# A CHROMATOGRAPHIC METHOD OF DETERMINING SERUM KALLIKREIN AND PREKALLIKREIN LEVELS IN DOGS

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A chromatographic method of determination of kallikrein and prekallikrein [1, 3, 4] was developed in order to measure the activity of these components in human blood serum. The principle of the method was based on measurement of BAEE (N-benzoyl-L-arginine ethyl ester) esterase activity of kallikrein after separation of the enzyme and its precursor from other serum proteinases with substrate specificity close to that of kallikrein. When experimental models of pathological states are created, it is often necessary to estimate the degree of activation of the prekallikrein–kallikrein system in the blood serum of animals. Data in the literature on the physicochemical properties of these components, isolated from the blood of experimental animals, are extremely limited. There is reason to suppose that the isoelectric point of kallikrein and prekallikrein in rabbit [2], porcine [5], or bovine [6] blood plasma is a little lower than that in man, so that adsorption of these components on ion-exchanges takes place under rather different conditions. Accordingly, to determine kallikrein activity and the prekallikrein content in an animal's blood serum by the chromatographic method, the optimal conditions for separation of these components from other trypsin-like blood proteinases must be chosen.

The possibility of using a chromatographic method for determining kallikrein and its precursor in dog's blood serum was studied in the investigation described below.

## EXPERIMENTAL METHOD

Kallikrein and prekallikrein were determined [1, 4] on a 10-ml column (1 × 20 cm) filled with DEAE-Sephadex A-50, equilibrated with 0.01 M Na-phosphate buffer, pH 7.0. The protein concentration in the unadsorbed fraction was estimated spectrophotometrically at 280 nm.

Optimal conditions for kallikrein and prekallikrein determination were chosen on the basis of the method in [3] with some modifications.

Kallikrein and prekallikrein were determined in protein fractions obtained by stepwise elution of proteins adsorbed on DEAE-Sephadex, in samples containing 0.5-1.0 ml of eluate. The result was expressed in international units (i.u.), i.e., as the number of nanomoles of BAEE hydrolyzed by the enzyme in 1 min before and after treatment with trypsin. The kininogenase activity of the test fractions was estimated as the quantity of kinins formed on incubation of the trypsin-activated fraction with human blood plasma heated to 56°C for 2 h, for which purpose 0.6-ml aliquots were taken at intervals of 20-30 min for 90 min at 37°C. Proteins were precipitated with 12.5% TCA and the liberated kinins were tested on the isolated rat uterine cornu.

## EXPERIMENTAL RESULTS

The following values of activity of kallikrein and concentration of prekallikrein in dog's blood serum were obtained by the chromatographic method of determination in the column version, strictly under the conditions suggested in [1, 4] for human blood serum.

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TABLE 1. Properties of Human and Canine Kallikrein and Prekallikrein

| Parameter | Human | Canine |
|---|---|---|
| Kallikrein, i.u./ml serum | 14 | 29 |
| Prekallikrein, i.u./ml serum | 340 | 14 |
| Protein content in unadsorbed fraction, optical units at 280 nm | 15 | 1,920 |

TABLE 2. Conditions of Inhibition of BAEE Esterase Activity

| Experimental conditions | NaCl concentration in eluting buffer, M | | | | | |
|---|---|---|---|---|---|---|
| | 0,05 | 0,10 | 0,15 | 0,20 | 0,25 | 0,30 |
| Protein concentration, optical density units at 280 nm | 0,790±0,134 | 0,935±0,175 | 1,020±0,197 | 1,130±0,163 | 1,350±0,117 | 1,550±0,203 |
| Initial BAEE esterase activity, i.u./ml serum | 39 | 19 | 39 | 39 | 9 | 19 |
| BAEE esterase activity after treatment with trypsin, i.u./ml serum | 55±18 | 85±12 | 170±42 | 720±42 | 1000±36 | 1030±49 |
| Inhibition of BAEE esterase activity by LSI, % of maximum (200 μg per sample) | 70—100 | 70—100 | 60—80 | 30—40 | 30—40 | 30—50 |
| Inhibition of BAEE esterase activity by LTI, % of maximum (200 μg per sample) | 10—20 | 10—20 | 10—20 | 30—40 | 40—50 | |
| Inhibition of BAEE esterase activity by ε-ACA, % of maximum (200 μg per sample) | 0 | 0 | 3 | 1 | 2 | 4 |

As Table 1 shows, the concentration of the protein fraction unadsorbed on DEAE-cellulose was almost 10 times less in canine than in human blood serum. The concentration of prekallikrein, determined by this method, was very low compared with that found in man.

