J. Clin. Chem. Clin. Biochem.
Vol. 18, 1980, pp. 395–401

Radioimmunoassay of Human Urinary Kallikrein

Determination of Human Urinary Kallikrein, II.

By *K. Mann, W. Göring, W. Lipp, B. Keipert, H. J. Karl,*

Medizinische Klinik II (Leitung: Direktor Prof. Dr. P. Paumgartner) Klinikum Großhadern, München

R. Geiger and *E. Fink*

Abteilung für Klinische Chemie und Klinische Biochemie (Leitung: Prof. Dr. H. Fritz) in der Chirurgischen Klinik der Universität München

(Reiceived November 6, 1979/January 30, 1980)

Summary: A radioimmunoassay for the determination of human urinary kallikrein was developed. The sensitivity of the assay was 0.5 µg/l. Dose-response curves of human submandibular and parotid saliva, sweat, pancreatic juice and bile paralleled the standard curve obtained with purified human urinary kallikrein. Substances with similar antigenic determinants were also found in human serum, ascites, seminal plasma, amniotic fluid, cervical mucus, tears, liquor and faeces, but not in human breast milk and gastric juice. Moreover, immunoreactive material was detected in the urine of guinea pigs, orangoutangs and chimpanzees, but not in the urine of rats, cats and rabbits. Porcine acrosin and kallikrein, as well as bovine trypsin and chymotrypsin, showed no cross reactivity.

Radioimmunoassay für Kallikrein im Harn des Menschen. Bestimmung von menschlichem Harnkallikrein, II.

Zusammenfassung: Ein Radioimmunoassay zur Bestimmung von menschlichem Harnkallikrein wurde entwickelt. Die untere Nachweisgrenze der Methode betrug 0,5 µg/l. Die Dosis-Wirkungskurven für Speichel der Submanibularis- und Parotisdrüse, von Schweiß, Pankreassaft und Galle lagen parallel zu denen von gereinigtem menschlichen Harnkallikrein. Substanzen mit ähnlichen Antigendeterminanten fanden sich auch in menschlichem Serum, Ascites, Seminalplasma, Amnionflüssigkeit, Cervixmucus, Tränen, Liquor und Stuhl, jedoch nicht in der Muttermilch und im Magensaft. Kreuzreagierendes Material wurde auch im Urin von Meerschweinchen, Orang Utan und Schimpansen, dagegen nicht im Urin von Ratte, Katze und Kaninchen entdeckt. Auch ergab sich keine Kreuzreaktion mit Akrosin oder Pankreaskallikrein vom Schwein sowie Trypsin und Chymotrypsin vom Rind.

Introduction

Various methods for the determination of urinary kallikrein[1]) are available. Biological assays such as the blood pressure response of dogs (1) to intravenous injection of urine, the vasodilator effect on the perfused hindleg of a dog and the direct effect of urine on the isolated rat uterus (2, 3) are not suitable for routine purposes.

Other methods are based on the hydrolysis either of synthetic substrates[1]), such as Bz-Arg-OEt (4), Tos-Arg-OMe (5), Cbz-Tyr-ONp (6), Ac-Phe-Arg-OEt (7), *D*-Val-Leu-Arg-NHNp (8) or of the natural substrate kininogen (9, 10, 11). These assays are not specific for kallikrein and, because of the presence of inhibitors and other proteases, do not allow the direct measurement of kallikrein in tissues or body fluids other than urine.

This report describes a radioimmunoassay for the determination of human urinary kallikrein which partly overcomes these problems. The lower limit of detection is 0.5 µg/l. Immunologically identical material was found in the saliva of the submandibular and parotid gland, pancreatic juice, bile and sweat; partially cross reacting material was detected in serum, seminal plasma,

[1]) Abbreviations:
Bz-Arg-OEt: Nα-benzoyl-*L*-arginine ethyl ester
Tos-Arg-OMe: Tosyl-*L*-arginine methyl ester
Cbz-Tyr-ONp: Carbobenzoxy-*L*-tyrosine-*p*-nitrophenyl ester
Ac-Phe-Arg-OEt: Acetyl-*L*-phenylalanyl-*L*-arginine ethyl ester
D-Val-Leu-Arg-NHNp: *D*-valyl-leucyl-arginine-*p*-nitranilide
KE: Kallikrein units
KIE: Kallikrein inhibitor units
Enzymes: Kallikrein (EC 3.4.21.8)

0340-076X/80/0018-0395$2.00

ascites, amniotic fluid, cervical mucus, tears, liquor and faeces.

