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Originals

Renal Calculi – Urate as a Urokinase Inhibitor

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Summary. The formation of renal calculi is one of the most widely studied urinary ailments. Spectrophotometric analysis of urinary inhibition on the urokinase/plasmin system revealed a significant difference between subjects with and without renal calculi ($P < 0.001$). The percentage urokinase/plasmin inhibition in the two groups was 77.1% for those with, and 47.1% for those without renal calculi. Because of the significant ($P < 0.015$) positive correlation (r = 0.762) between the percentage inhibition of urokinase/plasmin and urinary urate concentration, the inhibition of urate on urokinase/plasmin and on plasmin and urokinase was investigated. The urokinase/plasmin system was inhibited up to 94.4% with 0.5 mM urate. Inhibition occurred with both low and high molecular weight urokinases. In order to determine which enzyme of the complex was inhibited, each was investigated independently and a 50% inhibition of urokinase activity was obtained with 4 mM urate. It was found that the urine of stone formers contained a higher concentration of urate than the urine of healthy subjects and that at the same time there was a decrease in urinary urokinase activity in these patients.

Key words: Plasmin – Renal calculi – Urinary urate – Urokinase

Introduction

It is generally known that urinary urate plays a role in urolithiasis. In the treatment of renal stones the urinary urate concentration is reduced with allopurinol or by diet [2, 3]. However, the process of kidney stone formation is not clear. Hallson et al. [7] attempted to clarify the importance of urate in renal stone formation, and their evaporation studies showed that urate did not influence the formation of calcium oxalate crystals in whole urine of normal subjects. This observation ruled out the possibility that uric acid might "salt out" calcium oxalate as a result of hyperuricosuria or that it may be responsible for the nuclei of calcium oxalate stones. However, the question remains as to whether urate contributes in any way to the formation of urinary stones. We postulated that low activities or decreased production of urinary urokinase or plasmin may increase the urinary uromucoid concentration, thus inducing stone formation. In support of this hypothesis, it has been reported [12] that the urine of patients with renal stones has a higher trypsin inhibition than that of normal subjects. However, the possible existence and nature of urokinase inhibitors were not discussed. We therefore investigated the total inhibition of urokinase/plasmin in the urine of subjects with and without renal stones.

Materials and Methods

Reagents and Chemicals

All reagents were of the "Analar" grade. Merck and BDH supplied the reagents sodium phosphate, EDTA, sodium azide, uric acid, Triton X-100 and Carbowax 6000. Sigma Chemical Co. supplied the substrates plasminogen (human plasma), D-valyl-L-leucyl-L-lysine p-nitroanilide, Benzoyl-L-valyl-glycyl-L-arginine p-nitroanilide (Chromozym UK) and L-pyroglutamyl-glycyl-L-arginine p-nitroanilide (S-2444), the enzymes plasmin (human plasma) and human kidney urokinase (U 8627 and U 5628), as well as the inhibitors antipain, leupeptin and aprotinin. High molecular weight human urinary urokinase (HMW-UK) was obtained from the Green Cross Co., Osaka, Japan, and the Institute Choay in Paris, France, also supplied human urokinase.

Determination of Urokinase Kinetics

The activity of urokinase coupled to plasmin was assayed according to a modified method of Wiman et al. [14]. 400 µl activator reagent was added to 418 µl of 0.1 M sodium phosphate buffer, pH 7.3, containing 10 mM EDTA, 0.1 g/l sodium azide and 0.1 g/l Triton X-100. The activator reagent was composed of 1.0 µM plasminogen

and 0.6 mM D-valyl-L-leucyl-L-lysine p-nitroanalide, which was dissolved in 0.1 M sodium phosphate buffer. The reaction was initiated by the addition of urokinase (E.C. 3.4.21.31). After 2 min at 37 °C, the enzyme activity was estimated from the product concentration recorded at 405 nm on a Hitachi Spectrophotometer 150-20 attached to a data processor. The molar extinction coefficient for p-nitrophenol was taken as 9,620 M^{-1} cm^{-1} [13]. The blank consisted of a buffer with an activator reagent. The total reference volumes (buffer), blank and sample in each of the cuvettes were 828 µl. In a separate assay, the urokinase purity was investigated by adding of the urokinase inhibitor antipain [15].

