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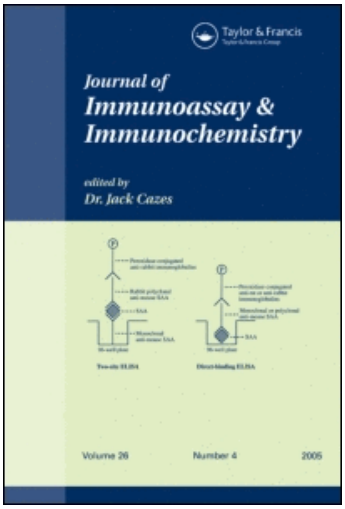

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Direct Radioimmunoassay of Rat Urinary Kallikrein: Its Application to the Determination of Active and Inactive Kallikrein Concentration after HPLC Analysis

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DIRECT RADIOIMMUNOASSAY OF RAT URINARY KALLIKREIN : ITS APPLICATION TO THE DETERMINATION OF ACTIVE AND INACTIVE KALLIKREIN CONCENTRATION AFTER HPLC ANALYSIS.

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ABSTRACT

Rat urinary kallikrein (RUK) was purified to apparent homogeneity by a three-step procedure and antibodies were raised in rabbits. Renal kallikrein exists as an active and an inactive form. A specific radioimmunoassay (RIA) was developped to measure directly the total kallikrein. The antibody used in this radioimmunoassay recognized both forms. No cross reactivity was detected with trypsin, esterase A or human urine. When iodinated rat urinary kallikrein was used, the detection range was between 0.125 and 16 ng with 6.5% intraassay variation and 8.1% between assay variation. Intrarenal kallikrein was measured in renal tissue after homogeneisation and solubilisation. Correlations between this RIA and the kininogenase activity or the amidolytic activity were highly significant. Since kallikrein exists as an inactive precursor the direct measurement of the total immunoreactive protein differs from activities determinations. An HPLC ion exchange system has been developped to separate active and inactive forms directly from urine, with a recovery of 79 ± 11%. This procedure permits measurement of inactive forms. Rat urine contains as much inactive kallikrein as active kallikrein.

"KEY WORDS" : Active rat urinary kallikrein. Inactive rat urinary kallikrein. HPLC separation. Direct kallikrein radioimmunoassay.

INTRODUCTION

Kallikreins are serine proteases. Plasma kallikrein (EC 3-4-21-34) and glandular kallikrein (EC 3-4-21-35) have different amino-acid composition, molecular weight, catalytic properties, immunolo-

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gic characteristics, and localization. Glandular kallikreins are found in salivary glands, pancreas, kidney (1-2), alimentary tract (3), spleen (4) and brain (5). The physiologic significance of renal kallikrein is still uncertain. There is evidence that glandular kallikrein is involved in local blood flow regulation (6), enzyme or peptide processing (7-8), electrolyte and water excretion (9), and blood pressure regulation (10). It has also been hypothetized that reduction in the activity of the renal kallikrein-kinin system could be a possible pathogenic factor in the development of renovascular hypertension (10). Measurement of the components of this system have been either by radioimmunoassay of the generated kinins, or by assay of the esterase activity on synthetic substrates. Although kinin RIA is the most sensitive and specific assessement of active kallikrein, it is now apparent that only 30 to 50% of the kallikrein is excreted in an active form (11). Total kallikrein can be measured following trypsin activation, but this method is not suitable for tissue homogenates because of the large amount of other proteins and the possible presence of inhibitors. Direct radioimmunoassay of immunoreactive glandular kallikrein appears to be a reliable method to measure total kallikrein (12), and it is the only means of detecting glandular kallikrein in plasma because of the presence of plasma kallikrein which has the same kininogenase activity.

We describe here a direct RIA for rat urinary kallikrein (RUK).

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MATERIEL AND METHODS

Preparation of Antigen. Rat urinary kallikrein was purified by a three step procedure including ion exchange chromatography, affinity chromatography on Aprotinin bound to Agarose, and Sephadex G100 filtration as previously reported (13). The preparation was further characterized by HPLC analysis, PAGE electrophoresis and electrofocusing and stored frozen at -80°C.

Preparation of Antisera. Antibodies were raised in 3 male fauves de Bourgogne rabbits immunized with purified urinary kallikrein. Each rabbit received a first multiple site injection in the back of

30 ug of pure kallikrein (1ml) emulsified with 1ml of complete Freund's Adjuvant (Difco). Every 6 weeks, a booster injection was given. Antisera were collected one week after each booster injection. Antisera were stored frozen at -20°C.

