# METHOD OF DETERMINATION OF PLASMA KALLIKREIN INHIBITORS IN MAN AND ANIMALS

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UDC 612.129:577.152.34/-064

KEY WORDS: blood plasma; kallikrein inhibitors; method of determination.

Kallikrein inhibitors reflect the state of the kinin and the other connected humoral systems of the body. To determine plasma kallikrein inhibitors, aminolytic methods with chromogenic substrates [8], enzymic [3, 9], and immunochemical [5] methods are used. A radioimmunologic method [11] is being developed. Enzymic methods, based on inhibition of proteinase activity, are the most popular. However, they are nonspecific relative to the kallikrein of blood plasma, for they determine the antiesterase [3, 9] or antiproteolytic [2] actitivity of the blood plasma (serum). Immunochemical methods have not found a place in laboratory test practice for they require highly specific antisera. The results of determination of the antitryptic activity of plasma [4] characterize the antikallikrein activity of the blood indirectly and only very approximately. Aminolytic methods are relatively specific and expensive.

Evidence has increasingly been obtained in recent years that the plasma kallikreins have no esterase or proteolytic activity and that they have only one specific property, their kininogenase activity. Hence it follows that all the methods currently used in clinical practice to determine plasma kallikrein inhibitors give no idea of the true antikallikrein capacity of the blood, but reflect the activity of inhibitors of other proteinases and esterases. The absence of a specific method of determination of kallikrein inhibitors is due to the fact that no highly purified standard preparations of kallikrein or high-molecular-weight kininogen, essential for the biochemical reaction, are available for the investigator. Since kininase and kallikrein inhibitors are inactivated by heating plasma acidified to pH 3.0, but since a functional single complex consisting of prekallikrein, kallikrein, and high- and low-molecular-weight kininogens, essential for the kininogenase reaction [6, 7], is preserved, a real opportunity is presented for obtaining a "standard" preparation of this protein complex from dried donors' plasma.

The object of this investigation was to develop a method of determination of the specific antikallikrein activity of blood plasma, including known ($\alpha_2$-macroglobulin, $\alpha_1$-antitrypsin, antithrombin-III, C'-inactivator) [1, 10] and unknown inhibitors of kallikrein [12].

## EXPERIMENTAL METHOD

The method is based on determination of the ability of inhibitors of the test plasma to inhibit the kininogenase activity of kallikrein. The following stages were developed in experiments in vitro: 1) preparation of the standard containing kallikrein and kininogen; 2) detection of kininogenase activity of prekallikrein; 3) selection of substances effectively inhibiting the action of kininases of the test plasma; 4) selection of the quantity of plasma causing 30-50% inhibition of the kininogenase activity of kallikrein; 5) determination of the conditions for conduct of the analysis and assay of the activity of kallikrein inhibitors.

Work to develp the method was carried out with blood plasma from 78 donors, 18 clinically healthy persons aged from 18 to 50 years, 28 clinically healthy children up to 3 years of age, 72 patients (children and adults) with various diseases, and also laboratory animals (18 rabbits, 21 rats, and seven cats).

The following reagents were used: bradykinin triacetate (from Reanal, Hungary), unithiol (5% solution in ampuls), EDTA (Trilon B), kaolin, and Tris (hydroxymethylaminomethane) of USSR manufacture.

V. F. Vladimirskii Moscow Regional Clinical Research Institute. (Presented by Academician of the Academy of Medical Sciences of the USSR A. D. Ado.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 91, No. 5, pp. 623-626, May, 1981. Original article submitted November 14, 1980.

0007-4888/81/9105-0695$07.50 

TABLE 1. Increase in Antikallikrein Activity of Blood Plasma (in μg bradykinin/ml/h) during Storage at 2-4°C (M ± m)

| Source of plasma | Blood plasma | | | | | | |
|---|---|---|---|---|---|---|---|
| | fresh | stored for | | | | | |
| | | 1 day | 2 days | 1 week | 2 weeks | 1 month | 5 months |
| Regular adult donors (n=38) | 4,55±0,47 | 5,74±0,49 | 8,92±0,61 | 14,04±0,59 | 27,51±0,63 | 32,83±0,68 | 143,2±5,16 |
| Clinically healthy adults (n=18) | 10,08±0,81 | — | — | — | — | 32,75±0,70 | 143,9±4,96 |
| Clinically healthy children under 3 years of age (n=28) | 30,00±0,62 | — | — | — | 60,0±0,82 | — | — |

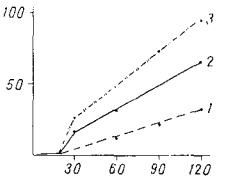


Fig. 1. Activity of kallikrein inhibitors of donors' plasma depending on their concentration and duration of contact with kallikrein. Abscissa, duration of contact of kallikrein inhibitors from donors' plasma at 37°C (in min); ordinate, inhibition of kininogenase activity of kallikrein (in %). 1) 0.01 ml plasma, 2) 0.02 ml, 3) 0.04 ml.