Material and Methods

Human urinary kallikrein used for radioiodination and as a standard was purified as described (12). Complete and incomplete *Freund*'s adjuvant was purchased from Difco Lab. Detroit, bovine serum albumin from Roth, Karlsruhe, rabbit serum from Behringwerke Marburg, Sephadex G-100 from Pharmacia Uppsala, Sweden, Biogel P-6 and P-60 from Bio-Rad, München, *D*-Val-Leu-Arg-NHNp from Kabi Sweden; Aprotinin (Trasylol 20000 KIE/ml) and pig pancreatic kallikrein (1300 KE/mg) were kindly provided by Bayer AG, Wuppertal, pig acrosin by *Müller & Fritz* (24). Crystalline bovine trypsin and chymotrypsin were purchased from Novo Industri.

Sample collection

24-hour urine samples of male and female patients and normal persons were collected. Urine samples of orangoutangs and chimpanzees were gifts of the zoological garden of Munich. Submandibular and parotid saliva, gained by selective catherizations of the glandular ducts, was collected before and after oral administration of 2 ml 100 g/l citric acid. Pancreatic and duodenal juice was collected by a duodenal catheter in an X-ray controlled position, or by a pancreatic fistula, which had arisen postoperatively. Bile fluid was collected externally by a postoperatively indwelling T drain of the bile duct, amniotic fluid by amniocentesis in the first trimenon, ascites by transabdominal puncture of patients with decompensated liver cirrhosis. Samples of serum and seminal plasma were obtained from healthy volunteers. All samples were centrifuged at 1000 *g* for 20 minutes and immediatelly frozen at – 40 °C.

Buffers

Buffer A 0.4 mol/l KH_2PO_4, Na_2HPO_4, pH 7.5

Buffer B 0.05 mol/l KH_2PO_4, Na_2HPO_4, 0.15 mol/l NaCl, 200 mg/l merthiolate, pH 7.5

Buffer C 0.05 mol/l KH_2PO_4, Na_2HPO_4, 0.15 mol/l NaCl, 2 g/l bovine serum albumin, 200 mg/l merthiolate, pH 7.5

Buffer D 0.01 mol/l KH_2PO_4, Na_2HPO_4, 0.15 mol/l NaCl, 100 g/l bovine serum albumin, 200 mg/l merthiolate, pH 7.5

Buffer E 0.01 mol/l KH_2PO_4, Na_2HPO_4, 0.1 mol/l NaCl, 10 g/l bovine serum albumin, 0.01 mol/l EDTA, 200 mg/l merthiolate, pH 7.5

Kallikrein activity

Kallikrein activity was measured using the substrate *D*-Val-Leu-Arg-NHNp (8).

Immunization

0.6 mg kallikrein in 1 ml saline was mixed with 2 ml complete *Freund*'s adjuvant and injected intradermally (two paravertebral areas) into (2.5–3.5 kg) white New Zealand rabbits. Intravenous booster injections were given 21 and 28 days after the initial injection. Antisera were collected by cardiac puncture 10 days later and stored at – 80 °C. A working solution was prepared by diluting the antiserum with buffer D. The specificity of the antiserum was checked by immunodiffusion, immunoelectrophoresis and inhibition studies as described (13). Precipitating antirabbit IgG serum was produced in goats. 1 mg rabbit γ-globulin (Serva Heidelberg), 5 mg *mycobacterium tuberculosis*, 1 ml saline and 1 ml *Freund*'s complete adjuvant were homogenized and injected intradermally at 8 sites. Booster injections were given twice at intervals of 4 weeks using incomplete *Freund*'s adjuvant. 8 days after each injection serum was tested for antibody titer by the double diffusion test of *Ouchterlony* and by radioimmunoassay.

Radioiodination

Kallikrein was iodinated by a modification of the chloramine-T method (14) described by *Erhardt* et al. (15). 2.5 µg human urinary kallikrein in 5 µl buffer B, 40 µl Na ^{125}I solution (14.8 MBq = 0.4 mCi) in buffer A were mixed and the reaction was started by addition of 10 µl (30 µg in buffer B) chloramine T. After 20 s the reaction was stopped by addition of 20 µl (60 µg) sodium metabisulfite and 200 µl buffer C. The reaction mixture was than transferred to a column with 50 µl of a 10 g/l potassium iodide solution, the column was eluted with buffer B (40 ml/h). 1.1 ml fractions were collected in disposable polypropylene tubes containing 200 µl buffer C. Two radioactive peaks were eluted. The fraction representing the maximum of the first peak was rechromatographed on a Biogel P-60 column, 1.5 × 60 cm, equilibrated with buffer D, and eluted with buffer B (40 ml/h). The radioactivity of the fractions was determined and 3–4 fractions each of the ascending, the middle and the descending part of the peak corresponding to the elution volume of were pooled, diluted with buffer E to a final radioactivity of about 200000 counts/min × ml and frozen in 10 ml fractions at – 40 °C.