If it is assumed that canine kallikrein and prekallikrein have a lower isolectric point than human, and that they can be adsorbed on an anion-exchange resin, it must be expected that an increase in the ionic strength of the buffer solution could probably cause desorption of these proteins, so that they could be determined in the unadsorbed fraction.

The conditions for obtaining kallikrein and prekallikrein from canine blood serum in the protein fraction unadsorbed on DEAE-Sephadex and for separating these components from other trypsin-like serum enzymes were chosen on the basis of a "test tube" method of determining kallikrein and prekallikrein in human blood serum [3]. For this purpose, 2.5-ml portions of a thick suspension of DEAE-Sephadex, equilibrated with 0.025 M Na-phosphate buffer, pH 7.0, were poured into test tubes, and 0.25 ml of serum was added to each. The contents were slowly mixed for 10 min, after which 3 ml of original buffer solution containing different concentrations of NaCl (0.05, 0.10, 0.15, 0.20, 0.25, and 0.30 M) was added to each tube. After mixing again for 10 min, the ion-exchanger was removed by filtration and the volume of filtrate made up to 5 ml with the original buffer solution. The protein fractions thus obtained were assayed for BAEE esterase activity, the increase in BAEE esterase activity after treatment with trypsin, and the action of soybean inhibitor (STI) on BAEE esterase activity after treatment with trypsin.

The fraction whose BAEE esterase activity was most sensitive to the action of STI was subjected to further analysis: Its sensitivity was determined to the action of lima bean trypsin inhibitor (LTI) and ε-aminocaproic acid (ε-ACA), and its kininogenase activity was measured.

Table 2 shows that the initial BAEE esterase activity of fractions obtained at different ionic strengths was between 10 and 40 i.u./ml serum. After activation with trypsin BAEE ester-

ase activity rose sharply, possibly indicating the presence of precursors of enzymes activated by trypsin in these fractions.

Since sensitivity to the action of STI and resistance to LTI and ε-ACA are among the important properties of kallikrein, these inhibitors were used to identify the kallikrein in the fractions after treatment with trypsin. Table 2 shows that BAEE esterase activity most sensitive to the action of STI was located in fractions eluted by buffer containing 0.05, 0.1, and 0.15 M NaCl. The proportion of the STI-sensitive fragment in protein fractions eluted with a higher ionic strength fell sharply (to 30-40%). Meanwhile, the quantity of enzyme whose activity was inhibited by LTI and ε-ACA in these fractions increased. LTI inhibited BAEE-esterase activity of fractions eluted with 0.05, 0.1, and 0.15 M NaCl by 10-20%, equivalent to an activity of between 5 and 30 i.u./ml serum. In fractions with 0.2 M NaCl, the STI-sensitive activity was 210-290 i.u., and with an increase in ionic strength this activity rose progressively.

The degree of inhibition of BAEE esterase activity by ε-ACA, as Table 2 shows, increased with an increase in ionic strength of the eluting buffer. Activity sensitive to this inhibitor was completely absent from fractions containing 0.05 and 0.1 M NaCl; in the fraction with 0.15 M NaCl ε-ACA inhibited about 5 i.u. of esterase activity, whereas on elution with buffer containing 0.25 M NaCl the fraction of ε-ACA-sensitive enzyme increased to 20 i.u.

It can thus be concluded from the data in Table 2 that to determine kallikrein and prekallikrein it is necessary to use the protein fraction eluted from BAEE-Sephadex by buffer solution containing 0.15 M NaCl. It was this fraction which contained the highest BAEE-esterase activity inhibited by STI and resistant to LTI and ε-ACA.

To confirm the presence of prekallikrein in this fraction, its ability to liberate kinins from the specific substrate was tested after activation with trypsin. In the course of incubation for 1 h with heated human blood plasma, 1 ml of the fraction actually liberated 1.2 μg-equivalents of bradykinin. The results thus indicate that, first, the chromatographic method is suitable for determining prekallikrein and kallikrein in canine blood serum and, second, the conditions of separation of the proenzyme from other proteinases are rather different from those in human blood serum [3]. A description of the method of determining kallikrein and its precursor in canine blood serum is given below.

