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Measurement of Kallikrein Activity in Urine of Rats and Man Using a Chromogenic Tripeptide Substrate

Validation of the Amidolytic Assay by Means of a Bradykinin Radioimmunoassay

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Summary: Urinary kallikrein activity in rats and man was measured by an amidolytic assay using the chromogenic tripeptide *D*-valyl-leucyl-arginine-*p*-nitroanilide (S 2266). The sensitivity of the assay permits the measurement of 3.5 to 250 mKU of purified porcine pancreatic kallikrein. The intraassay coefficient of variation was 1.6 ± 0.3% and the interassay coefficient of variation 7.4 ± 0.9%. No changes in kallikrein activity were found in urine samples kept for 7 days at 4 °C or 14 months at −20 °C. Repeated freezing and thawing reduced kallikrein activity. Kallikrein activity of the same urine samples was also estimated by means of a bradykinin radioimmunoassay, which determines the kinins released during incubation of urine with partially purified dog kininogen (kininogenase activity). The urinary kallikrein activity assayed by means of these two methods showed a high correlation with coefficients of $r = 0.9314$ for rat urine ($p < 0.001$) and $r = 0.8002$ for human urine ($p < 0.001$).

Anwendung eines chromogenen Tripeptids als Substrat für die Bestimmung der Kallikrein-Aktivität im Urin von Ratte und Mensch
Vergleich des amidolytischen Assays mit dem Bradykinin Radioimmunoassay

Zusammenfassung: Die Kallikrein-Aktivität im Urin von Ratte und Mensch wurde durch einen amidolytischen Assay bestimmt. Als Substrat diente das chromogene Tripeptid *D*-Valyl-Leucyl-Arginin-*p*-nitroanilid (S 2266). Die untere Nachweisgrenze für glanduläres Kallikrein (Schweinepankreas-Kallikrein) lag in dem angewandten Verfahren bei 3,5 bis 250 Millieinheiten der biologischen Aktivität. Die Variationskoeffizienten betrugen in der Serie 1,6 ± 0,3%, zwischen den Serien 7,4 ± 0,9%. Die Kallikrein-Aktivität im Urin blieb sowohl über 7 Tage bei 4 °C als auch über 14 Monate bei −20 °C unverändert meßbar. Wiederholtes Einfrieren und Auftauen verminderte die Kallikrein-Aktivität. Die Bestimmung der Kallikrein-Aktivität im Urin wurde gleichzeitig sowohl mit dem amidolytischen Assay als auch mit einem Radioimmunoassay für Bradykinin durchgeführt; durch letzteren wird die Kininogenase-Aktivität des Kallikreins gemessen. Die mit beiden Verfahren erhaltenen Werte der Kallikrein-Aktivität im Urin korrelierten eng miteinander. Es ergaben sich Korrelationskoeffizienten von $r = 0{,}9314$ ($p < 0{,}001$) für die Messungen im Rattenurin und $r = 0{,}8002$ ($p < 0{,}001$) für die Bestimmungen im Menschenurin.

Introduction

Glandular kallikreins are serine proteases, which cleave kallidin from high and low molecular weight kininogen. Urinary kallikrein belongs to the glandular kallikreins and is identical to renal kallikrein (1) which is secreted into the distal tubular fluid. The content of kallikrein in urine can be estimated either by means of the esterolytic activity it exerts on substrates like Tos-*L*-Arg-O-Me or Bz-*L*-Arg-O-Et or by assaying the kinins released during the incubation with kininogen. For the latter determination, either a bioassay (2) or a radioimmunoassay for bradykinin have been used (3, 4). Direct kallikrein radioimmunoassays have also been developed (5, 6,

[1]) *Abbreviations*
Bz-*L*-Arg-O-Et: Nα-benzoyl-*L*-arginine ethyl ester
Tos-*L*-Arg-O-Me: Tosyl-*L*-arginine methyl ester
H-*D*-Val-Leu-Arg-*p*-nitroanilide: H-*D*-valyl-leucyl-arginine-*p*-nitroanilide
KU: kallikrein units
KIU: kallikrein inactivator units

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7, 8). A synthetic chromogenic amide substrate for kallikrein is now available (H-*D*-Val-Leu-Arg-*p*-nitroanilide, Kabi GmbH, München). The cleavage of the chromophore *p*-nitroaniline from the tripeptide is caused by the amidolytic activity of glandular kallikrein. The kinetics of this enzymatic reaction are similar to those of kallikrein with its natural substrate (9). Other serine proteases including plasma kallikrein can also cleave the synthetic substrate, but have much lower specific activity than glandular kallikrein (10). Therefore, this assay is more selective than the esterolytic assays, and the determination of glandular kallikrein activity by the amidolytic assay is easier and less time-consuming than by bioassay or radioimmunoassays. To study the validity of the newly developed method, the enzyme concentration in urine determined by this method was compared to the concentration measured by the well established bradykinin radioimmunoassay (4) and the correlation of data obtained with both methods was estimated.

