

PARALOGISM IN THE INTERPRETATION OF THROMBOGRAM OBTAINED WITH THE THROMBIN GENERATION ASSAY OF HUMAN BLOOD PLASMA

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Recording thrombin generation in blood under the action of coagulation initiators provides important early information about the state of the hemostasis system. Thrombin generation analysis (TGA) developed to determine the endogenous clotting potential and anticoagulant activity of blood plasma is extremely important diagnostic and prognostic method. However, the main problem of the TGA method is that in the descending part of the thrombogram, an error appears in the conversion of thrombin activity to its concentration. By studying the temporal relationship between thrombin and plasmin activity, and clot structure during its formation and dissolution in human blood plasma we propose another option for calculating the parameters of the thrombogram obtained by the TGA method.

Key words: *thrombin generation assay, thrombin concentration, thrombogram parameters calculation.*

Thrombin, as the central enzyme of the coagulation system, ensures integrity and normal functioning of the cardiovascular system and body as a whole [1, 2]. The activity of the enzyme at each moment of coagulation system activation reaction characterizes the efficiency of its work and related processes of thrombus formation and stabilization, systems of protein C, kallikrein-kinin and thrombin-activated fibrinolysis inhibitor (TAFI), effect on platelets, endothelial cells of the vessel wall. Recording generation of thrombin activity under action of blood coagulation initiators – tissue factor and aPTT reagent, provides important early information about the state of hemostasis system. The thrombin generation analysis (TGA) method, developed on this basis, makes it possible to determine the endogenous clotting potential of blood plasma, its balance with the anticoagulant activity of plasma, and the ability to adequately respond to disturbances in this balance [1, 2]. Given this diagnostic and prognostic value, the method for determining thrombin generation in the plasma and blood of patients is of extreme importance at this time.

By studying the temporal relationship between thrombin and plasmin activity and clot structure

during its formation and dissolution in human blood plasma, we saw an opportunity to propose another option for calculating the parameters of the thrombogram obtained when analyzing thrombin generation by the TGA method. The article presents the results of constructing a thrombogram, taking into account the concentration of thrombin generated up to the inflection point.

Materials and Methods

The experiments used the tissue factor preparation TromborelS from Siemens (Germany), thrombin – reagent from Sigma (USA). S2238 peptide substrate with a p-nitroanilide label was purchased from Chromogenix, the preparation APTT (activated partial thromboplastin time) and 25 mM CaCl₂ solution – reagents from Granum (Ukraine).

Permission to conduct research with human blood was obtained from the Ethics Commission of Palladin Institute of Biochemistry of NAS of Ukraine (No 6, 25.09.2024), and informed consent was obtained from each donor. Blood samples from healthy donors were collected in 3.8% sodium citrate (1 part sodium citrate and 9 parts blood, pH 7.4). Plasma was separated from blood cells within 1 h

of blood collection by centrifugation of the latter at 1500 g for 20 min. Plasma aliquots were stored at -20°C .

Blood plasma coagulation was studied by turbidimetric method by recording the turbidity of the fibrin clot at 405 nm on an SF 2000 spectrophotometer [3]. The clot was formed in plastic cuvettes, to which were sequentially added (259 or 159 μl) 0.02 M HEPES buffer containing 0.15 M NaCl, 0.005% Tween-20, pH 7.4, 40 μl blood plasma, 1 μl TromborelS reagent, 100 μl 25 mM CaCl_2 and 100 μl 2.71 mM S2238. The plasma coagulation process was initiated by adding CaCl_2 and TromborelS. The final volume of the reaction mixture was 400 μl . The results were processed using the standard statistical program Excel. The average values of the parameters and their standard deviations were determined.

Results and Discussion

Fig. 1 and Fig. 2 show the results of the study of thrombin generation in the blood plasma of donors and patients *in vitro*, and the thrombogram of thrombin concentration dependence in the reaction medium on the reaction time using the TGA method.