Preparation of labeled Antigen. Pure kallikrein was labeled with 125-iodine according to the chloramine T method (14). Three ug kallikrein (10ul) was allowed to react with 500 uCi of NaI (Amersham 100 mCi/ml) in phosphate buffer, 500 mmol/L, pH 7.4 (20 ul). The reaction was initiated by the addition of 10 ul of Chloramine T in phosphate buffer, 50 mmol/L, pH 7.4. After 1 min of gentle stirring, at room temperature the reaction was stopped with 40 ul of metabisulfite in phosphate buffer, 500 mmol/L, pH 7.4. Iodinated protein was separate from free 125-iodine by Sephadex G100 filtration and then analysed on HPLC gel filtration.

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Radioimmunoassay protocol.

a) Kallikrein concentration : incubations were performed in duplicate in phosphate buffer saline, 10 mmol/L, pH 7 with NaCl 140 mmol/L, plus BSA 10 g/L. The incubation medium consisted of 0.1ml of RIA buffer, 0.1ml of labeled antigen (approximatively 10,000 cpm), 0.1ml of standard (0.125-16 ng/tube) or unknown samples, 0.1 ml of a 1/60,000 dilution of antiserum. The final volume was separated from the 125-I kallikrein by the polyethylene glycol method (15). One percent bovine gamma globulin (200 ul) and PEG 200 g/L (1 ml) were added to each tube and mixed. Following centrifugation (3000 rpm for 30 min at 4°C) the supernatant was aspirated and radioactivity of the unwashed precipitate was counted in an autogamma (M 578 spectrometer PACKARD). Results were calculated using the Logit-Log linearization of the standard curve (16) and expressed in ng/tube of kallikrein.

b) Kallikrein kininogenase activity : This activity was determined as previously described (17) using 125-I Tyr 8-Bradykinin as tracer and the nonapeptide Bradykinin as standard. An aliquot of sample

was incubated for 30 min with citrated dog plasma as kininogen source and generated kinins were quantified with a kinin radioimmunoassay. Results were expressed in ng/tube of Bradykinin generated per minute (ng BK/tube/min).

Preparation of kidney extract :

Under pentobarbital anesthesia, the aorta was canulated in the abdominal portion and ligated between the heart and the kidneys in order to wash the kidneys in situ with phosphate buffered saline. Kidneys were then excised and stored frozen until used. In a preliminary protocol, preweighed kidneys were homogeneized in TRIS-HCL buffer, 200 mmol/L, pH 8.2 (10 ml/g tissue v/w) using a POTTER refrigerated system. After homogeneisation deoxycholic acid was added to reach 5 g/L final concentration, and incubated for one hour at 4°C. The incubate was then centrifuged (30 min, 3000xg). The supernatant was used to measure kallikrein concentration and activity. Dialysis of the samples prior to measurement to remove deoxycholic acid did not induce any significant difference in the results.

HPLC analysis of active and inactive kallikrein in rat urine :

The HPLC analysis were conducted on an LKB Model equipped with a 2150 Pump and 2158 Uvicord spectrophotometer controlled with a 2152 programable controller.

Anion exchange HPLC was carried out with a column of ULTROPACK TSK DEAE 5 PW (7.5 x 75 mm) provided by LKB. A salt gradient was used at a flow rate of 0.5 ml/min and 0.5 ml fractions were collected. Gel permeation HPLC was performed with a LKB ULTROPACK precolumn TSK SWP (7.5 x 600 mm). The flow rate was 0.5 ml/min with sodium phosphate buffer, 100 mmol/L, pH 6.8 containing sodium chloride 100 mmol/L. A calibration curve was established with a mixture of standard proteins. Retention time was plotted against the logarithm of the molecular weight. Inactive kallikrein was detected after activation with trypsin. For this, 2.5 ug of Trypsin (DIFCO) were added to the sample and incubated for 20 min at 37°C in sodium phosphate buffer, 100 mmol/L, pH 7.8, and the action of trypsin was

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stopped with 20 ug of Soy bean Trypsin inhibitor (STI Sigma). After a 15 min incubation, the activity was measured either by kininogenase activity or by amidolytic activity (18).

RESULTS

Purification of kallikrein and characterisation of the antiserum.

- Rat urinary kallikrein was purified to homogeneity and was characterized as previously reported (13) by the following criteria :

1) it displayed two bands on SDS disc gel electrophoresis with molecular weight 28,000 and 29,000 respectively.