Radioimmunoassay conditions

Human urinary kallikrein standard samples, human urinary [^{125}I] kallikrein, unknowns and goat antirabbit IgG were diluted in buffer E. 3.3 ml/l of rabbit serum were added to the human urinary [^{125}I] kallikrein solution. 0.1 ml buffer E, 0.1 ml labelled human urinary kallikrein (approximately 20000 counts/min) and 0.1 ml antiserum in an appropriate dilution were pipetted into disposable reaction tubes (1.8 × 5.5 cm). Under routine conditions the dilution was 1:32000.

The reaction mixture was incubated at 22 °C for 24 hours and, in order to separate the bound and free kallikrein, 0.1 ml of the second antibody (antirabbit γ-globulin, produced in goats) was added in a dilution of 1:30, mixed and left at 4 °C for another 4–8 hours. After centrifugation at 2000 *g* for 30 min, the supernatant was decanted, the tube was wiped with filter paper and the radioactivity of the precipitate measured. All calibration samples were set up in triplicate (tab. 1). Standard curves were constructed by plotting the fraction of bound radioactivity, B-N/B_0-N (B: counts bound, B_0: counts read for zero dose, N: non specifically bound counts) against the dose of human uriuary kallikrein either in a logit-log mode or in a linear-log mode. The assays were evaluated by a computer program employing the spline approximation method (16). To test the accuracy of the radioimmunoassay samples of human urine, pancreatic juice and saliva of the parotid gland were

Tab. 1. Scheme of human urinary kallikrein radioimmunoassay.

	Sample	Zero binding	Unspecific binding	Total activity
	(ml)	(ml)	(ml)	(ml)
	B	B_0	N	T
Buffer E	0.1	0.2	0.2	–
Standard or sample	0.1	–	–	–
Anti-human urinary kallikrein antibody (rabbit) dil. 1:32000	0.1	0.1	–	–
Human urinary [^{125}I] kallikrein (0.1 ng) + rabbit serum dil. 1:300	0.1	0.1	0.1	0.1
	24 h at 22 °C			
Anti-rabbit γ-globulin antibody (goat) dil. 1:30	0.1	0.1	0.1	–
	8 h at 4 °C			
	Centrifugation: 2000 *g*, 30 min			

measured in three different concentrations (urine: 2.9; 2.6; 3.3; pancreatic juice: 16; 9.5; 11.5; parotid saliva: 46; 53; 52 μg/l) in six different assays. The same solutions were used as "internal standard" in all assays performed.

Specificity

Bovine trypsin and chymotrypsin (0.01–1.0 g/l), porcine acrosin and pancreatic kallikrein (1–100 mg/l) and urine samples from orangoutang and chimpanzee were studied for cross reactivity.

Results

Iodination and binding of human urinary [^{125}I] kallikrein to antisera

After labelling by the chloramin -T method the reaction mixture was immediately transferred to a Biogel P-6 column, which effectively separated the labelled kallikrein from free iodine. The fraction with the highest specific activity was rechromatographed on Biogel P-60. In one experiment all fractions were tested for binding with excess antibody. The results are shown in figure 1. The maximum binding was found for fraction 29. Therefore, fractions no. 28 to 31 were pooled and diluted to the final concentration (20000 counts/min) for the use in radioimmunoassay. Reproducible results were obtained for up to 6 weeks. The specific activity of this human urinary [^{125}I] kallikrein preparation was 1.48 MBq/μg (40 μCi/μg). 1 ml of the solution contained approximately 1 ng of human urinary [^{125}I] kallikrein. The maximum binding of the pooled fractions was 57%. Sufficiently high antibody titers were found in all 7 rabbits. The titers expressed as 50% binding of 0.1 ng labelled human urinary kallikrein were $1:4 \times 10^4$ to $1:2 \times 10^5$ (fig. 2). With dilutions of the different antisera, yielding a B/T ratio of 30–38%, identical slopes and positions of the standard curves were obtained.

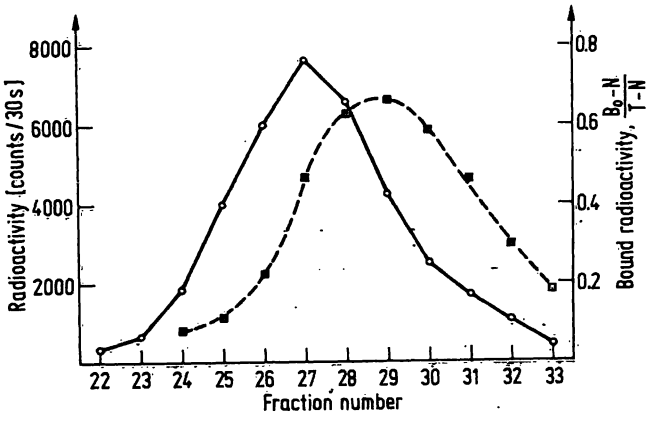

Fig. 1. Rechromatography of desalted human urinary [^{125}I] kallikrein on a Biogel P-60 column (1.5 × 60). Fraction volume: 1.6 ml, elution buffer: B, flow rate 40 ml/h. ■—■ specific binding of human urinary [^{125}I] kallikrein for antibody at human urinary kallikrein antiserum dilution 1:1000, conditions see methods; o—o radioactivity/tube × 30 s.

