

EXPERIMENTAL WORKS

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doi: <https://doi.org/10.15407/ubj96.02.019>DETERMINATION OF THROMBIN AND PLASMIN ACTIVITY
USING THE TURBIDIMETRIC ANALYSIS OF CLOT FORMATION
AND DISSOLUTION IN HUMAN BLOOD PLASMAA. UDOVENKO[✉], Ye. MAKOGONENKO, O. HORNYTSKA,
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Based on the turbidimetric curve of formation and dissolution of a blood plasma clot initiated by the activated partial thromboplastin time reagent, a method for determining the coagulation component of thrombin activity and fibrinolytic activity of plasmin is proposed. The activity of thrombin was calculated by the value of the lag period, and plasmin by its amidase activity at the moment of complete dissolution of the clot. At the end of the lag period, about 0.45% of the available prothrombin was activated, and at the moment of complete dissolution of the clot 1.05% of the available plasminogen was activated. This method makes it possible to determine the ratio of the thrombin generation rate to that of plasmin, the time of clot formation to the time of its dissolution, as well as the overall hemostasis potential and coagulation and fibrinolytic components and their ratio.

Key words: thrombin generation, plasmin generation, coagulation, fibrinolysis, global hemostasis assay.

Thrombin (EC 3.4.21.5) and plasmin (EC 3.4.21.7) are the final enzymes of the activation pathway of the coagulation system and fibrinolysis of human blood plasma, respectively. The concentrations of these enzymes serve as indicators of the functional readiness of each system to respond to disturbances in the hemostatic system. Furthermore, the ratio of the rates of plasma clot formation and dissolution in vitro serves as an indicator of the equilibrium between the coagulation and fibrinolysis systems. [1, 2]. Thrombin, apart from its primary role in transforming fibrinogen into fibrin, performs various additional functions. It activates platelets by cleaving its protease-activated receptors; expedites the internal pathway of the coagulation cascade by activating FXI, FV and FVIII; triggers the activation of endothelial cells; influences the protein C system and thrombin activatable fibrinolysis inhibitor and exerts an effect on the permeability of the blood-tissue barrier [3]. The multifunctionality of thrombin has led to the development of numerous methods for determining its concentration in

blood plasma, primarily relying on the amidase activity of the enzyme towards peptide substrates with p-nitroanilide (pNA) and fluorescent labels [2, 4-7]. However, it was shown that the curve of thrombin amidase activity does not coincide with the curve of growth of turbidity, which reflects the formation of the fibrin clot structure in the process of blood plasma coagulation [6-9]. During the lag period, the amidase activity of thrombin diminishes as the peptide substrate competes with fibrinogen for the active center of thrombin. Conversely, the peptide released by thrombin in μM concentrations can inhibit the formation of protofibrils and their lateral association, potentially by impeding the interaction of A-a sites during desA-fibrin polymerization. Through its multisite interaction with fibrinogen, after cleavage of fibrin peptides A, thrombin remains bound to the fibrin substrate – including monomers, oligomers and protofibrils while retaining its activity towards fibrinogen [8]. Therefore, the method of determining the speed of thrombin formation and its concentration based on its amidase activity does not reflect the

thrombin activity and concentration formed at the initial stage of blood plasma coagulation in which protofibrils are formed and the activity of thrombin is directed only to the formation of fibrin and fibrin protofibrils. In the lag period, the thrombin formed by the coagulation system shows maximum activity in relation to the fibrinogen substrate, while the rate of protofibril formation depends on the thrombin concentration. Therefore, the goal of our research was to develop a method for determining the coagulation component of thrombin activity, which is formed by the blood plasma coagulation system during the lag period (τ), based on the turbidimetric curve (TDC) of the blood plasma clot formation and dissolution. Simultaneously, the analysis of the dissolution phase of the blood plasma clot by the fibrinolytic system has allowed for the development of a method to determine the concentration of formed plasmin based on its amidase activity. Methods for determining the thrombin and plasmin concentration make it possible to characterize the state of the hemostasis system, the level of the balance of the activity of its coagulation and fibrinolytic links in vitro at the level of enzyme activity and predict the possible direction of changes in human blood plasma in normal conditions and in various pathologies [2, 9, 10].