Standard Curve of Urokinase Activity

A standard activity curve of urokinase coupled to plasmin was obtained by incubating different amounts of urokinase in a stirred waterbath for 90 min at 37 °C. This assay was done in the same phosphate buffer, pH 7.3, and activator reagent which were previously used for the urokinase kinetics determination. After the desired incubation time, the reaction was stopped immediately by inserting the tubes in ice and adding 100 µl of a 50% acetic acid solution to all the tubes [8]. The urokinase activity was expressed in International Units (IU). One IU equals 0.5 nmoles [1].

Urine Specimens

Morning urine was collected in plastic tubes from 7 adult male patients with calcium oxalate stones but without urinary tract infection, and from 14 healthy male subjects without stones. To determine the stability of the urokinase inhibitor system in urine, fresh urine samples were maintained at room temperature and at −15 °C [14] both with and without an equal volume of sodium acetate (1 mol/l at pH 3.9). At times intervals (days) the urinary urokinase activity was determined from the standard curve.

Spectrophotometric Determination of Total Urokinase/Plasmin Inhibition

The total inhibition of the urine on urokinase/plasmin was assayed according to the spectrophotometric method described under the heading *Determination of Urokinase Kinetics.* However, to determine the influence of urinary inhibitors on the coupled urokinase system, the activity of urokinase was first obtained by running a "reference". The reference consisted of a blank (buffer with activator reagent) which was scanned for 40–60 min at 37 °C before urokinase (0.16–0.19 IU) was added. The enzyme activity was then monitored for about 50 min. The blank rate value was subtracted from the rate value obtained in the presence of the enyzme. The urokinase activity in the presence of urine was determined next. This was known as "sample". This sample assay was done exactly as the reference, except that 10 µl urine were added to the blank from the start. The same amount of urokinase used in the reference was added after 40–60 min. This spectrophotometric procedure allowed the effects of urinary urokinase to be eliminated so that the degree of urinary inhibition could be calculated.

Correlation Studies

The spectrophotometric procedure described above was carried out to determine the correlation between the total percentage inhibition of the coupled urokinase system and urinary urate concentrations. The urinary urate determinations were kindly done by the Institute of Chemical Pathology, University of Pretoria.

Sodium Dodecyl Sulphate (SDS) Gradient Gel Electrophoresis

The electrophoresis was carried out on a Pharmacia GE-2/4 electrophoresis apparatus at 150 V. Polyacrylamide gradient gel slabs, 4–30%, were prepared in a Pharmacia GM-1 gradient mixer using a peristaltic pump. Potassium persulphate and 3-dimethylaminopropionitrile were used as catalysts and the buffer was 0.09 M Tris, 0.08 M borate, 3 mM EDTA and 0.2% SDS at pH 8.3. The samples investigated were completely dissociated by heating at 95 °C for 5 min in the presence of 1% SDS and 1.25% dithiothreitol. For the determination of the molecular mass of the separated proteins, the following marker proteins were used: phosphorylase b, albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor and lactalbumin. Protein bands were stained with 0.2% Coomassie brilliant blue R 250 in a 45% methanol and 10% acetic acid solution. Destaining was achieved with 7% acetic acid.

Urate Inhibition Studies

Freshly prepared 8.28 mM Na-urate solution at pH 7.3 was used in all these studies. Various aliquots were added to the enzyme assays of urokinase/plasmin, plasmin and urokinase. The same method was originally used for the urokinase/plasmin and urokinase assays described in the section *Spectrophotometric Determination of Total Urokinase/Plasmin Inhibition*, except that urate was added to the reaction mix instead of urine (Method A). However, this method was time consuming, and since the urokinase activity decreased after a couple of hours, the methodology was changed. The same procedure described in the section *Determination of Urokinase Kinetics* was applied where all reagents were present at the beginning of the assay and the reaction was allowed to continue only for 30 min (Method B).

The plasmin activity determinations were done according to the spectrophotometric assay method described by Cs-Szabo et al. [4], except that the phosphate buffer, pH 7.3, as described in [14] was used.

