SIMULTANEOUS DETERMINATION OF PRECURSORS OF KALLIKREIN, PLASMIN, AND THROMBIN AND THEIR INHIBITORS IN HUMAN BLOOD PLASMA

O. A. Gomazkov and N. V. Komissarova

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Prekallikrein, plasminogen, and prothrombin of human blood plasma are separately activated by kaolin, streptokinase, or thromboplastin. Measurement of the TAME-esterase (TAME: N-α-tosyl-L-arginine methyl ester) activity of each of the enzymes thus produced and its changes during incubation of the plasma with the activator can be used to estimate the content of kallikrein, plasmin, and thrombin and their inhibitors. Evidence is given that under the conditions of determination as described activation is specific for each enzyme and does not affect the level of the other precursors. The method is developed in modifications enabling the values of seven indices to be obtained with 0.4-0.7 ml of blood plasma.

KEY WORDS: kallikrein; plasmin; thrombin; their precursors and inhibitors; method of simultaneous determination.

Kallikrein, plasmin, and thrombin are key enzymes in functionally linked blood systems concerned with the regulation of hemostasis and vascular tone. Activation of prekallikrein in vitro takes place by contact between plasma and negatively charged surfaces, especially with kaolin; plasminogen is activated by streptokinase and prothrombin by exposure to thromboplastin and Ca^{++}. The esterase activity of the enzymes rises under these circumstances parallel with the increase in the specific – kininogenase, fibrinolytic, or plotting – activity [5, 7, 9].

DESCRIPTION OF METHOD

I. Activation of Precursors of the Enzymes. The method is based on that suggested for determination of the kallikrein precursor and its inhibitor [4]. Native blood plasma is activated by kaolin, thromboplastin, or streptokinase with two periods of incubation. In the first period (1 min) maximal activity of the enzyme formed from the precursor is determined, and in the second period (5, 30, or 60 min) activity of the inhibitor is determined (Fig. 1). Preliminary experiments showed that these periods are optimal for detecting the maximal level of the precursors and activity of the inhibitors of kallikrein, thrombin, and plasmin.

Blood plasma in a volume of 0.2 ml is activated by an equal volume of kaolin mixture at 25°C, and 0.1 ml aliquots are taken 1 and 5 min later for determination of prekallikrein and kallikrein inhibitor [1]. To determine the plasminogen content the plasma is activated with streptokinase [2]. Samples of 0.1 ml are taken after 1 and 60 min for further tests. Prothrombin is activated at 37°C. The incubation mixture contains: 0.05 ml plasma, 0.05 ml 0.9% NaCl, 0.2 ml of thromboplastin suspension diluted in accordance with the instruction, and 0.1 ml of 1% $CaCl_2$ solution. After incubation for 1 and 30 min, the reagents for determination of esterase activity are added to the samples. These samples are for estimation of the prothrombin and thrombin inhibitor. When 3-methyl-2-benzothiazolone hydrazone (MBTH) is used as reagent in the final stage of the determination the quantity of plasma and activators must be smaller: 0.02 ml plasma, 0.05 ml thromboplastin, and 0.03 ml 1% $CaCl_2$ solution.

Institute of General Pathology and Pathophysiology, Academy of Medical Sciences of the USSR, Moscow.
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TABLE 1. Level of Precursors of Kallikrein, Plasmin, and Thrombin and of Their Inhibitors in Human Blood Plasma and Serum Determined by the Esterase Method

Test object and method of determination	Initial activity	Prekallikrein	Kallikrein inhibitor	Plasminogen	Plasmin inhibitor	Prothrombin	Thrombin inhibitor	No. of determinations
Blood plasma (method with chromotropic acid)	3,9±1,15	62,1±10,7	0,9±0,03	69,2±2,0	0,28±0,07	69,6±5,7	1,25±0,27	8
Blood plasma (method with MBTH)	2,9±0,6	61,7±2,4	0,88±0,07	81,8±5,5	0,04±0,02	77,7±4,7	1,44±0,07	12
Blood serum (method with MBTH)	13,3±5,4	74,0±3,8	0,55±0,05	90,7±9,5	0	17,0±4,3	0,19±0,013	4

Legend. 1) Enzyme activity expressed in μmoles hydrolyzed TAME per ml plasma or serum per hour. Values of inhibitors given in conventional units [4]. 2) Activation time for determination of inhibitors of kallikrein 5 min, plasmin 60 min, and thrombin 30 min (see "Method").