When comparing the experimentally obtained curve of fluorescent signal growth, Fig. 1, and its transformed form – thrombogram, Fig. 2, draws attention to the fact that on thrombogram at point of inflection (peak height), there is a transition from an increase in the concentration of thrombin to its decrease. At the same time, in Fig. 1, after inflection point, magnitude of fluorescent signal continues to increase, which indicates an increase in total activity of thrombin and, therefore, concentration of

thrombin. It is obvious that during transformation of the activity of generated thrombin after inflection point using calibration curve into concentration of thrombin in reaction medium, decrease in increment in thrombin activity (increase in thrombin concentration) was represented as a decrease in its concentration in the medium. It is obvious that a logical error occurred during calculation of thrombogram. It consists in the fact that up to the inflection point of curves, the growth of thrombin activity and the growth of thrombin concentration change unidirectionally – they increase. After inflection point, increment in thrombin activity decreases. To preserve the logic of unidirectional behavior of thrombin activity and thrombin concentration, the decrease in increment in thrombin concentration after the inflection point, Fig. 1 was interpreted as a decrease in total concentration of thrombin in the medium, Fig. 2. In addition, the concentration of thrombin generated in system before inflection point was not taken into account when constructing segment of thrombogram after inflection point.

As a result of this error, form of thrombin concentration curve versus time (thrombogram) is violated as shown in Fig. 3, and an erroneous conclusion follows that thrombin concentration in reaction medium after inflection point decreases as a result of TFPI α , PCa, ATIII and α 2-MG action, and not increases, as shown in Fig. 1, Fig. 3. Moreover, a contradiction arises in that at the same time interval the total activity of thrombin increases, Fig. 1, and the total concentration of thrombin tends to zero, Fig. 2. Therefore, descending part of thrombogram after inflection point cannot be considered, Fig. 2,

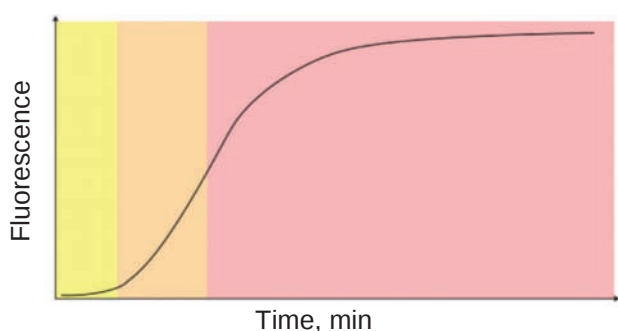


Fig. 1. The change in the fluorescent signal over time, reflecting the generation of thrombin in blood plasma, which is initiated by tissue factor and CaCl_2 (yellow – initiation phase, orange – amplification phase, and resulting phase) [1] with permission

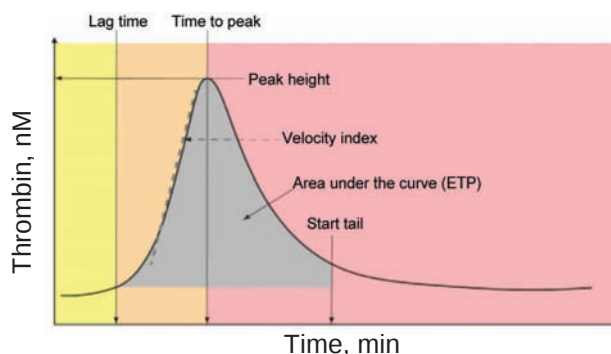


Fig. 2. Thrombogram and its parameters, including ETP (endogenous thrombin potential), built on basis of thrombin amidase activity curve formed in the reaction medium [1] with permission

as a stage of inhibition of thrombin generation by inhibitors, since only the growth of thrombin activity decreases, and the total activity and concentration of thrombin continue to increase, Fig. 1, Fig. 3.

The proposed interpretation of graphs can be supplemented by the following considerations. The curve in Fig. 1 represents the dynamics of the release of label from fluorescent substrate by thrombin, which depends not only on the thrombin concentration but also on the concentration of thrombin substrates, namely fibrinogen (Fg) and fluorescent substrate, which is probably exhausted by the time the curve reaches the plateau, as in Fig. 3 is exhausted S2238. Presented in Fig. 2, the first derivative of fluorescent signal growth gives the activity of thrombin at each moment of reaction. To convert thrombin activity to concentration, it is necessary to use the calibration curve of thrombin activity in relation to ZGGR-AMC substrate (or pNA-substrate) vs thrombin concentration or the specific activity of thrombin standard in relation to ZGGR-AMC substrate. Therefore, the thrombogram in Fig. 2, representing dependence of thrombin activity on time, cannot adequately reflect the changes in thrombin concentration in the cuvette *in vitro*. After passing the inflection point, label growth begins to decrease, probably due to depletion of the fluorogenic substrate supply, increased thrombin autolysis, and, unlikely, action of thrombin inhibitors. Therefore, the calculation of thrombin concentration based on values of thrombin activity is erroneous, as it does not include the value of thrombin activity (read concentration) at moment of inflection point of the reaction. In this regard, the descending part of thrombogram does not indicate a decrease in the increment in thrombin activity, but a decrease in concentration of thrombin, which is an unintentional error, Fig. 2, Fig. 3. As a result, the endogenous thrombin potential (ETP) as well as the height of thrombin concentration peak is determined with an error.