Statistical Analysis

Since small samples were involved, the inhibition data were analysed for differences among subjects with and without renal stones by employing the Mann-Whitney Test, which is a nonparametric procedure [11].

Results

To determine the linearity of the coupled urokinase system, it was necessary to establsih its kinetic pattern. Different urokinase concentrations were used (Fig. 1). The reaction was linear for at least 150 min with 21.6×10^{-2} IU and lower, whereas at a higher concentration (32.4×10^{-2} IU) the reaction was linear for only 120 min. The inhibition obtained with 1 µM antipain reflected the assay specificity for urokinase. In accordance with the times found, an in-

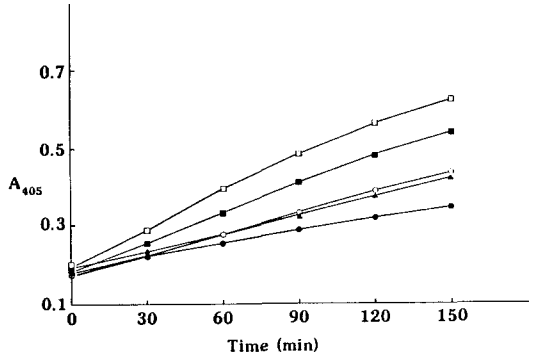

Fig. 1. Spectrophotometric reaction time course of different urokinase concentrations at 37 °C: no urokinase (●), 10.8 x 10^{-2} IU (0), 21.6 x 10^{-2} IU (■), 32.4 x 10^{-2} IU (□) and 21.6 x 10^{-2} IU with 1 μM antipain (▲). The conditions are described in the section *Determination of Urokinase Kinetics* under Materials and Methods

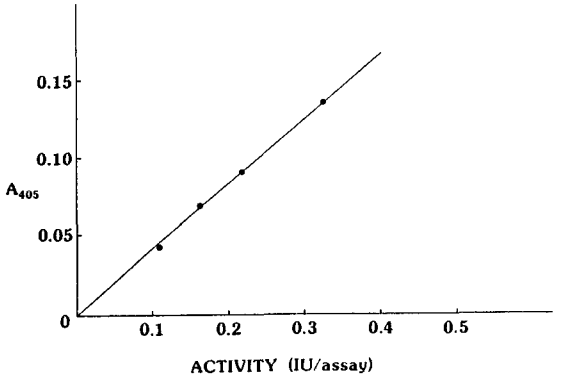

Fig. 2. Standard curve of urokinase activity with plasminogen as the substrate. The incubation time was 90 min and was carried out in a stirred waterbath at 37 °C. The reaction was stopped with 50% acetic acid

cubation period of 90 minutes was decided on for future urokinase determinations.

Since our studies were aimed at determining the urinary inhibition on the coupled urokinase/plasmin system, the stability of the urinary inhibition was initially monitored using urokinase. As an increase of urokinase activity with time should indicate any instability of the inhibitor(s), a standard curve was constructed for the different urokinase activities (Fig. 2). Enzyme activity studies were carried out in the presence and absence of a pH 3.9 acetate buffer at different temperatures (Table 1). Acidification of urine caused elevated enzyme activity at both room temperature and at −15 °C, but the enzyme activity of whole urine remained stable for 4 days at both room temperature and −15 °C.

Different levels of total urinary inhibition were compared spectrophotometrically in subjects with and without renal calculi (Table 2). Elevated inhibitor modification on the urokinase/plasmin system was observed in the presence of urine obtained from stone formers. In contrast, urine from healthy subjects showed a lower level of inhibition.