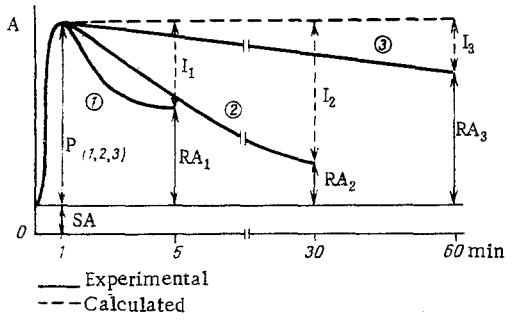

Fig. 1. Diagram representing determination of precursors of kallikrein, thrombin, and plasmin and their inhibitors during specific activation in blood plasma. Abscissa, incubation time of plasma with activator (in min); ordinate, enzyme activity. SA) Spontaneous (initial) activity of plasma; $P_{(1,2,3)}$ – level of activated precursor of enzyme; $RA_{(1,2,3)}$ – residual activity of enzyme after blocking by inhibitor; $I_{(1,2,3)}$ – index of inhibitor; 1) kallikrein, 2) thrombin, 3) plasmin. Continuous line shows experimentally determined values, broken line calculated values.

II. Incubation of Activated Samples with the Substrate. To measure esterase activity of the enzymes, N-α-tosyl-L-arginine methyl ester (TAME) was chosen as the substrate, for it is hydrolyzed by kallikrein, plasmin, and thrombin at about the same rate and does not undergo spontaneous hydrolysis over a wide range of pH values; the pH-optimum for the reaction with this substrate was similar for all three enzymes. The measured values of K_m for kallikrein, plasmin, and thrombin are $1.36 \cdot 10^{-2}$ M [4], $5.4 \cdot 10^{-3}$ M, and $8.5 \cdot 10^{-3}$ M respectively. The reaction products are not competitive inhibitors.

Esterase activity was determined by incubating 0.1 ml of activated plasma with TAME in 0.1 M phosphate buffer, pH 7.6, at 37°C for 30 min. The final concentration of TAME in the 1-ml sample was $5 \cdot 10^{-2}$ M.

III. Spectrophotometric Determination of the Reaction Products. The methanol formed as a result of hydrolysis of TAME is oxidized by $KMnO_4$ to formaldehyde, which can be determined quantitatively by the color reaction with chromotropic acid [8]. The enzyme reaction is stopped by the addition of 0.5 ml 15% TCA. The principle of the colorimetric determination, carried out on a 0.5-ml sample, is similar to that described for kallikrein [4].

The methanol formed in the reaction with TAME can also be determined by the use of the other reagent MBTH [6]. This method, which is more sensitive than the hydroxamate method [3] or the method with chromotropic acid [8], was used by the writers to study the plasma kallikrein, plasmin, and thrombin and their inhibitors. Advantages of this method are that minimal quantities of plasma can be used (0.4 ml is sufficient for all

determinations) and low values of esterase activity of the enzymes can be detected. Samples measuring 0.2 ml are taken from the incubation mixture containing TAME (see section II); the reaction is stopped by the addition of 0.2 ml of 10% solution of $ZnSO_4$. The samples are then treated with 0.2 ml of a 1% solution of $KMnO_4$ in 10% $KHSO_4$, followed after 1 min by 0.2 ml of 1% solution of $NH_2OH \cdot HCl$ and 1.5 ml of a reagent consisting of equal volumes of 10% $KHSO_4$ and 1% MBTH solutions. After 20 min, 1.5 ml of a 2% solution of $NH_4Fe(SO_4)_2 \cdot 12H_2O$ is added to each sample. The samples are centrifuged and the supernatant is estimated spectrophotometrically at 635 nm for 60-100 min against a reagent control.