Materials and Methods

In the experiments was used: activated partial thromboplastin time (APTT) reagent from Granum (Ukraine) and recombinant tissue plasminogen activator (t-PA) from Boehringer Ingelheim (Germany). Plasminogen was isolated from blood plasma by affinity chromatography on Lys-Sepharose, plasmin was obtained by activating plasminogen with urokinase immobilized on Sepharose 4B and thrombin was a reagent from Sigma (USA). S2238 and S2251 pNA-labeled peptide substrates were purchased from Chromogenix. Fibrinogen was isolated from blood plasma of donors by the method [13].

Donor blood samples 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 one hour after blood collection by centrifuging the latter at 1500 g for 20 min. Aliquots of plasma were stored at -20°C.

Blood plasma coagulation was studied using the turbidimetric method by recording the turbidity of the fibrin clot at 405 nm [10]. The clot was formed in plastic cuvettes to which 0.02 M HEPES buffer containing 0.15 M NaCl, 0.005% Tween-20, pH 7.4,

40 μ l of blood plasma and t-PA was added sequentially to a final concentration of 75 IU/ml and 50 μ l of the APTT reagent. The plasma coagulation process was initiated by adding 25 mM CaCl_2 . The final volume of the reaction mixture was 400 μ l. The results were processed using the standard Excel statistical program. The average values of the parameters and their standard deviation were determined.

Results and Discussion

To determine the thrombin and plasmin concentrations, turbidimetric curves of blood plasma clot formation and dissolution were used, initiated by the APTT reagent and recorded at 405 nm using a spectrophotometer, Fig. 1. Determination of thrombin activity was carried out on the basis of the parameter τ , and plasmin formation by measuring its amidase activity directly in the cuvette at the moment of complete dissolution of the clot.

The parameter τ characterizes the time of protofibrils formation and is expressed in seconds [10]. The stage of protofibril formation in the process of plasma clot formation, initiated by the APTT reagent, is intricate. It involves the sequential activation of the internal pathway of the coagulation system, prothrombin activation leading to thrombin formation, cleavage of fibrin peptides A from fibrinogen by thrombin resulting in fibrin desA formation, activation of FXIII by thrombin, self-assembly of desA fibrin molecules leading to the formation of double-stranded protofibrils with partial stitching of FXI-IIa along the γ - γ chains of adjacent DD modules and the commencement of cleavage of fibrinopeptides B from the protofibrils [14]. The enzymatic transformations occurring during the stage of protofibril formation encompass nearly the entire coagulation system. It should be noted that the value of τ is also affected by such blood plasma factors as: the concentration of fibrinogen $\downarrow$, $\text{Ca}^{2+}\uparrow$, $\text{Cl}^-\downarrow$, $\text{Na}^+\uparrow$, as well as proteins included in the FXIIIa clot (α 2-AP, fibronectin, PAI-1) [14, 15]. The subsequent destiny of desA fibrin is determined by the amount of thrombin activity formed, specifically: under conditions of low thrombin activity and a high concentration of fibrinogen, desA fibrin forms complexes and oligomers with fibrinogen $\text{Fg}-(\text{desA})_n$ -Fg, which represent soluble fibrin and serve as markers of the activated state of the coagulation system [15, 16]. At a high concentration of thrombin, when fibrinopeptides B begin to be cleaved and fibrin oligomers reach a length of eight pairs of fibrin monomers, the next stage of fibrin polymerization begins – the process of lateral associa-

tion of protofibrils into fibrils and the formation of a three-dimensional clot structure [13, 14, 17].

Thus, the parameter τ serves as an integral indicator of the blood plasma hemostasis system's ability to activate prothrombin into thrombin, transform fibrinogen into fibrin and generate protofibrils suitable for the subsequent stage – the formation of a fibrillar thrombus network. An essential positive aspect of this approach is that, unlike determining the overall activity of thrombin as an enzyme using a peptide substrate with a p-nitroanilide or fluorescent label, we determine the specific coagulation component of thrombin activity directed only to the conversion of fibrinogen to fibrin.

It's important to note that the activity of the formed thrombin is determined by the rate of protofibril formation, which, in turn, is directly associated with the structure of the resulting clot [18]. With high thrombin activity, clots with thin fibrils are formed, which determines their greater resistance to fibrinolysis, with low thrombin activity clots form with thicker fibrils and less resistance to fibrinolysis. The proposed approach has an advantage over the determination of thrombin activity using a peptide substrate. While the amidase activity of thrombin enables the monitoring of its activity and concentration throughout its entire path of formation and existence in blood plasma, it cannot be directly correlated with the stages of fibrin clot formation or other functions of thrombin within the plasma [4-7, 9].