Considering lag period of curve (initiation stage), a very important point in TGA method is not taken into account – connection between stages of formation of the clot structure in blood plasma and phases of thrombin generation process. Comparing stages of clot formation, namely the formation of protofibrils, their lateral association and formation of a fibrillar structure, and initiation phase of prothrombin activation, it was found that the end of formation of clot structure coincides with the beginning of acceleration of thrombin generation, Fig. 3,

A. This means that all Fg as a substrate of generated thrombin in the cuvette turns into a fibrin clot and forms a solid phase – a network of fibrils of the clot, on which thrombin is sorbed while maintaining Fg-transforming and amidase activity [4]. In this phase, TFPI α does not have a significant inhibitory effect on activation of prothrombin, because: a) formation of FVIIa–TF, FVIIa–TF/FXa complexes [5] and activation of FIX in FIXa and FX in FXa, which essentially combines extrinsic and intrinsic pathways of prothrombin activation, occurs on phospholipid surface, and inhibition of thrombin by TFPI α – in plasma, in different phases of the heterogeneous system [6]; b) concentration of Fg in plasma is much higher than concentration of TFPI α – from ~900 to ~6000 nM (depending on dilution of plasma in microplate cell) and 2 nM [7], therefore thrombin, released from phospholipid surface, interacts first with Fg in plasma [8]. Although thrombin is formed in small concentrations, which is confirmed by the presence of a lag phase, Fig. 3, A, its action is directed only at Fg, as main substrate, and is sufficient for formation of a clot.

After complete Fg transformation into a fibrin clot, some excess thrombin appears, which switches to activation of FVIII, FV, and FXI with formation of tenase and prothrombinase complexes. The appearance of tenase and prothrombinase complexes causes a “burst” in rate of thrombin formation and a rapid increase in amidase activity, which is observed in Fig. 1 and Fig. 3 [9]. It is not clear at what stage of the thrombogram the amplification stage ends, but it is probably beyond the peak of thrombin activity.

A “burst” in the rate of thrombin generation after conversion of all fibrinogen to fibrin and clot formation may appear to be a loss of control over prothrombin activation since the concentration of Fg at this point is depleted. However, it can also be viewed as a biological mechanism used by coagulation system to form a strong boundary between damaged tissue and blood plasma or the surrounding environment. Thrombin in a high concentration forms a fibrin thin-fibrous clot with covalently included proteins: α 2-AP, PAI-1, fibronectin, cross-linked FXIIIa α C regions of fibrin, α Ib β 3 and other platelet proteins, which forms a “borderline” structure of the clot resistant to action of fibrinolytic system and other proteases, necessary for initiation of regenerative processes in damaged tissues.

It should be noted that, under *in vitro* conditions, thrombomodulin and heparin in donor plasma are normally absent in microplate cells [10]. There-