Material and Methods

Rat urine was collected in metabolic cages over a period of 24 hours. At the end of the collection period, urine volume was measured and the urine frozen in two portions and stored at −20 °C. For assaying kallikrein, urine was thawed at 4 °C, centrifuged for 10 minutes at 3000 *g* and then diluted 150 times by adding distilled water. Human urine was collected at 4 °C over 24 hours from normal volunteers and patients with essential hypertension. The urine was frozen at the end of the collection period and stored at −20 °C until measurement. After thawing and centrifugation, kallikrein activity was measured in urine undiluted or diluted twice with distilled water.

Kallikrein activity was estimated by bradykinin radioimmunoassay (3, 4) by incubating 100 µl diluted urine with partially purified kininogen from dog plasma (5 mg in 900 µl 0.1 mol/l Tris-HCl buffer) for 20 minutes at pH 8.5 and 37 °C in the presence of kininase inhibitor *o*-phenanthroline (3 mmol/l). The kininogen preparation used had neither kallikrein-like nor kininase activity under the conditions of the assay. An inhibition of added porcine kallikrein could not be detected. The incubation was stopped by the addition of 4 ml ethanol (990 ml/l) to 100 µl of incubate. After centrifugation and evaporation of the supernatant to dryness, the residue was dissolved in 600 µl 0.1 mol/l Tris-HCl buffer, pH 7.4 and kinin content was measured by radioimmunoassay as reported (4). Urinary kallikrein activity was expressed as µg bradykinin per minute incubation per ml urine. The intraassay coefficient of variation was 5.5 ± 1.5%, the interassay coefficient of variation 11.9 ± 1.3%. Antibradykinin serum crossreacted with lysyl-bradykinin (100%) and methionyl-lysyl-bradykinin (85.1%). However, methionyl-lysyl-bradykinin is not a disturbing factor, since it is not produced at the pH of the assay. The simultaneous measurement of lysyl-bradykinin (kallidin) and bradykinin reveals the actual kallikrein activity of the urine independently of its aminopeptidase activity. Low molecular weight kininogen also crossreacts with the antibody (0.7%), but this crossreaction does not disturb the measurement of kinins, since the kininogen remaining in the incubation medium is precipitated by the addition of ethanol. The blank of kininogen included in each assay was always free of kinin-like immunoreactive substances. No detectable crossreaction (< 0.001%) was found with peptides unrelated to the kinins (angiotensin I, angiotensin II, angiotensin III, substance P, neurotensin, Leu-enkephalin, saralasin, bradykinin potentiator B and C, eledoisin).

Urinary kallikrein activity was also determined by cleavage of the chromophore *p*-nitroaniline enzymatically from the chromogenic tripeptide substrate S 2266 (H-*D*-Val-Leu-Arg-*p*-nitroanilide, Kabi Diagnostika GmbH, München). The method used was slightly modified from that reported by *Amundsen* et al. (9). Urine was incubated with substrate (1.5 mmol/l in the presence or absence of aprotinin (50 KIU, Trasyloi®) for 30 minutes at 37 °C. Tris-HCl, pH 8.2 (0.2 mol/l) was used as buffer. The reaction was stopped by addition of 500 g/l acetic acid. The activity of urinary kallikrein was calculated according to *Amundsen* (9, 11) and given in units per liter (U/l), one unit being the amount of glandular kallikrein which cleaves one µmol substrate per minute incubation at the given conditions. Values of kallikrein activity are given as mean ± SEM. Significance was calculated by *Student*'s t-test; for correlations significance was tested against zero.