To construct the calibration curve, thrombin with a specific activity of 1000 NIH units per 1 mg of protein was used, which corresponded to the activity of 1 NIH unit per 1 μ g of thrombin or a concentration of 28 nM when dissolving 1 NIH unit in 1 ml of the reaction medium. The blood plasma coagulation reaction was carried out in 0.02 M HEPES buffer, pH 7.4, containing 0.15 M NaCl and 0.005% Tween-20, 10-fold diluted plasma, 6.2 mM CaCl_2 and was initiated by adding thrombin to the indicated concentration. Fig. 1 shows a typical result of the experiment, which presents a series of turbidimetric curves for determining the values of τ . The obtained values of τ were used to construct a calibration curve in the coordinates $1/\tau$ (V) – [Thr] (rate of protofibril formation in s^{-1} vs thrombin concentration, which is expressed in NIH units of thrombin activity in 1 ml of the reaction medium), Fig. 2.

It should be noted that thrombin activity is linearly related to the rate of protofibril formation ($1/\tau$), and thrombin activity can be considered as the rate of fibrinogen to fibrin conversion (mainly desA fibrin) during the lag period (τ). Table 1 shows the values $\tau_1 = 92.4 \pm 5.83 \text{ s}^{-1}$, corresponding to thrombin activity of 0.114 ± 0.0071 NIH units in 1 ml, which corresponds to 3.17 nM of protein. The concentration of prothrombin in the medium under our conditions is 1.42 μ M. The concentration of the formed thrombin is $(3.17 \text{ nM}/1420 \text{ nM}) \cdot 100\% = 0.22\%$ of the available prothrombin. The presence of t-PA in the

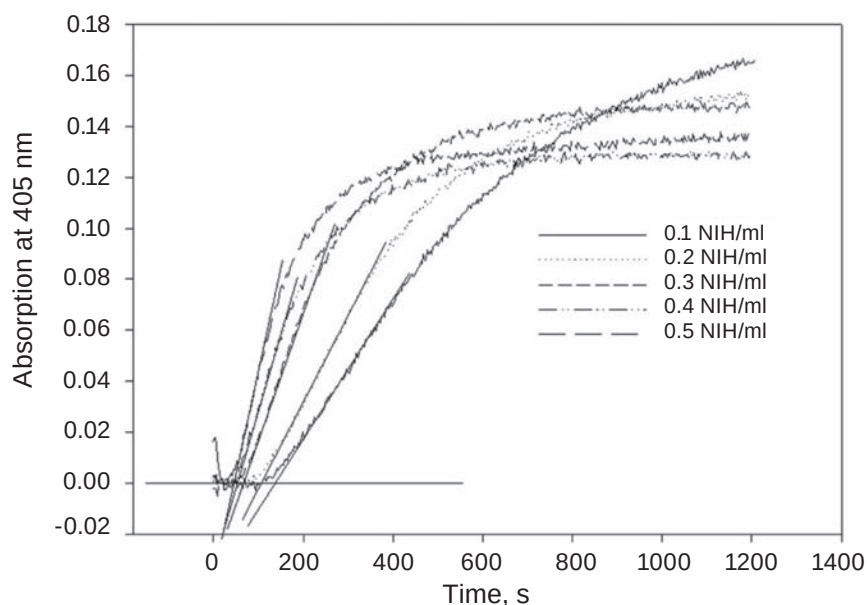


Fig. 1. Turbidimetric curves obtained by activation of donor blood plasma coagulation by a series of standard thrombin concentrations from 0.1 to 0.5 NIH units in 1 ml of reaction medium