## EXPERIMENTAL RESULTS

It was found that dried donors' plasma or "pooled" serum remaining after various analyses could serve as the source of kallikrein and kininogen (its high- and low-molecular-weight forms). The kininogenase activity of kallikrein and prekallikrein of dried donors' plasma under the activation conditions used corresponded to 2-4 ng bradykinin/mg protein/min. Prekallikrein can be detected by contact between plasma, neutralized after heating in an acid medium with kaolin (500 μg/ml), or with powdered glass (250 mg/ml). Tests with three kininase inhibitors – EDTA, 8-hydroxyquinoline, and unithiol (2,3-dimercaptopropane sulfonate) showed that unithiol was the most effective. In a concentrations of 500 μg/ml incubation mixture it caused 100% inhibition of activity of total blood plasma kininase. Antikallikrein activity must be measured only in recently centrifuged plasma. Depending on the state and species of the individual, the quantity of plasma required for testing may vary from 0.0005 to 0.05 ml.

On the basis of the results the following technique is suggested for determination of the activity of plasma kallikrein inhibitors. Immediately before the analysis a standard mixture of kallikrein with kininogen is prepared. A weighed sample of dried plasma corresponding to the number of tests is dissolved in distilled water and diluted 1:1 with physiological saline in the proportion of 100 mg dried plasma to 1 ml distilled water and 1 ml physiological saline. The solution of plasma is treated with 0.35 ml of 1N HCl, heated for 20 min to 61°C, cooled to 2-4°C, and neutralized with 0.315 ml of 1N NaOH at the same temperature, treated with an equal volume of cold Tris-HCl-buffer, pH 7.6-7.8 (1.7 ml), and stood in a cold bath until addition to test plasma.

The kininase inhibitor (0.1 ml of a 5% solution of unithiol, diluted 1:10 – 500 μg), and the freshly centrifuged test plasma or the same volume of physiological saline (control test) are poured into a test tube, 650 μg kaolin in 0.1 ml Tris-HCl buffer or 250 mg of powdered chemically pure glass, and 1.0 ml of the cold kallikrein standard with kininogen are added. The samples are allowed to stand for 5-10 min at 20°C and incubated at 37°C for 1 h. The reaction is stopped by the addition of 0.5 ml of 10% TCA, the protein precipitate is separated by centrifugation, and the supernatant neutralized with 0.5N NaOH to pH 7.0-7.2. To prevent adsorption of the kinins 0.1 ml of 0.04M oxalic acid is added and the quantity of kinins is determined in the TCA filtrate by measuring contraction of an isolated organ (rat uterine cornu or strip of cat's jejunum). Synthetic brady-

TABLE 2. Activity of Kallikrein Inhibitors of Human and Animal Blood Plasma (in μg bradykinin/ml/h) during Interaction with Homologous and Heterologous Kallikrein (M ± m)

| Source of plasma kallikrein inhibitors | Kallikrein | | | |
|---|---|---|---|---|
| | human | rabbit | rats | cats |
| Healthy children under 3 years old (n = 28) | 30.0 ± 0.54 | 4.00 ± 0.27 | 14.96 ± 0.36 | 2.00 ± 0.11 |
| Clinically healthy adults (n = 18) | 10.08 ± 0.11 | 1.29 ± 0.14 | 1.31 ± 0.12 | 1.56 ± 0.16 |
| Rabbits (n = 18) | 19.60 ± 0.54 | 6.81 ± 0.49 | 5.00 ± 0.45 | — |
| Rats (n = 21) | 76.50 ± 1.02 | 62.50 ± 0.59 | 44.08 ± 0.93 | — |
| Cats (n = 7) | 14.40 ± 0.61 | — | — | 3.08 ± 0.29 |

TABLE 3. Activity of Kallikrein Inhibitors (in μg bradykinin/ml/h) in Patients' Blood Plasma, Fresh and Kept at 4°C (M ± m)

| Disease | Blood plasma | | |
|---|---|---|---|
| | fresh | kept at 4°C for | |
| | | 2 weeks | 5 months |
| | Children under 3 years | | |
| Pneumonia (n = 17) | 10.38 ± 0.65 | 35.0 ± 0.89 | 93.0 ± 2.12 |
| Bronchial asthma (n = 10) | 8.76 ± 0.53 | 39.67 ± 0.91 | 132.0 ± 4.82 |
| | Children aged 7-13 years | | |
| Chronic hepatitis (n = 13) | 7.30 ± 0.61 | 14.96 ± 0.68 | 98.6 ± 3.17 |
| | Adults | | |
| Chronic nonspecific obstructive bronchitis (n = 0) | 6.66 ± 0.41 | 16.80 ± 0.59 | 108.00 ± 4.94 |
| Chronic occupational bronchitis (n = 22) | 5.89 ± 0.38 | 12.30 ± 0.51 | 101.50 ± 3.87 |

kinin is used as the standard. The difference between the quantities of bradykinin found in the control and experimental samples is determined and, knowing the quantity of plasma taken for the sample which inhibits kininogenase activity of the standard by 30-50%, the absolute values of activity can be calculated.