Table 1. Stability of urinary urokinase inhibitor(s)

Time (days)	Room Temperature (24 °C)		Freeze (−15 °C)	
	Without acetate	With acetate	Without acetate	With acetate
0	8.8[a] (100)	14.6 (166)	–	–
4	9.2 (105)[b]	18.4 (209)	9.2 (105)	17.2 (196)
15	5.2 (59)	12.0 (136)	10.0 (114)	4.5 (51)

[a] expressed as IU/ml
[b] expressed as a percentage of control

Table 2. Spectrophotometrically determined urinary total inhibition of subjects with and without renal stones

Without stones		With stones	
Patients	Inhibition (%)	Patients	Inhibition (%)
1	34.72	1	100.00
2	37.82	2	70.88
3	62.69	3	61.45
4	57.13	4	67.77
5	57.26	5	82.51
6	59.95	6	73.78
7	40.41	7	83.01
8	53.58		
9	58.55		
10	48.45		
11	25.59		
12	48.02		
13	44.04		
14	31.51		

Table 3. Inhibition statistics of subjects with and without renal calculi

Subject	Average inhibition (%)	S.D.	Median	Range
Without stones	47.12	11.72	48.23	25.59– 62.69
With stones	77.06	12.72	73.78	61.45–100.00

These two groups differed significantly ($P < 0.001$). Other statistical data on the groups are in Table 3. We next studied the correlation between total percentage inhibition and urinary urate content (Fig. 3). A significant ($P = 0.015$) Spearman rank correlation coefficient of $r = 0.762$ was obtained.

To investigate this inhibition phenomenon, further urate inhibition studies were done. Human urokinase preparations

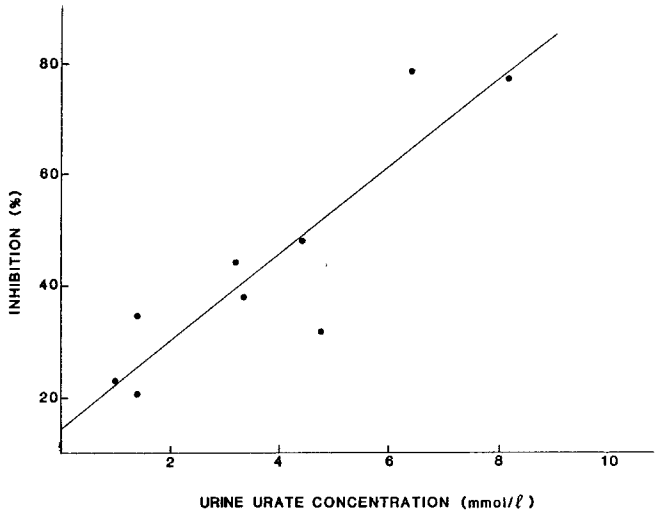

Fig. 3. The correlation between percentage levels of urokinase/plasmin total inhibition and the urinary urate concentration of 9 subjects. The determination of the total inhibition of urokinase/plasmin is described in the section *Spectrophotometric Determinations of Total Urokinase/Plasmin Inhibition* of Materials and Methods

Table 4. Inhibition of different urokinase preparations by urate

Company	Type	Activity (IU)	Urate concentration (mM)	Inhibition (%)
Sigma	LM-UK	0.155	0.1	33.3
			0.5	94.4
Choay	LM-UK	0.241	0.1	50.0
			0.2	85.7
Green Cross	HM-UK	0.318	0.5	28.5

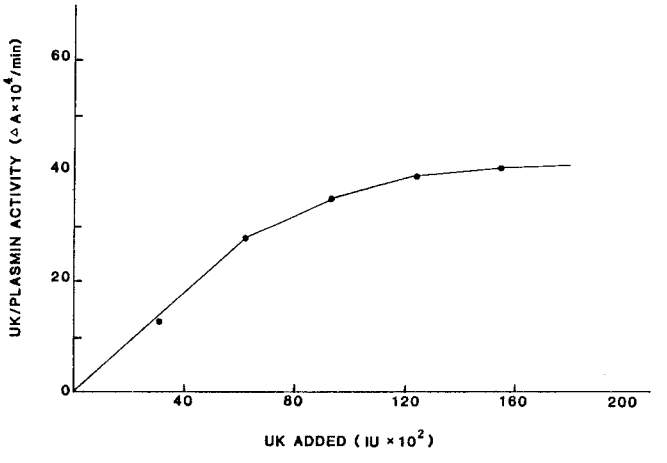

Fig. 4. Activity curve of urokinase/plasmin in the presence of plasminogen. For conditions, see Materials and Methods, *Urate Inhibition Studies*, Method B