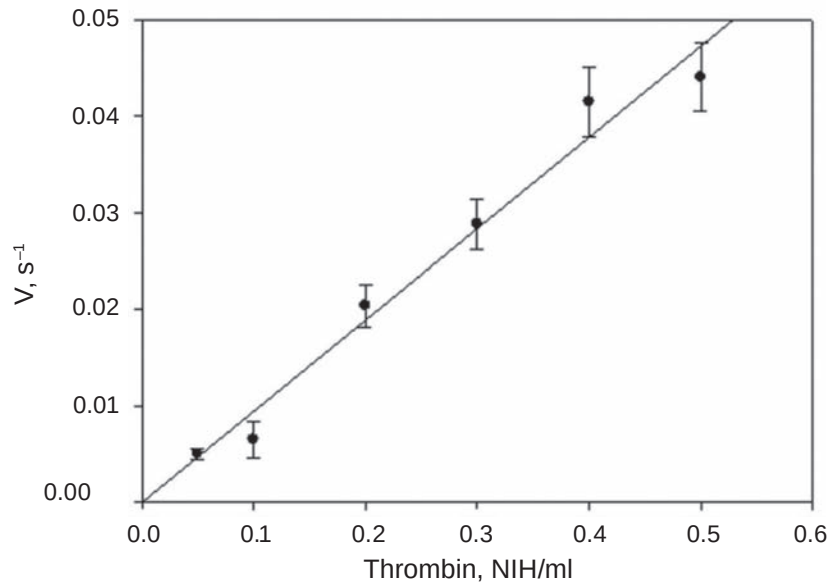


Fig. 2. The calibration curve built on the basis of the parameter τ_1 ($V = 1/\tau_1$) TDC of healthy donors' blood plasma clot formation and dissolution, to determine the concentration of thrombin in human blood plasma, ($n = 3$)

Table 1. The values of the turbidimetric curves parameters of the blood plasma clots formation and dissolution, initiated by the APTT reagent, in the absence of t-PA ($n = 4$)

τ_1 , s	V_1 , o.u./s	H, o.u.	τ_2 , s	CP, o.u.×s
90.40 ± 9.25	0.00410 ± 0.00019	0.096 ± 0.0091	161.0 ± 18.6	25.00 ± 2.69

Table 2. The values of the turbidimetric curves parameters of the blood plasma clots formation and dissolution, initiated by the APTT reagent, in the presence of t-PA ($n = 4$)

τ_1 , s	V_1 , o.u./s	H, o.u.	τ_2 , s	τ_3 , s	τ_{3s} , s	τ_{3f} , s	V_2 , o.u./s	Pm, $\mu\text{g/ml}$	OHP, o.u.×s	CP, o.u.×s	FP, o.u.×s
92.40	0.0052	0.089	181.0	731.0	549.0	198	-0.00032	1.250	17.50	25.00	7.82
$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$
5.83	0.0018	0.001	12.9	19.9	44.4	37	0.000072	0.086	2.22	2.69	1.77

reaction medium did not affect the activity of thrombin and the rate of formation of protofibrils.

Hence, employing the parameter τ from the turbidimetric curve of clot formation and dissolution enables the determination of the concentration, percentage of activated prothrombin and the average speed of prothrombin activation in blood plasma, initiated by the APTT reagent.

Determination of the concentration of plasmin formed in blood plasma, initiated by the APTT reagent, was intended to be performed using the parameter L. This parameter reflects the half-life of

the clot, and its value exhibits a linear dependence on the concentration of plasmin within the system of purified blood plasma proteins. This approach has proven successful in studying the activation process of Glu- and Lys-plasminogen within the forming fibrin clot [19]. However, the calibration curve for plasmin obtained in blood plasma had a very low slope and could not be used to estimate the plasmin activity that formed during the fibrinolysis system activation [20, 21].

Therefore, a simpler approach was chosen to determine the speed of plasminogen activation, the