Results

In the amidolytic assay, the amount of the chromophore, *p*-nitroaniline, cleaved during incubation was linearly related to the incubation time up to 120 minutes as well as to the amount of active enzyme. The results obtained by the amidolytic assay followed a straight line from 3.5 up to 250 milliunits kallikrein, using porcine pancreatic kallikrein as standard. The colour of liberated *p*-nitroaniline was stable for at least 4 hours (absorbance after stopping: 0.341 ± 0.03, absorbance after 255 minutes storage at room temperature: 0.359 ± 0.029, n:3). The intraassay coefficient of variation was 1.6 ± 0.3%, the interassay variation 7.4 ± 0.9%. The kallikrein activity in rat urine remained unchanged during storage for 7 days at 4 °C (1st day: 2.03 ± 0.24; 3rd day: 2.10 ± 0.45; 7th day: 2.18 ± 0.48 U/l), when storage time was prolonged, all urines tested lost their activity rapidly (11th day of storage at 4 °C: 1.00 ± 0.14 U/l, $p < 0.005$). Repeated freezing and thawing caused a decrease in activity of urinary kallikrein, which was already significant after the first freezing ($p < 0.01$, fig. 1). During storage of urine at −20 °C, no changes of kallikrein activity were observed over a period of 14 months (0.38 ± 0.07 U/U_{vol} vs.

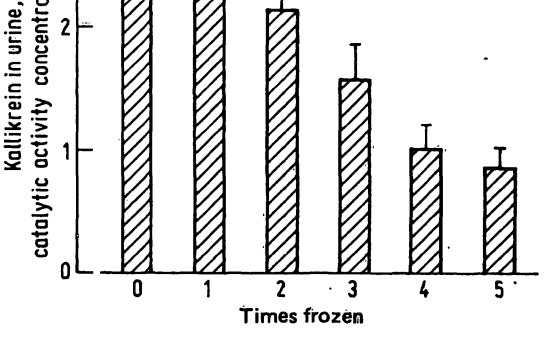

Fig. 1. Loss of urinary kallikrein catalytic activity in urine of rats (n = 8) after freezing and thawing, which was repeated fivefold. Values are given as means ± SEM.

0.41 ± 0.07 U/U_{vol}, n:10). The measurement of urinary kallikrein in rats by the amidolytic assay and by bradykinin radioimmunoassay was performed in urine of normotensive rats, spontaneously hypertensive rats, rats with deoxycorticosterone treatment (200 mg/kg in silicone pellets s.c.), rats with renovascular hypertension (aortic ligature) and rats with salt-loading (20 g/l NaCl as drinking fluid). The well established changes in urinary kallikrein excretion such as enhancement by deoxycorticosterone acetate (12, 13) or reduction by salt-loading (14, 15) were detected by both methods with the same degree (tab. 1). The correlation coefficient of the kallikrein activity for all groups of rats investigated obtained by both methods are given in table 2. The regression line calculated from the values obtained in the normotensive control rats was $y = 0.042 x + 0.07$ (fig. 2), the slope of the regression line was the same in all groups investigated. Also a high correlation between both methods was found in urine of normotensive and hypertensive humans (tab. 2).

Tab. 1. Comparison of urinary excretion determined by amidolytic assay and by bradykinin radioimmunoassay in rats with stimulated renal kallikrein-kinin system by deoxycorticosterone acetate (200 mg/kg in silastic subcutaneously over 14 days) and with suppressed activity of renal kallikrein by salt-loading (20 g/l NaCl as drinking fluid over 7 days). Values are given in U/l (mean ± SEM).

Investigation group	Salt-loading		Deoxycorticosterone	
Determination	Control (n = 13)	20 g/l NaCl (n = 13)	Control + 10 g/l NaCl (n = 10)	Deoxycorticosterone + 10 g/l NaCl (n = 8)
Amidolytic assay S 2266	1.23 ± 0.10	0.69 ± 0.04	0.59 ± 0.08	1.21 ± 0.14
		$p < 0.001$		$p < 0.001$
Bradykinin radioimmunoassay	25.50 ± 1.88	14.62 ± 0.76	11.75 ± 2.66	25.13 ± 3.66
		$p < 0.001$		$p < 0.01$