A mixture is prepared of 3 ml of a thick suspension of BAEE-Sephadex A-50, equilibrated with 0.02 M phosphate buffer, pH 7.0, with 0.25 ml serum diluted 1:2 with the same buffer solution. The mixture is stirred for 10 min, 3 ml of 0.2 M phosphate buffer, pH 7.0, containing 0.15 M NaCl is added, the mixture is again carefully stirred (3-5 min), and is then filtered on a large-pore glass filter. The volume of filtrate is made up to 5 ml with 0.02 M phosphate buffer. Four aliquots, each of 0.5-1 ml, are taken from 5 ml of the filtrate. To the two of them intended for kallikrein determination, 1-1.5 ml of 0.05 M Tris-HCl, pH 8.0, and 1 ml of $1.5 \times 10^{-3}$ M BAEE solution are added. The increases in optical density in the course of 15 min are measured on a spectrophotometer at 253 nm. A sample containing 2 ml Tris-HCl and 1 ml $1.5 \times 10^{-3}$ M BAEE solution is used as the control. To the two tubes containing aliquots of enzyme intended for prekallikrein determination, 0.1 ml of 0.1 M Tris-HCl, pH 8.8, and 0.1 ml trypsin (1 mg in 30 ml of 0.02 N HCl) are added. After incubation at 25°C for 15 min trypsin activity is inhibited by ovomucoid (0.1 ml in a concentration of 2 mg/ml) and 10 min later the volume of the samples is made up to 2 ml with 0.05 M Tris-HCl, pH 8.0. After addition of 1 ml of BAEE solution, optical density measurements begin. The concentrations of kallikrein and prekallikrein are expressed in i.u. (the number of nanomoles BAEE hydrolyzed during 1 min) in 1 ml blood serum and calculated by the equation

$$\frac{D_{253} \times 3 \times 5 \times 1000}{15 \times 1.1 \times 0.25 \times V} = \text{number of units (i.u.) in 1 ml blood serum,}$$

where $D_{253}$ is the increase in optical density in the sample during 15 min, 3 the volume of the sample in the cuvette (in ml), 5 the volume of filtrate (in ml), 15 the reaction time (in min), 1.1 the difference in optical density for 1 mM solutions of bovine albumin and BAEE, 0.25 the volume of blood serum taken for analysis (in ml), and V the volume of the aliquot taken from the filtrate (in ml).

Our measurements made by the method described above showed that 1 ml of blood serum from a healthy dog contained kallikrein activity which varied from 0 to 64 i.u. and prekallikrein in an amount which varied from 127 to 187 i.u.

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# A METHOD OF SIMULATING ACUTE TRANSIENT CORONARY INSUFFICIENCY IN RATS

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The attention of cardiologists has been drawn to one typical form of pathology of cardiac activity, namely, acute transient coronary insufficiency (ATCI), which is characterized by reduction of the coronary blood flow followed by its (postischemic) renewal.

As a rule, experimental models of ATCI are formed in one of three ways: tightening and subsequent loosening of a ligature or application and tightening of a clamp on the coronary artery [2-5, 9, 13]; stimulation of sympathetic nerve endings or centers, or administration of sympathomimetics [8]; a combination of measured occlusion of the coronary artery (critical stenosis of the vessel) with a transient increase in the work load on the heart, for example, by imposing a high rate of cardiac contraction [14]. In acute experiments, the first method is used most often. However, it has at least two disadvantages. First, this method is accompanied by severe mechanical injury to the coronary artery and myocardium when the ligature is tightened and subsequently loosened, and second, it is impossible by this method to produce a reversible disturbance of the coronary blood flow because of trauma to the coronary artery by the ligature and subsequent thrombosis. The method suggested in this paper is largely free from these disadvantages.

To protect the coronary artery from mechanical injury, a rectangular plastic plate is applied to the myocardium. The plate is cut out of a sheet of stiff plastic (polystyrene or celluloid, for example), its corners are rounded by means of a saw, and two holes are drilled 1 mm from the edges and 3 mm apart (by means of an intramuscular injection needle, rotation of which creates a drilling movement). The plate can be used over and over again.

Preparation of the animal for the operation, thoracotomy, and access to the heart and coronary artery follow the procedure described in [1]. After the ligature has been taken beneath the coronary artery (silk or "supramide" threads with an atraumatic needle are best) its ends are taken through the holes in the plate, which is placed on the myocardium in the projection zone of the coronary artery (in rats, the artery runs in the substance of the myocardium at a depth of 0.5-1.0 mm). The ligature is tied with a double or triple slip knot on the upper surface of the plate. The lumen of the artery is closed by firm compression of the vessel with the thread against the undersurface of the plate when the knots of the ligature are tightened. Loosening the ligature (in the case of repeated occlusion) or its removal (in the case of a single occlusion) can be done by loosening the knots with forceps (in the first case) or cutting the ligature (in the second case) on the upper surface of the plate (Fig. 1B, C). Injury to the myocardium and artery is slight because all the most traumatic manipulations (tightening the knots of the ligature, loosening them or cutting) are carried out on the protective plate and not directly on the surface of the myocardium or the wall of the artery.

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