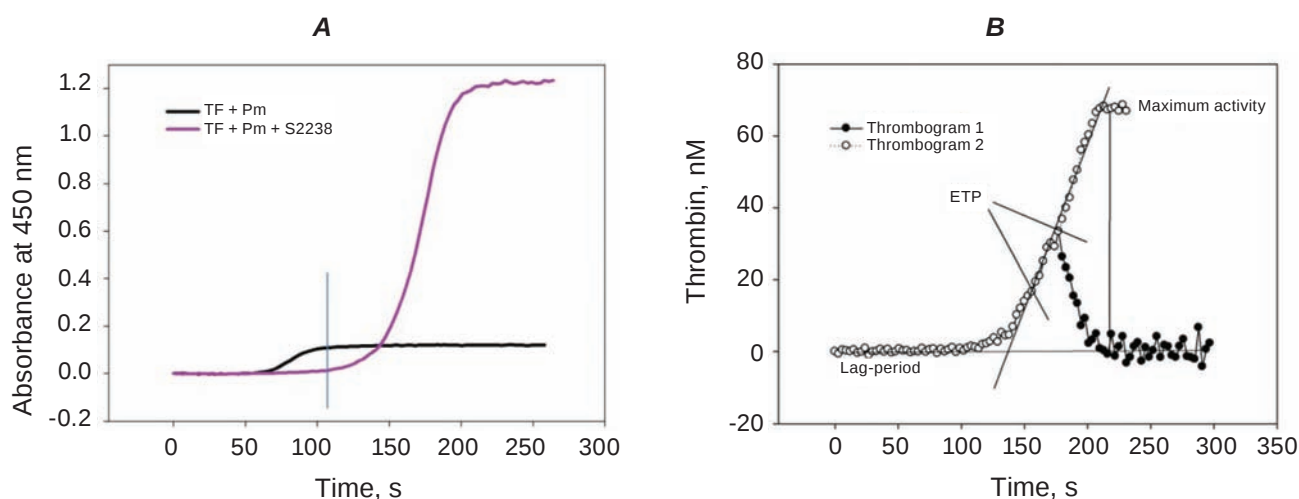


Fig. 3. Turbidimetric curve of donor blood plasma coagulation initiated by TF in the presence of CaCl_2 and curve of *p*-nitroaniline label release from thrombin substrate S2238 (red line), obtained under the same conditions (A). Thrombograms constructed on the basis curve of the *p*-nitroaniline label release curve from thrombin substrate S2238, using the traditional TGA calculation (thrombogram 1) and taking into account the error in the calculation of thrombin concentration after the inflection point of the label growth curve (thrombogram 2) (B)

fore, downward section of the curve in Fig. 2 could not be fully explained only by the effect of PCa and ATIII. Moreover, $\alpha 2$ -MG preserves amidase activity of thrombin after its capture in complex [11].

Attention is drawn to the fact that the second phase of prothrombin activation – amplification, unfolds in plasma inside the fibrin clot formed in cells of microplate or, presumably, in a clot formed in a damaged vessel, on its walls, or at the site of tissue damage and bleeding *in vivo*. Obviously, despite high concentrations of thrombin observed *in vitro*, one cannot expect much release of excess thrombin outside the clot *in vivo*, because clot fibrin, platelets, components of glycocalyx, and other proteins will retain it in forming thrombus, and thrombin molecules, that got into plasma outside the clot will be inactivated by ATIII-He, $\alpha 2$ -MG and bound by others blood plasma components [12].

Conclusions. Thus, the main problem of the TGA method is that in the descending part of the thrombogram, Fig. 2, an error appears in the conversion of thrombin activity to its concentration. The thrombin concentration obtained in the right part of the thrombogram is an increment in thrombin con-

centration in the entire system and total thrombin concentration increases, Fig. 3, A. Against the background of fluorescent signal growth after inflection point, the increment in thrombin activity in this part of the curve decreases, which thrombogram registers as a decrease in the concentration of thrombin in the system, Fig. 2. Therefore, these concentrations of thrombin must be added to concentration of the “peak height” point, which ends the left shoulder of thrombogram, Fig. 3, to obtain the true value of ETP, thrombin concentration, its maximum value and to calculate values of other parameters of thrombin generation curve in human blood plasma.

Conflict of interest. The authors have completed the Unified Conflicts of Interest form at http://ukrbiochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

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ПАРАЛОГІЗМ В ІНТЕРПРЕТАЦІЇ ТРОМБОГРАМИ, ОТРИМАНОЇ З ВИКОРИСТАННЯМ МЕТОДУ АНАЛІЗУ ГЕНЕРАЦІЇ ТРОМБІНУ В ПЛАЗМІ КРОВІ ЛЮДИНИ

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Визначення активності тромбіну в крові, що активується ініціаторами згортання, дає важливу ранню інформацію про стан системи гемостазу. Метод аналізу генерації тромбіну (TGA), розроблений для визначення ендogenous потенціалу згортання та антикоагулянтної активності плазми крові, є надзвичайно важливим діагностичним і прогностичним інструментом. Однак основна проблема методу ТГА полягає в тому, що в низхідній частині тромбограми з'являється помилка в процесі перерахунку активності тромбіну в його концентрацію. Вивчення зв'язку у часі між активністю тромбіну та структурою згустку в процесі його утворення та розчинення в плазмі крові людини, дозволило запропонувати інший варіант розрахунку параметрів тромбограми, отриманої методом ТГА.

Ключові слова: метод генерації тромбіну, концентрація тромбіну, розрахунок параметрів тромбограми.

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