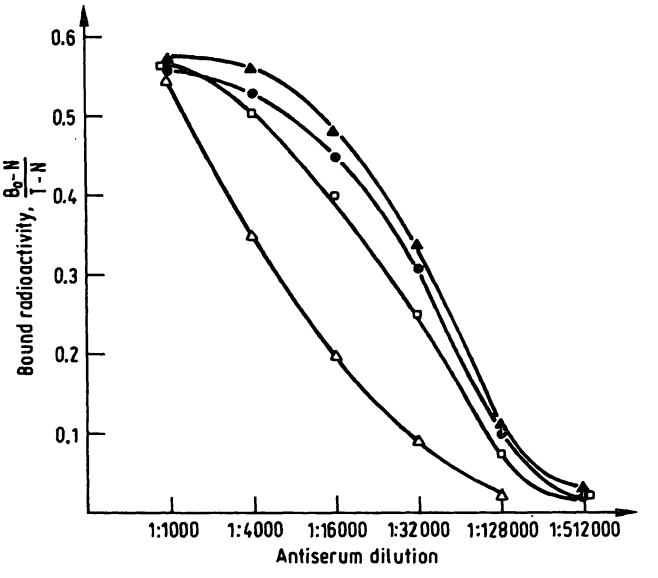

Fig. 2. Titration curves of 4 rabbit anti-human urinary kallikrein sera. The tubes, containing 0.2 ml buffer E, 0.1 ml anti-human urinary kallikrein serum dilution and 0.1 ml human urinary [^{125}I] kallikrein (20000 counts/min) were incubated at 22 °C for 24 h. Bound human urinary [^{125}I] kallikrein was precipitated by adding 0.1 ml of the dilution (1:30) of goat antirabbit-γ-globulin serum. The antiserum dilutions given indicate the final dilution. Rabbit no. ▲—▲ 4, □—□ 5, ●—● 6, △—△ 7.

Time and temperature dependence of the antigen antibody reactions

In order to find the optimal incubation conditions for the radioimmunoassay the time dependence of the binding of human urinary [^{125}I] kallikrein to the antibody was studied at 4, 22 and 37 °C. The results are summarized in figure 3. The highest binding rate was observed at 22 °C, equilibrium was reached after 48 hours. The increment of binding between 24 and 48 h was only 0.09 of maximum binding. Therefore, 24 h incubation was used for routine purposes. The kinetics of the reaction with goat antirabbit IgG serum was studied at 4 and 22 °C. The equilibrium of precipitation of radioactivity was reached after 8 h, optimal binding was obtained at 4 °C.

Standard curves and sensitivity

The sensitivity of the radioimmunoassay can be increased by preincubating unlabelled antigen with the antibody before the labelled antigen is added. By means of 48 hours "cold preincubation" the lower limit of detection was reduced from 0.9 to 0.4 μg/l, the 50% intercept from 10.2 to 2.5 and the slope from – 1.1 to – 1.3 under routine assay conditions (fig. 4).

Precision

The intraassay coefficient of variation (N = 6) was 4–7% for urine (range 2.9 to 46 μg/l), for saliva of the parotid gland 3–8% (range 3.3 to 52 μg/l) and for pancreatic juice 4–8% (range 2.6 to 53 μg/l).

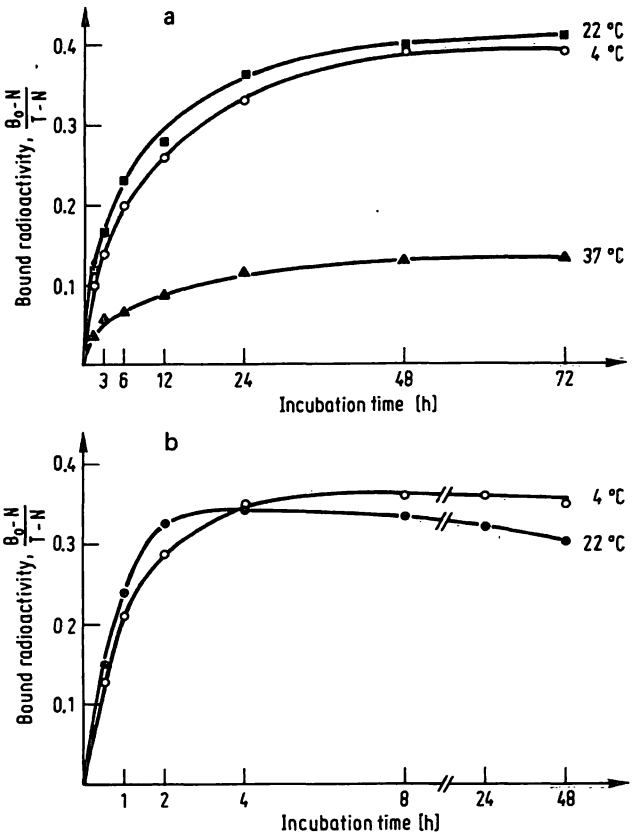

Fig. 3. Time and temperature dependence of the binding of human urinary [^{125}I] kallikrein to the anti-human urinary kallikrein antibody (a) and anti-γ-globulin antibody (b) resp.