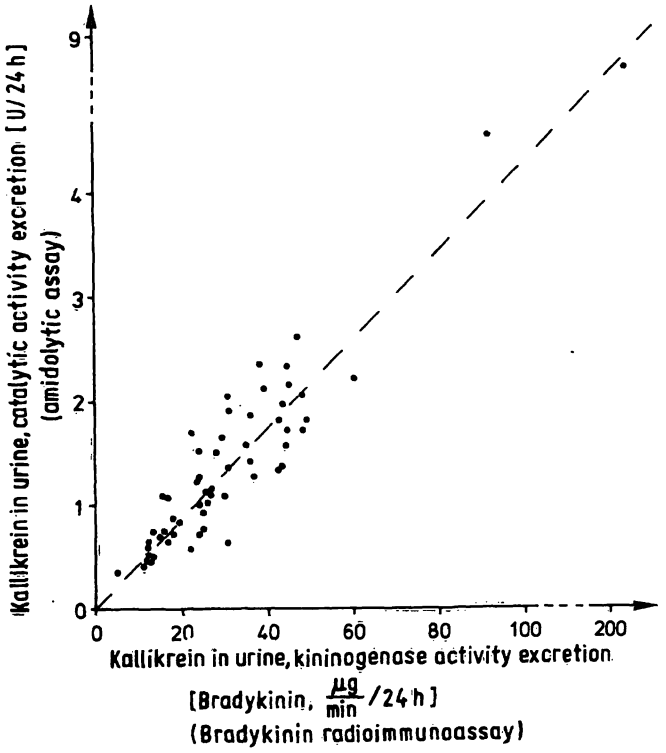

Fig. 2. Correlation of urinary kallikrein activity measured simultaneously by bradykinin radioimmunoassay (abscissa) and by amidolytic assay (ordinate) in untreated normotensive Wistar rats. The coefficient of correlation is $r = 0.9623$ ($n = 58$; $p < 0.001$) and the regression line is $y = 0.042 x + 0.07$.

Tab. 2. Coefficients of correlation between the values of urinary kallikrein activity measured by both the bradykinin radioimmunoassay and the amidolytic assay in urine of rats and man.
Significance for all groups tested: $p < 0.001$.

Group	Number of samples	Coefficient of correlation
Rats	185	0.9314
Normotensive control rats	58	0.9623
Spontaneously hypertensive rats	20	0.9361
Renal hypertensive rats	45	0.9421
Salt loading (20 g/l saline)	26	0.9303
Deoxycorticosterone	36	0.8699
Man		
Normotensive	36	0.8002
Hypertensive	25	0.8177

Discussion

The amidolytic assay using the chromogenic tripeptide substrate H-*D*-Val-Leu-Arg-*p*-nitroanilide (S 2266) is a convenient and sensitive method for the measurement of urinary kallikrein activity. The assay may be considered as being selective for glandular kallikrein, since the absorption of the sample is read against a blank, in which kallikrein is inhibited by aprotinin (Trasylol®),

which does not inhibit urokinase or bacterial enzymes (9). Plasma kallikrein has a much lower specific activity for the chromogenic substrate (10). Thus, plasma kallikrein would not disturb the measurement of urinary (renal) kallikrein, even when small amounts of the big molecule of plasma kallikrein could filter through the glomeruli. In a previous investigation (8), the activity of purified porcine pancreatic kallikrein measured by the amidolytic assay was compared with the one determined in a newly developed esterolytic assay, and a strong correlation between the values was found.

In urine, kallikrein activity was stable for seven days at 4 °C and for 14 months at −20 °C. However, with every freezing and thawing, the urine lost some kallikrein activity. Thus, it is advisable to avoid freezing and thawing of urine before measurement if technically possible, in order to test for the real activity of kallikrein activity in urine.

Both the kininogenase and the amidolytic assays measure the kallikrein-like activity, but not the absolute amount of renal kallikrein in urine. It cannot be excluded that kallikrein measurement by either method is differently affected by the presence of kallikrein inhibitors. However, the high correlation between kininogenase and amidolytic activity observed suggests that both methods measure the same enzyme. The correlation was also found in widely different experimental conditions such as salt-loading, mineralocorticoid excess and renovascular hypertension as well as spontaneous hypertension in rats, and in low and high concentrated urine. The sensitivity of the amidolytic assay for glandular kallikrein is as high as that of the esterolytic assays, but the latter methods (Tos-*L*-Arg-O-Me, Bz-*L*-Arg-O-Et) are less specific (4, 16) than the assay of kininogenase activity of kallikrein, while the amidolytic assay appears to be as specific as the kininogenase assay. The amidolytic assay is less difficult to perform than the radioimmunoassays and does not require expensive equipment. So the amidolytic assay is adequate for the measurement of urinary kallikrein activity in urine of both experimental animals and man.

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