2) on isoelectric focussing, two main components of PI 3.90 and 4.08 were separed.

3) the specific activity of purified kallikrein was 909 ug kinin/min/mg of protein when purified rat High Molecular Weight kininogen was used as substrate and 39.8 umoles of S2266 hydrolysed/min/mg of protein when estimated by the amidolytic assay.

4) the radioactivity was eluted as a single peak after Gl00 filtration and HPLC gel permeation.

- Three out of three immunized rabbits produced antibodies with suitable titers for RIA and immunohistological studies (Figure 1). The antibody K3 was used at a final dilution of 1/60,000 to obtain a 35% total binding of freshly iodinated rat urinary kallikrein. Using Ouchterlony double immuno-diffusion analysis one single precipiting line was observed with rat urine and rat kidney homogenate, but not with rat esterase A fraction, human urine, or porcine pancreatic kallikrein. The antiserum produced 100% inhibition of the kininogenase activity of purified RUK and or rat urine but only 80% inhibition of the amidolytic activity of rat urine.No inhibition was observed with human urine.

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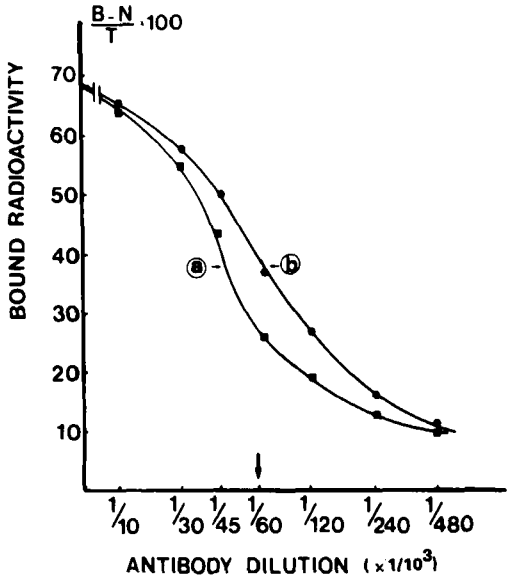

Figure 1 : Titration curve of rabbit anti-kallikrein antiserum. Curve a, with 125-I-Kallikrein alone. Curve b, in the presence of 1 ng of unlabelled standard kallikrein. The arrow indicates the optimal dilution of antisera used for final RIA conditions, this dilution corresponds to the maximum deviation between curves a and b.

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Iodination of rat urinary kallikrein.

The incubation mixture was purified on Sephadex G100. Two peaks of radioactivity were observed (Figure 2). The five 1 ml fractions of the first peak showed maximal binding with an excess of antibody. The radioactivity precipitated in the second peak was not different either in the presence or in the absence of antibody and was 8 to 12% of the total added counts. The five fractions showing the major specific binding in the initial peak were stored at -20°C, and were stable for more than 2 months. The specific radioactivity was 51 Ci/g.

Characteristics of the RIA.

The standard radioimmunoassay curve shown in Figure 3 results from 10 differents experiments. The interassay coefficient of varia-

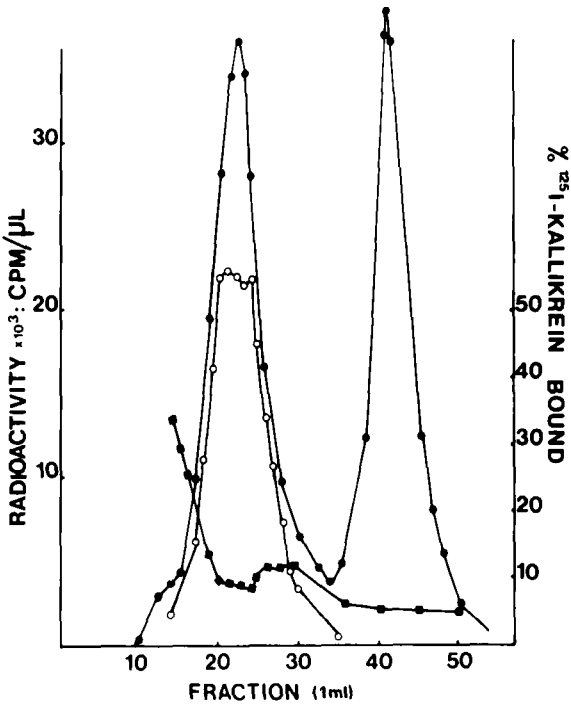

Figure 2 : Purification of iodinated rat kallikrein on Sephadex G100. Elution was performed in phosphate buffer, 50 mmol/L, pH 7.4 with NaCl 140 mmol/L and B.S.A. 5 g/L. (●——●) distribution of the total radioactivity ; (○——○) radioactivity bound specifically to the antisera ; (◆——◆) non specific binding.

tion was 8.1%, the intraassay coefficient was 6.5%, and the recovery of added pure kallikrein was 94 ± 8%. The limit of detectability of the assay corresponding to a B/Bo of 93 ± 3% was 0.125 ng/tube (4.3 fmoles of kallikrein). The detection range was from 0.125 to 16 ng/tube of kallikrein.