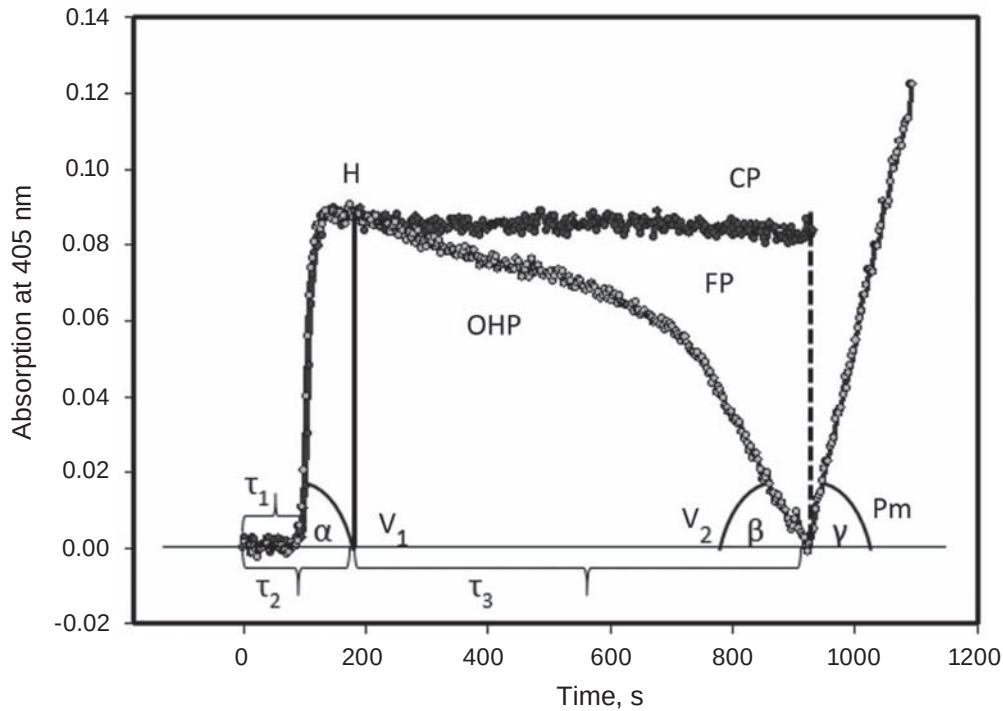


Fig. 3. Determination of the thrombin and plasmin activity formed during the fibrin clot formation and dissolution in blood plasma in the presence of added t-PA and the peptide substrate S 2251 (H-D-Val-Leu-Lys-pNA), using the τ parameter and plasmin amidase activity at the moment of complete dissolution of the clot (V). τ_1 – lag period of clot formation, τ_2 – clot formation time, τ_3 – clot dissolution time, V_1 – the rate of protofibrils lateral association (equal to $\tan \alpha$), H – maximum clot turbidity, dashed vertical line – the moment of introducing the plasmin substrate into the dissolved clot, V_2 – maximum clot dissolution rate (equal to $\tan \beta$), P_m – amidase activity of the formed plasmin (equal to $\tan \gamma$). CP , OHP and FP are the coagulation, overall and fibrinolytic potential of the blood plasma hemostasis system, which is equal to the area under the coagulation curve (CP), formation and dissolution (OHP) of the clot and their difference (FP), respectively

activity and concentration of plasmin formed in a blood plasma clot during its formation and complete dissolution by the fibrinolytic system, Fig. 3. Formed plasmin activity was determined at the moment when the three-dimensional plasma clot structure was completely destroyed and the optical density in the cuvette reached zero. At this moment, the amidase substrate S2251 was added to the cuvette to a concentration of 0.3 mM and the increase in absorbance at 405 nm was recorded for approximately two minutes. The linear dependence of the medium optical density growth on time indicates the end of the plasminogen activation process in the dissolved plasma clot by tissue plasminogen activator, which is associated with the destruction of the clot fibrillar structure and the loss of structures that stimulate plasminogen activation [22, 23], as well as with the absence of the plasmin inhibition process [19, 20]. Further calculation of the plasmin activity, which remained active at the time of clot

complete dissolution, is carried out based on data on the standard plasmin specific amidase activity. It was established that the amidase activity of such plasmin on the S2251 substrate with fully activated active centers and without plasminogen residues is 33 o.u. at 405 nm of p-nitroaniline released by the enzyme per 1 mg of standard plasmin protein in one minute. The concentration of plasmin at the time of measurement will be:

$$C = \frac{\Delta E_{405} \text{ o.u.}}{N_{\min} * 0.033 \text{ o.u.}/(\frac{\mu\text{g}}{\text{ml}}) * \text{min}} = \frac{\Delta E_{405}}{N * 0.033} \mu\text{g/ml},$$

where $N_{\min}$ – time of measurement of plasmin activity in minutes; ΔE_{405} o.u. – growth of the optical density in the cuvette during the measurement; $0.033 \text{ o.u.}/(\mu\text{g/ml}) \cdot \text{min}$ – specific amidase activity of 1 $\mu\text{g/ml}$ of plasmin per 1 min with S-2251 (H-D-Val-Leu-Lys-pNA).