The interassay coefficient of variation of identical samples (N = 6) containing 2,9, 16, 46 µg/l, each measured at 6 different occasions, was 4–13%.

Specificity

Cross reaction of other serine proteinases with anti-human urinary kallikrein serum was tested under radioimmunoassay conditions. No cross reaction was observed for bovine trypsin and chymotrypsin, pig pancreatic kallikrein and pig acrosin, rat urine, homogenate of rat tubules (17), rabbit and cat urine. In contrast, cross reacting material was found in the urine of guinea pigs, orangoutangs and chimpanzees. In human samples immunologically active material was found in the saliva of submandibular and parotid gland, pancreatic juice, bile, sweat, serum, ascites, seminal plasma, amniotic fluid, cervical mucus, tears, liquor and faeces, but not in gastric juice and breast milk.

A necessary though not sufficient criterion of immunological identity is parallelity of dose response curves of the sample and the standard substance measured radioimmunologically. This criterion was fulfilled for human urine, saliva of the submandibular and parotid gland, pancreatic juice, bile and sweat (fig. 5, 6). The kallikrein content in urine was 0–300 µg/l, in pancreatic juice 300–12000 µg/l and in saliva of the submandibular and parotid gland 400–2000 µg/l.

Dose-response curves of serum, ascites, seminal plasma, amniotic fluid, cervical mucus, tears, liquor and human

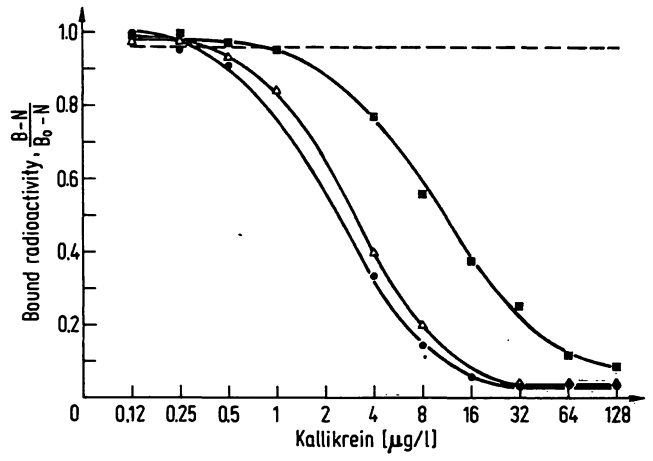

Fig. 4. Influence of preincubation time of unlabelled human urinary kallikrein on standard curves. Conditions see Table 1.
- ■—■ Incubation of standard, anti-human urinary kallikrein serum and tracer for 24 h.
- △—△ Preincubation of standard and anti-human urinary kallikrein serum for 24 h, followed by 24 h incubation after addition of tracer.
- ●—● Preincubation of standard and anti-human urinary kallikrein serum for 48 h, followed by 24 h incubation after addition of tracer.
- --- lower limit of detection.

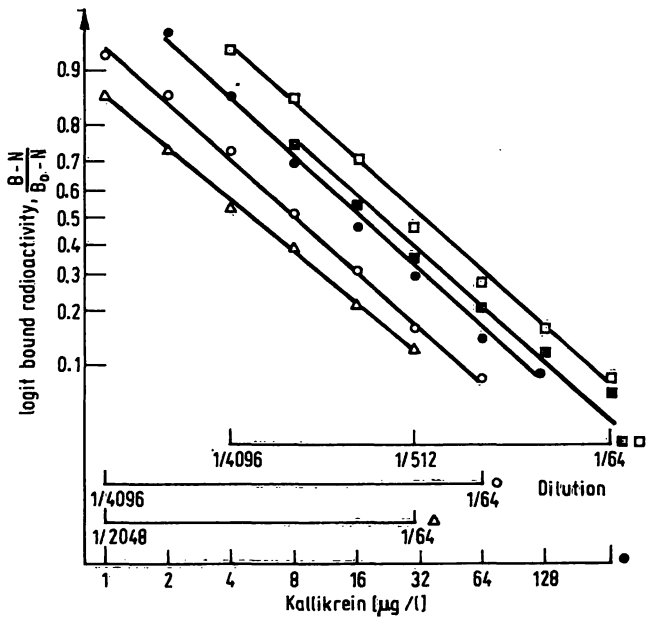

Fig. 5. Dose response curves of purified human urinary kallikrein and human body fluids in the human urinary kallikrein radioimmunoassay. The reactivity is identical. Results are expressed in the logit-log transformation, normalized to maximum counts bound (B_0).
●—● standard human urinary kallikrein, ■—■ human urine, □—□ parotid saliva, △—△ submandibular saliva, ○—○ pancreatic juice.