The displacement curves obtained with rat urine and renal homogenate were parallel with the rat urinary kallikrein standard curve. Furthermore the addition of Trypsin, Human urine and semi

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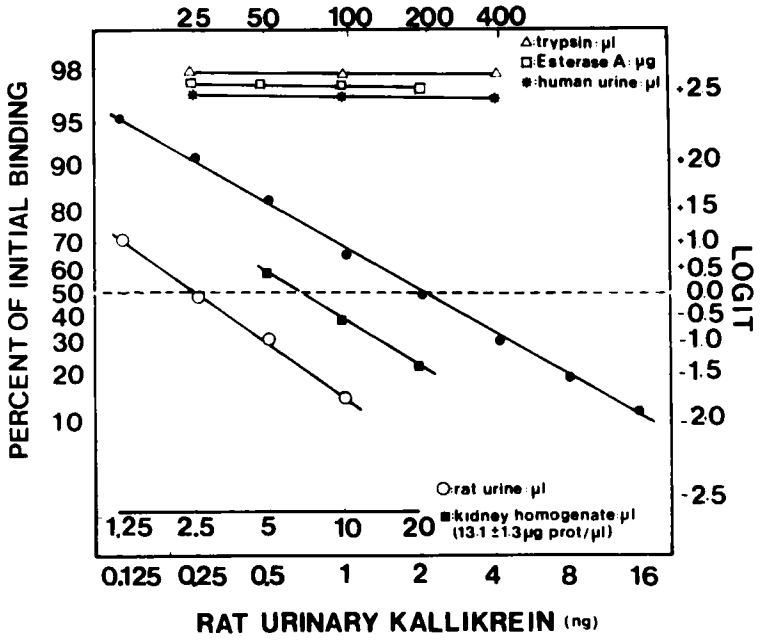

Figure 3 : Logit-Log transformation of the standard curves (n = 6) of rat urinary kallikrein.

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purified rat esterase A did not induce significant displacement of the standard curve.

Measurement of kallikrein concentration in kidney homogenate.

To validate the extraction protocol we have measurement the kallikrein concentration in 14 kidney extracts.The results shown in Figure 4 and Table 1 indicated that the kallikrein concentration was proportional and linear to the weight of kidney extracted. Kallikrein represented 9.6 ± 1.7 ug of kallikrein/g of total solubilized protein.

Comparison of the direct radioimmunoassay, amidolytic assay and kininogenase assay.

Correlation with the amidolytic activity is shown in Figure 5. The kallikrein concentration was very well correlated with the kini-

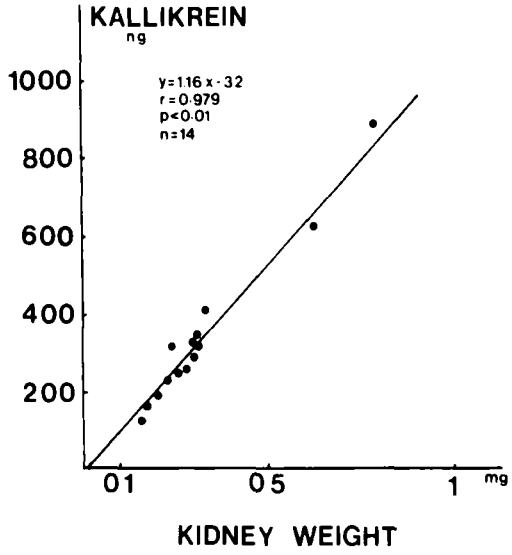

Figure 4 : Relation between the weight of kidney extracted and the amount of immunoreactive kallikrein detected (n = 14).

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nogenase activity before and after trypsin treatment. Furthermore the trypsin treatment had no influence on the direct measurement of kallikrein. Although the observed correlation with the amidolytic assay was highly significant ($r = 0.849$ $p<0.01$), intercept (+ 31.8) was significantly different from zero suggesting the presence of an amidase activity not due to kallikrein.