In four independent experiments based on TDC, the following values of τ and P_m were found for thrombin and plasmin, respectively: $92.40 \pm 5.83 \text{ s}^{-1}$ i $1.25 \pm 0.086 \text{ } \mu\text{g/ml}$, which corresponded to their molar concentrations of 3.17 and 14.7 nM, Fig. 3. Considering that the concentration of prothrombin and Glu-Pg in the reaction medium was 1428 and 1400 nM, respectively, the % concentration of activated thrombin and plasmin to that of their proenzymes was 0.22 and 1.05%, respectively. The ratio of thrombin to plasmin concentration was $\sim 1:4.68$. For the formation of protofibrils, it turned out to be sufficient to activate $\sim 0.22\%$ of the available prothrombin, which is close to the previously obtained values [9], and for the complete dissolution of the clot – 1.05% of the Glu-Pg concentration. The clot formation time was 180 s, and the clot dissolution time by the fibrinolytic system was 731 s, their ratio was 1:4.06. The average rate of prothrombin activation in the lag period was 0.31 nM/s, and the average rate of plasminogen activation was 0.020 nM/s. The ratio of the rate of prothrombin activation to such plasminogen is 1.5:1, but the ratio of fibrinogen to fibrin transformation time to such fibrin dissolution which is 1:4.04 and close to the thrombin to plasmin concentration ratio.

As presented in Fig. 3, turbidimetric curves make it possible to determine hemostatic potential and its components in human blood plasma. One of the important parameters is the CP/FP ratio, which characterizes the equilibrium state in the hemostasis system and is equal to $25:7.82 = 3.2$. It is worth noting that the ratio of thrombin to plasmin concentration is 4.63 which close in magnitude to the ratio CP/FP and can potentially be independent parameters characterizing the state of equilibrium systems of human blood plasma hemostasis.

Conclusions. The development of methods for determining the thrombin and plasmin activity, formed during the clot formation and dissolution in human blood plasma, enables the quantitative assessment of the coagulation component of thrombin activity at the initial stage of clot formation and the fibrinolytic activity of plasmin at the moment of complete clot dissolution. The obtained parameters are important kinetic characteristics of the clot formation and dissolution processes in human blood plasma, namely: parameters determine the speed of prothrombin activation, the concentration of thrombin at the end of protofibril formation; the activity and concentration of plasmin at the point

of clot three-dimensional structure complete dissolution, the rate of plasminogen activation and destruction of the clot structure by plasmin; the time of clot three-dimensional structure formation and dissolution, namely the slow and fast stages. It is shown that the ratio of thrombin activity to plasmin activity is close in magnitude to the rate ratio of fibrinogen into fibrin transformation to such fibrin dissolution. The value of the coagulation and fibrinolysis potentials ratio and the time of clot three-dimensional structure formation and dissolution is also important. The proposed methods of quantitative assessment of the balance between the coagulation and fibrinolysis systems in blood plasma in model systems in vitro can complement the existing methods [1, 2, 24-30].

Ethical committee approval. Study was approved by ethical committee of Palladin Institute of biochemistry of NAS of Ukraine, 25.05.23, N 6.

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

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ВИЗНАЧЕННЯ АКТИВНОСТІ ТРОМБІНУ І ПЛАЗМІНУ НА ОСНОВІ ТУРБІДИМЕТРИЧНОГО АНАЛІЗУ ФОРМУВАННЯ І РОЗЧИНЕННЯ ЗГУСТКУ В ПЛАЗМІ КРОВІ ЛЮДИНИ

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На основі турбідиметричної кривої формування і розчинення згустку плазми крові, ініційованого АЧТЧ реагентом, запропонова-

но метод визначення коагуляційної компоненти активності тромбіну та фібринолітичної активності плазміну. Активність тромбіну розраховували за величиною лаг-періоду, а плазміну – за його амідазною активністю в момент повного розчинення згустку. Знайдено, що швидкість формування протофібрил лінійно корелювала з активністю тромбіну. На кінець лаг-періоду активувалося біля 0,22% наявного протромбіну, а в момент повного розчинення згустку – 1,05% наявного Pg. Метод дозволяє визначити відношення швидкості генерації тромбіну до такої плазміну, часу формування згустку до часу його розчинення, а також загальний гемостатичний потенціал, його коагуляційну та фібринолітичну компоненту та їх відношення.

Ключові слова: генерація тромбіну, генерація плазміну, згортання, фібриноліз, метод загального гемостазу.

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