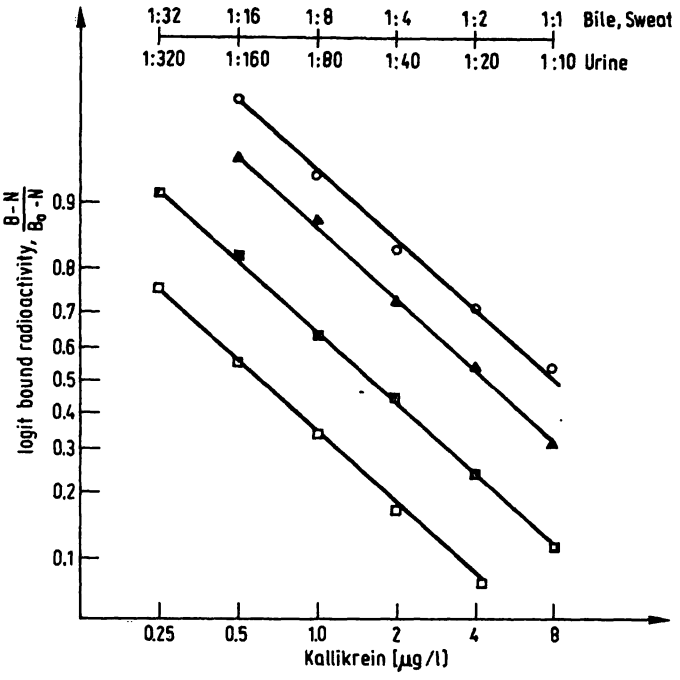

Fig. 6. Dose response curves of human urinary kallikrein and human body fluids in the human urinary kallikrein radioimmunoassay. Standard and samples were preincubated for 48 h, before labelled human urinary kallikrein was added, followed by a incubation time of 24 h. The reactivity of human urinary kallikrein and body fluids is identical.
■—■ standard human urinary kallikrein, □—□ bile, ○—○ sweat, △—△ urine.

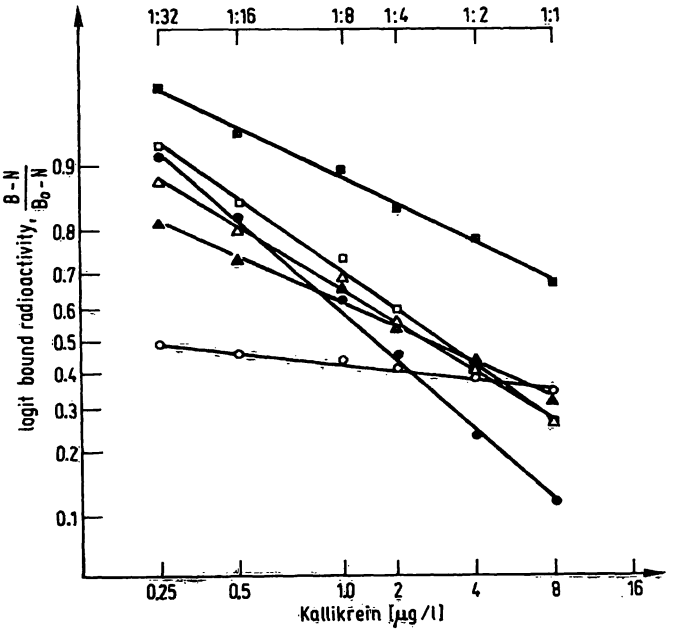

Fig. 7. Dose response lines for ●—● standard human urinary kallikrein, ■—■ amniotic fluid, ▲—▲ seminal plasma, □—□ ascites, △—△ serum, ○—○ chimpanzee urine.

faeces, urine samples of guinea pigs, orangoutangs and chimpanzees were neither parallel to the human urinary kallikrein standard curves nor to each other (fig. 7).

Recovery test

Several recovery tests have shown the good precision of the method. Different quantities of purified human urinary kallikrein were added to antigen-free rat urine, in order to measure the added and recovered kallikrein ratio. (tab. 2).

Stability

The storage of pancreatic juice, saliva and urine at 4 °C and 22 °C from 4 to 68 hours, as well as freezing and thawing four times, had no influence on the kallikrein content measured by the radioimmunoassay (tab. 3).

