In our method, the “MRCF” was used as a measure of both; the coagulant activity and the concentration of fibrinogen, since it is dependent on both reacting factors; the thrombin generated during coagulation and the sample fibrinogen.

A straight line relationship was obtained by plotting the “MRCF” values against the “CA” values, of plasma coagulated with a constant concentration of thromboplastin. The relationship was shown by 20 different plasma samples, coagulated at a constant plasma concentration, as well as, by plasma samples at variable concentrations (Fig. 5). These results show that plasma samples, at constant or variable concentrations, coagulate with a constant ratio between the “MRCF” and the “CA” values. Since the “MRCF” value was calculated as “CA”/“TCF” (Fig. 1) the “TCF” of these samples should be constant within a normal range. The “TCF” values are shown in Table 1, with a range from 1.55 to 1.65, and a mean of 1.605 min. In other words, during coagulation of plasma samples, the effect of thrombin, generated at maximum rate, and measured as “MRCF”, is increased by the increase in fibrinogen to give a constant “TCF” value. This means that there is a certain balance between the coagulant activity of a plasma sample and its fibrinogen concentration. This conclusion is in accordance with previously reported results suggesting the existence of a balance of activities among various components of the coagulation system, resulting in a constant fibrin-forming potential of plasma samples, measured as the product of maximum thrombin activity and fibrinogen concentration (Grannis and Kazal 1965). It was also shown, by the same investigators, that the increase in plasma fibrinogen concentration was accompanied by a decrease in the maximum thrombin, observed during the thrombin activity curve. The decrease in maximum thrombin was caused by elevated antithrombin activities.

An increase in coagulant activity, simulated by increasing thromboplastin, increased the “MRCF” only, and a decrease in coagulant activity, simulated by increasing heparin, decreased the “MRCF” only (Fig. 8), except for the small
changes in "CA" discussed before.

The addition of fibrinogen to variable plasma concentrations (Fig. 7) and to plasma at different levels of coagulant activity (Fig. 8) caused an increase in both the "MRCF" and the "CA" values. The increase in the "MRCF" values was much less compared to the effect of increasing plasma concentration, since the increase in fibrinogen was not accompanied by a corresponding increase in coagulant activity. At low coagulant activities the effect of increasing fibrinogen gave rise to straight line relationships, however it gave rise to curved lines at higher coagulant activities. This was expected since the relationship between the "MRCF" and coagulant activity was not a straight line as shown in Fig. 1. In general, the slopes of the lines representing the increase in fibrinogen concentration reflected the level of activity of the plasma, to which fibrinogen was added.

The addition of fibrinogen to plasma decreased the "PCT" and increased the "TCF" (Figs. 9, 10 and 11). The decrease in the "PCT" can be explained by the protective effect of fibrinogen on thrombin. Thrombin formed during coagulation reacts preferentially with its natural substrate fibrinogen, and is bound to it by two binding sites. The bound amount of thrombin was reported to be directly proportional to the concentration of fibrinogen and protected from inactivation by antithrombin III (Liu et al. 1979).

The prolongation of the "TCF" caused by the addition of fibrinogen denotes an increase in fibrinogen concentration without a corresponding increase in coagulant activity. This may explain the reported increase in the thrombin clotting time, of plasma samples with high fibrinogen content (Fricke and McDonagh 1983). Coagulation of plasma with thrombin does not show a significant period of "PCT", because clot formation starts very rapidly. Therefore, the mentioned prolongation of the "TCF" will be measured as prolonged clotting times.

The addition of heparin decreased the "MRCF", and prolonged the "PCT" and the "TCF" (Fig. 11). The effect was not only dependent on the concentration of heparin added, but was also affected by the fibrinogen content of the sample (represented by the "CA" value). Heparin was more effective on samples with lower fibrinogen content (Fig. 12). The addition of fibrinogen to samples with heparin reversed the effect of heparin; it increased the "MRCF" and decreased the "PCT". The "TCF" was not proportionally decreased because the added fibrinogen prolongs the "TCF".

In conclusion, the determination of the "MRCF" and "CA" can be used to compare the coagulant activity of plasma samples with normal values, when samples are coagulated at a constant plasma concentration (0.3 ml/cuvette), with a constant thromboplastin concentration (0.6 μl/cuvette). The middle part of curve A (Fig. 12) represents the change of "MRCF" by normal changes in fibrinogen content. A rise in the coagulant activity of plasma samples will cause values to increase in the direction of the curves B and B', depending on the sample
fibrinogen content. Similarly, a decrease in coagulant activity, or administration of heparin, will cause the values to decrease in the direction of curves C and C'. Plasma samples with fibrinogen content higher than normal, will give values that increase in the direction of the curve D. Accordingly, the coagulant activity of a plasma sample can be compared with normal samples, by relating its “MRCF” to the “MRCF” shown by curve A, at the “CA” value of the sample.

The coagulant activity determined using a weak stimulus, can be used as a measure for the cascade magnification power. The cascade magnification power determines the rate at which thrombin is generated. The clot formation effect of the generated thrombin is opposed by the plasma antithrombin, and at the same time protected from antithrombin by fibrinogen. Accordingly, in the presence of a certain concentration of antithrombin and fibrinogen, the cascade magnification power may be a contributing factor in the formation of intravascular thrombus. A weak stimulus which is not enough to cause an intravascular thrombus, may do so when the cascade magnification power is higher than normal. The magnification power of plasma from patients with intravascular thrombus should be compared with normals.

**References**