HPLC analysis.

The recovery of the activity injected on the column was 79 ± 11% (n=9) and was dose dependant (Figure 6). The choosen NaCl gradient from 0 to 300 mmol/L allowed a reproducible separation of active (fraction 1) and trypsin activatable kallikrein (fraction 2, Figure 7). In the active fraction, the amidolytic activity (0.4 ± 0.04 umoles/min) represented 82 ± 8% of total activatable kallikrein (0.51 ± 0.06 umoles/min) mean while it represented only 25 ±

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TABLE 1

Relation between the weight of kidney treated and the proteins and kallikrein content(n = 14).

Kidney weight (mg)	Total protein (mg)	Total kallikrein (ng)	Protein (mg/mg Tissue)	Kallikrein ng/mg Tissue	Kallikrein ng/mg Protein
180	15.6	161.5	0.086	0.897	10.35
212	21.7	184	0.102	0.867	8.47
226	32.5	321.8	0.14	1.424	9.90
267	24.8	248	0.09	0.915	9.87
280	31.3	253	0.112	0.903	8.08
156	19.8	129.4	0.126	0.802	6.53
287	36.5	334.4	0.127	1.16	9.16
237	29.6	232.5	0.127	0.981	7.85
305	33.15	345	0.108	1.130	10.40
328	28.03	405	0.085	1.230	14.44
298	27.6	288	0.092	0.966	10.43
310	31.2	319	0.100	1.029	10.22
615	68.4	630.4	0.111	1.024	9.21
774	91.4	892	0.118	1.15	9.75
		mean +SEM	0.109 $\pm$ 0.016	1.042 $\pm$ 0.159	9.618 $\pm$ 1.73

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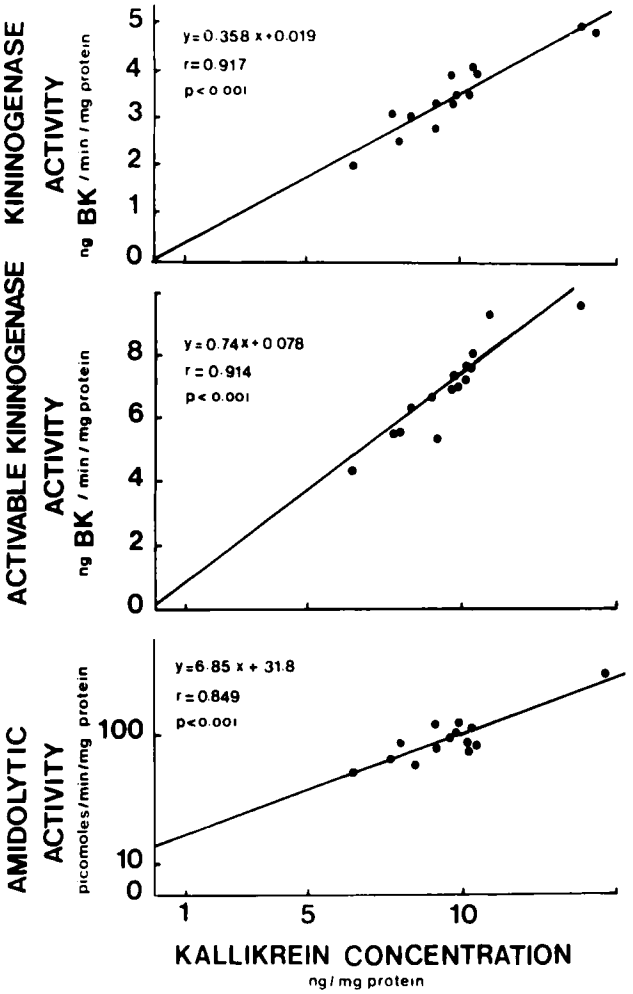

Figure 5 : Comparison of the direct detection of kallikrein with three different assessments of kallikrein activity : direct kininogenase activity, reflecting the active kallikrein ; trypsin activatable kininogenase activity, representing the total kallikrein activity ; and the amidolytic activity on the synthetic substrate Val-Leu-Arg-PNA.