Discussion

The radioimmunoassay technique, which is sensitive and specific, should be very suitable for studies on the physiological and pathophysiological role of glandular kallikreins. In the same species, the different glandular kallikreins can be measured by a radioimmunoassay for

Tab. 2. Recovery test.

Human urinary kallikrein added (µg/l)	Human urinary kallikrein recovered (µg/l)	N	Recovery (%)
50	52.9 ± 2.6	8	105.8 ± 4.9
5	6.6 ± 1.03	8	130 ± 1.5

Concentrations of purified human urinary kallikrein indicated above were added to 1 ml of antigen free rat urine and measured by radioimmunoassay.

Tab. 3. Stability of human glandular kallikrein during storage determined by radioimmunoassay.
Results are given as per cent binding $(\frac{B-N}{B_0-N} \cdot 100)$.

	0 h	4 h	68 h	4 h	68 h	Freezing and thawing
		22 °C		4 °C		4 times
U 1	87.5	79.6	85.6	78.8	83.3	84.9
2	94.8	86.8	90.6	86.4	93.0	92.0
3	61.7	56.2	61.3	53.1	59.8	59.7
P 1	12.6	9.3	10.9	9.6	11.6	12.3
2	7.7	7.8	8.1	7.1	10.2	8.5
3	5.7	5.9	7.1	5.9	6.9	5.9
4	8.8	8.9	10.3	9.1	10.4	8.7
S 1	50.8	48.4	52.1	47.6	53.0	51.5
2	15.4	15.0	16.2	15.2	17.6	16.8
3	39.9	39.5	41.7	37.8	42.5	45.6
4	16.7	16.0	18.0	14.7	18.3	17.8

U = urine, P = pancreatic juice, S = saliva

Samples were stored at different temperatures and for different time intervals.

any one of them, owing to the high immunological similarity or even identity of the glandular kallikreins in one species (18, 19, 20, 21). In order to measure human glandular kallikreins we developed a radioimmunoassay for urinary kallikrein.

By use of the chloramine T method, human urinary kallikrein was iodinated to a specific activity sufficiently high for use in this assay. The maximal binding of the tracer to the antibody was 0.62 and no essential loss of binding was observed for up to six weeks. Best incubation conditions for the measurement of human urinary kallikrein in respect to time and temperature were found to be similar to those described for the radioimmunoassay of pig pancreatic kallikrein (18). The sensitivity of the assay was 0.9 µg/l and could be increased about twofold by the technique of "cold preincubation".

The high specificity of the radioimmunoassay was demonstrated by employing various proteinases and biological samples. Some cross reactivity was found when urine of chimpanzees (fig. 7), orangoutangs and guinea pigs was measured. However, the dose response curves did not parallel the human urinary kallikrein standard curves. These observations can be explained by partial immunological identity of the glandular kallikreins of these species. No crossreactivity at all was detected for the serine proteases, bovine trypsin and chymotrypsin and porcine acrosin. Final proof of non-cross reactivity of the various human serine proteases is not yet available; however, in analogy to the results obtained by radioimmunoassay of pig pancreatic (18) and rat submandibular kallikrein (20), it may be expected that human proteinases other than tissue kallikreins do not interfere in the radioimmunoassay of human urinary kallikrein. Therefore, in contrast to the assays based on the enzymatic activity of kallikrein, the radioimmunological measurement of kallikrein is possible in the presence of other proteases. A further advantage of the radioimmunoassay method is that storage of biological samples at 4 and 22 °C up to 68 h, as well as freezing and thawing (tab. 3), have no influence on the measured kallikrein concentration, whereas a rapid decrease of the enzymatic activity is known to occur in frozen samples (11).

The radioimmunoassay was used to measure kallikrein concentrations in biological samples. The dose response curves obtained for serum, ascites, seminal plasma, amniotic fluid, cervical mucus, tears, liquor and faeces did not parallel the standard curve. Therefore, a direct quantitative determination of glandular kallikrein in these samples is not possible. Similar results were obtained when radioimmunoassays for pig pancreatic kallikrein (22) and rat urinary kallikrein (23) were applied to the measurement of glandular kallikrein in serum and plasma. Dose response curves paralleling the human urinary kallikrein standard curve were found for human urine, saliva, bile, pancreatic juice and sweat, thus allowing the measurement of the kallikrein concentration in these samples.

Several explanations for non-parallelity are possible, for example:

a) inhibitors present in the samples occupy antigenic sites of [^{125}I] kallikrein thus reducing the binding to the antibody and interfering in the radioimmunoassay;

b) prekallikrein or modified kallikrein with immunologically different properties interfere;

c) other cross reacting antigens, not yet characterized, are present in the samples;

d) non-specific interferences.

All these possible interferences and methods for their elimination have to be investigated further. Final proof of the presence of glandular kallikrein in the various biological samples has to come from additional evidence, not obtained by immunological methods.