It was found that keeping the plasma (serum) in a refrigerator at 4°C leads to an increase in activity of the kallikrein inhibitors (Table 1).

The results in Table 1 show that after keeping the plasma for 1 and 2 days at 4°C activity of the kallikrein inhibitors was increased by 30 and 98% respectively. Kallikrein inhibitors of freshly centrifuged plasma from healthy children under 3 years of age were 3 times as active as kallikrein inhibitors of clinically healthy adults. Activity of kallikrein inhibitors in plasma from regular donors was only one-half to one-third that of healthy subjects. However, when the plasma was kept in a refrigerator its antikallikrein capacity increased up to values characteristic of clinically healthy persons.

Enzyme activity of the kallikrein inhibitors was shown to depend on their concentration and to appear more slowly than the activity of kallikrein itself. The inhibitory action of the inhibitors was preceded by a latent period of 20 min (Fig. 1). Activity of kallikrein inhibitors in plasma was 10-15% lower than in blood serum because of dilution of the plasma with solutions of anticoagulants. Species differences in the content and activity of plasma kallikrein inhibitors from man and laboratory animals were detected (Table 2).

The results in Table 2 show that during interaction with kallikrein of the same species the levels of activity of inhibitors from healthy human subjects and rabbits were very close to one another, whereas rat plasma kallikrein inhibitors are 4 times as active as cat plasma kallikrein inhibitors and only one-third as active as human kallikrein inhibitors. During interaction with kallikrein of a different species (foreign) the following general rule was observed: human kallikrein inhibitors inhibit the kininogenase activity of animal kallikrein only 1/2-1/15 as strongly as those of the same species, and animal kallikrein inhibitors inhibit human plasma kallikrein 2-5 times more strongly. This fact explains why the tissue kallikrein inhibitors of animals (Contrykal, Gordox, Trasylol, etc.) are used therapeutically in clinical practice.

Activity of kallikrein inhibitors was determined by the method developed above in blood plasma of adults and children with diseases of the bronchopulmonary and hepatobiliary systems, in the fresh state and kept at 4°C (Table 3).

Comparison of the activity of kallikrein inhibitors in sick and healthy children and in adults (Tables 2 and 3) shows that the existence of an inflammatory and inflammatory-allergic condition, or its exacerbation, in the lungs and liver are accompanied by changes in their concentration in fresh plasma. As a rule the activity of kallikrein inhibitors falls under such conditions. The degree of fall corresponds to the severity of the disease and the ability of the patient to compensate. If the plasma is kept in the refrigerator the increase in activity of kallikrein inhibitors in plasma from patients also remains low by contrast with that from healthy donors (compare Tables 1 and 3). If the prognosis of the disease is poor, activity of the inhibitors does not increase during keeping of the plasma at 4°C. Determination of kallikrein inhibitors is thus of both diagnostic and prognostic value. In addition, in conjunction with the levels of plasma kallikrein activity, it can be used as an objective criterion for deciding whether to administer antiproteases such as Contrykal, Trasylol, or Gordox, and as a laboratory control to monitor the times of administration of these substances.

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# CONTACT TRANSDUCER FOR CONTINUOUS MEASUREMENT OF THE DIAMETER OF BLOOD VESSELS

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UDC 615.471:611.13/14-087.73

KEY WORDS: blood vessels; measurement of diameter; contact transducer.

When responses of blood vessels to nervous and humoral influence are studied, transducers enabling the diameter of the tested vessel to be recorded continuously are extremely effective [1]. The same technical problem arises also when the mechanical properties of blood vessels are studied. Since blood vessels characteristically exhibit viscoelastic behavior (especially under conditions of activation of the smooth muscles of the vessel wall), recording deformation of the wall in response to a change in tension must also be undertaken continuously. A number of special transducers have been introduced to solve this problem. Foremost among them are external contact transducers of various types, catheter transducers, enabling the internal diameter of the vessel to be measured, and no-contact transducers (ultrasonic and optical). Examples of the use of such instruments can be found in [1-4]. Most methods can be successfully used on comparatively large arteries (over 1.0 mm in diameter). More recently external contact transducers of cantilever type, described in [2] have become popular. An improved variant of a transducer of this type is suggested.

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