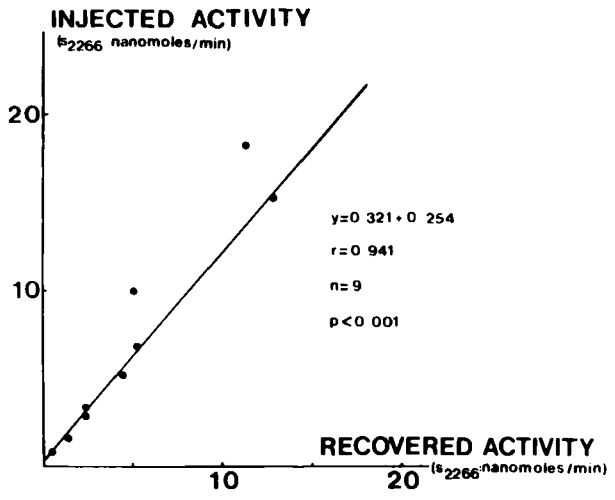

Figure 6 : Relation between the total activity injected on the HPLC ion exchange column and the activity recovered in the eluted fractions.

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7% in the inactive fraction (0.11 ± 0.02 umoles/min).However when kallikrein was assessed in these two fractions by its concentration using our direct RIA,no difference was observed (10.4 ng/tube in fraction 1 and 10.7 ng/tube in fraction 2).This last result showed that 50% of the total urinary kallikrein is eliminated in an inactive form.

DISCUSSION

The HPLC data indicate that we have produced antibody which recognizes both the active and inactive forms of kallikrein with a high degree of specificity. This antibody allowed the development of a direct radioimmunoassay against rat glandular kallikrein. We were able to quantify glandular kallikrein in various organs where immunohistological localisations have been already described. The characteristics of our radioimmunoassay is in agreement with previous reported assays (12,19,20). By using a combination of HPLC

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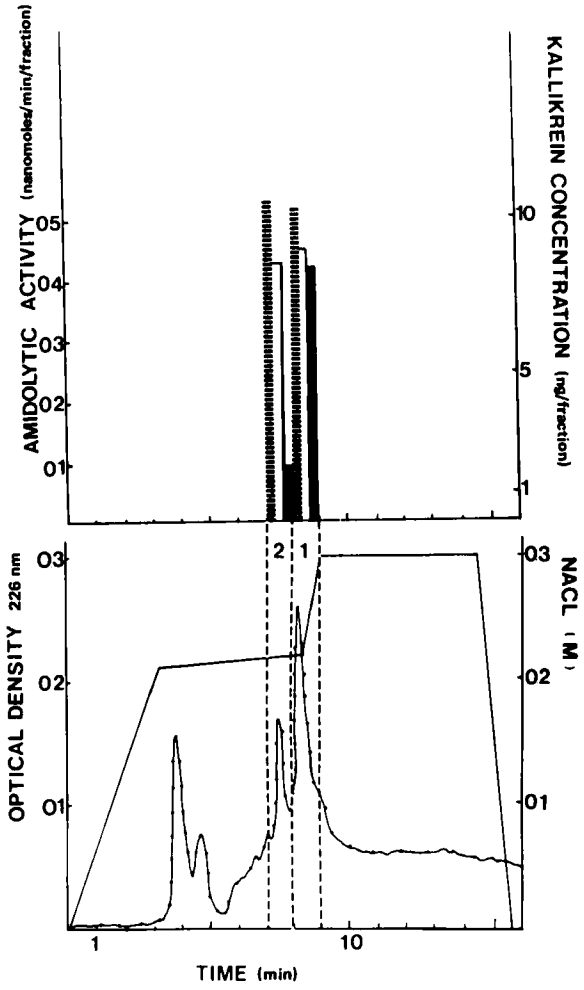

Figure 7 : HPLC analysis of the active and inactive kallikrein of 2ul rat urine. (●—●—●) optical density ; (——) salt gradient ; (▬) amidolytic activity ; (▭) amidolytic activity after trypsin treatment ; (▮▮▮▮▮▮▮▮) direct concentration of kallikrein.

separation with direct RIA, we provide a new protocol which allows the direct determination of active and inactive kallikrein. We confirm by these direct measurements that the urinary excretion of inactive kallikrein represents 50% of the total immunoreactive kallikrein excreted in normal rat urine. Untill now, active and inactive kallikrein were determined only by their kininogenase activities before and after trypsin activation, or estimated by the difference between the measurement of the total immunoreactive kallikrein and the direct kininogenase activity. Direct measurement of total immunoreactive kallikrein appears a suitable assay for assessing factors that could influence the synthesis of kallikrein and its precursors (21).

This new protocol could also be of interest in studies of the mechanism of activation of inactive kallikrein since. It is now possible to detect the disappearance of the inactive form.

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