The physiological and pathophysiological role of glandular kallikreins is still unknown to a large degree. The availability of a radioimmunoassay for human glandular kallikrein will provide the means to study possible relationships between pathologic states and the concentrations of glandular kallikreins in body fluids and tissues. Such studies should lead to a better understanding of the physiological function of the kallikreins.

Acknowledgements

This work was generously supported by Sonderforschungsbereich 51. We wish to thank very much Prof. Dr. *H. Fritz* for comments and interesting discussions.

References

1. Frey, E. K., Kraut, H. & Werle, E.: Kallikrein (Padutin) Stuttgart: Enke 1950, p. 96.
2. Beraldo, W. T., Aranjo, R. L. & Mares-Guia, M. (1966), Am. J. Physiol. *211*, 975–980.
3. Croxatto, H. R. & San Martin, M. (1970), Experientia *26*, 1216–1217.
4. Trautschold, J., Werle, E. & Schweitzer, G. (1974), in Methoden der enzymatischen Analyse (Bergemeyer, H. V. ed.) 3rd edn., 1071–1080, Verlag Chemie, Weinheim/Bergstraße.
5. Moriwaki, C., Inone, N., Hoijma, Y. & Moriya, H. (1971), Yâkugaku Zasshi *91*, 413–416.
6. Martin, C. J., Golubow, J. & Axelrod, A. E. (1959), J. Biol. Chem. *234*, 294–298.
7. Fiedler, F., Geiger, R., Hirschauer, Ch. & Leysath, G. (1978), Hoppe-Seyler's Z. Physiol. Chem. *259*, 1667–1673.

8. Claeson, G., Friberger, P., Knös, M. & Eriksson, E. (1978), Haemostasis *7*, 76–78.
9. Mann, K., Geiger, R. & Werle, E. (1976), Adv. Exp. Med. Biol. *70*, 65–73.
10. Marin-Grez, M. & Carretero, O. A. (1972), J. Appl. Physiol. *32*, 428–431.
11. Carretero, O. A., Oza, N. B., Piwonska, A., Ocholik, Th. & Scicli, A. G. (1976), Biochem. Pharmacol. *25*, 2265–2270.
12. Geiger, R., Mann, K. & Bettels, T. (1977), J. Clin. Chem. Clin. Biochem. *15*, 479–483.
13. Mann, K. & Geiger, R. (1977), Kininogenases – Kallikrein 4 (Haberland, G. L., Rohen, J. W. & Suzuki, T., eds.) Schattauer Verlag, Stuttgart–New York, 55–61.
14. Greenwood, F. C., Hunter, W. M. & Glover, J. (1963), Biochem. J. *89*, 114–123.
15. Erhardt, F., Marschner, J., Pickardt, R. C. & Scriba, P. C. (1973), Z. Klin. Chem. Klin. Biochem. *11*, 381–387.
16. Huber, W., Mann, K. & Karl, H. J. (1979), J. Clin. Chem. Clin. Biochem. *17*, 241–246.
17. Geiger, R. & Mann, K. (1976), Hoppe-Seyler's Z. Physiol. Chem. *357*, 553–558.
18. Fink, E. & Güttel, Ch. (1978), J. Clin. Chem. Clin. Biochem. *16*, 381–385.
19. Fritz, H., Fiedler, F., Dietl, T., Warwas, M., Truscheit, E., Kolb, H. J., Mair, G. & Tschesche, H. (1977), in: Kininogenases – Kallikrein 4 (Haberland, G. L., Rohen, J. W. & Suzuki, T., eds.) Schattauer Verlag, Stuttgart–New York, 15–28.
20. Proud, D., Bailey, G. S., Nustad, K. & Gautvik, K. M. (1977), Biochem. J. *167*, 835–838.
21. Amouric, M. & Figarella, C. (1979), in: Current Concepts in Kinin Research (Haberland, G. L. & Hamberg, U., eds.) Pergamon Press, Oxford and New York, 103–110.
22. Fink, E., Geiger, R., Witte, J., Biedermann, S. & Fritz, H. (in press). Proceedings of the Eigth Workshop Conference Hoechst Enzymatic Release of Vasoactive Peptides. Raven Press, New York.
23. Carretero, O. A., Scicli, A. G., Amin, V., Nichols, C., Ocholik, T. & Rabito, S. (1979), in: Proceedings of the International Symposium on Kinins. Plenum Press, New York and London.
24. Müller, W. & Fritz, H. (1978), in: Regulatory Proteolytic Enzymes and their Inhibitors (Magnuson, S., ed.), Pergamon Press, Oxford and New York, 173–179.

Dr. Reinhard Geiger,
Abteilung für Klinische Chemie
und Klinische Biochemie
in der Chirurgischen Klinik
der Universität München
Nußbaumstraße 20,
D-8000 München 2