The quantity of methanol formed after enzymic hydrolysis of TAME was determined from a calibration curve plotted for 1.0 μmole absolute CH_3OH when the method with chromotropic acid was used and for 0-0.5 μmole when the method with MBTH was used.

Tests were carried out with citrated blood donor's plasma; the plasma was frozen immediately after centrifugation and kept for not more than 2 weeks at −10°C. All operations before stopping the enzyme reaction were carried out in silicone-treated (or plastic) vessels.

The following reagents were used: TAME, N-α-tosyl-L-argininemethyl ester hydrochloride, Reanal (Hungary); streptokinase (Streptase), Behringwerke AG (West Germany); calcium-thromboplastin, Boehringer-Mannheim (West Germany) or laboratory thromboplastin, Kirov Blood Transfusion Research Institute; MBTH, 2,3-dihydro-3-methylbenzothiazole-2-hydrazone hydrochloride, Serva (West Germany); all other reagents were of Soviet manufacture.

Data for the content of kallikrein, plasmin, and thrombin precursors and their inhibitors in healthy human blood plasma obtained by the use of modifications with chromotropic acid and MBTH are given in Table 1. As Table 1 shows, the results obtained by the two alternative methods were the same. With both variants all seven indices could be determined in 0.4-0.7 ml plasma. However, the method with MBTH, by means of which much smaller amounts of methanol in the sample can be determined, is suitable for the estimation of esterase activity of kallikrein and other enzymes in biological material with low enzymic activity. Similar results for the quantity of kallikrein and plasmin precursors and inhibitors were obtained by investigation of blood serum. Activation of the clotting system and clot formation lead to considerable exhaustion of prothrombin. The initial esterase activity of the serum is considerably increased under these circumstances as a result of the thrombin formed.

Values for inhibitors of kallikrein, plasmin, and thrombin (expressed in conventional units) also are given in Table 1, according to earlier calculations [1, 4]. The activity of each of these enzymes in human blood is known to be controlled by several inhibitors. By the method as described only the total inhibitor activity with respect to each enzyme can be estimated.

Combined investigation of kallikrein, plasmin, and thrombin precursors is possible only if during specific activation of each of the enzymes no cross activation of another precursor takes place. Separate activation of human blood plasma by kaolin or streptokinase in vitro has been shown not to affect the level of the precursor of the "neighboring" enzyme [2]. Comparison of the results obtained for plasma and serum (Table 1) shows that activation of clotting and the sharp decrease in the prothrombin level are not significantly reflected in the values of arginine esterases activated by kaolin or streptokinase. In additional experiments with activation of plasma by kaolin after thromboplastin (activation time 1 min) the esterase activity as determined corresponded to the sum of the precursors of kallikrein and thrombin determined separately. With the concentrations and times chosen, activation in vitro is thus specific for each enzyme and does not affect the value of the precursor of another enzyme.

LITERATURE CITED

1. O. A. Gomazkov, N. V. Komissarova, et al., Kardiologiya, No. 6, 25 (1972).
2. N. V. Komissarova and O. A. Gomazkov, Byull. Éksp. Biol. Med., No. 2, 35 (1974).
3. M. E. Brown, J. Lab. Clin. Med., 55, 616 (1960).
4. R. W. Colman et al., Ann. Intern. Med., 71, 763 (1969).
5. G. Girey et al., J. Lab. Clin. Med., 80, 496 (1972).
6. H. Moriya, et al., J. Biochem. (Tokyo), 69, 815 (1971).
7. S. Sherry and W. Troll, J. Biol. Chem., 208, 95 (1954).
8. M. Siegelman et al., Arch. Biochem., 97, 159 (1962).
9. W. Troll and S. Sherry, J. Biol. Chem., 208, 85 (1954).