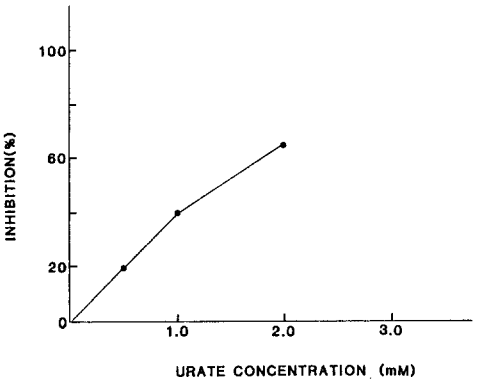

Fig. 5. Inhibition of urate on urokinase/plasmin system (0.344 IU). For conditions see legend of Fig. 4

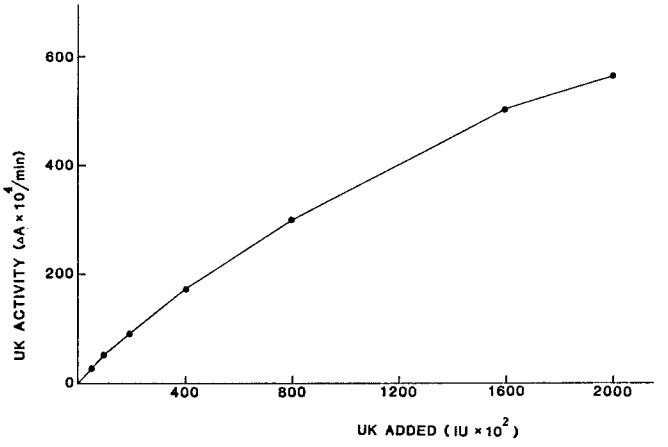

Fig. 6. Activity curve of urokinase (Sigma) in the presence of S-2444. For conditions, see Materials and Methods, *Urate Inhibition Studies*, Methods B

were obtained from three different companies, viz. Sigma, Choay and The Green Cross Co. Because the molecular weights of the first two preparations were unknown, SDS-PAGE was carried out. The results of the electrophoresis indicated that both urokinases had a low molecular weight (33,000), in contrast to the third prepreparation [9, 10], which is one of the high molecular weight urokinases (55,000). Preliminary research using Method A indicated elevated inhibition levels in the presence of urate. Both the low molecular weight urokinases (LM-UK) and the high molecular weight urokinase (HM-UK) were inhibited (Table 4). The effect of urate on the urokinase/plasmin system was studied next in the presence of Sigma LM-UK (Method B). A urokinase activity curve was first constructed to determine the enzyme concentration range for a linear response with the available substrate concentration (Fig. 4). Inhibition studies revealed that urate modified the acitivty of the urokinase/plasmin system (Fig. 5). The inhibitors of urokinase, antipain (0.5 μM) and leupeptid (0.5 μM) inhibited the system by 42.1% and 79.0% respectively.

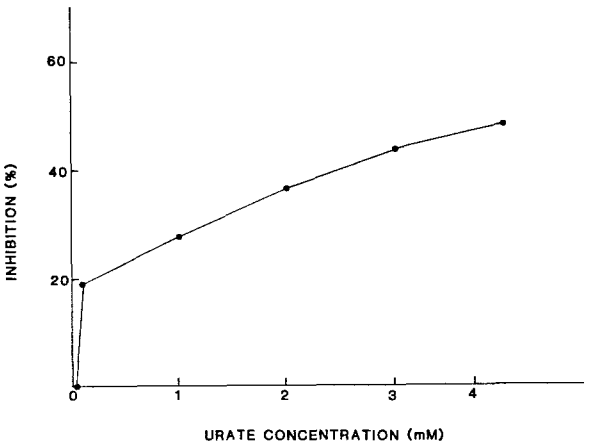

Fig. 7. Inhibition of urate on urokinase (Sigma, 0.388 IU). For conditions see legend of Fig. 6

As it then had to be determined which of the coupled enzymes was specifically involved in the inhibition, two consecutive inhibition studies were designed using plasmin and urokinase independently. The influence of urate on 0.199 μM plasmin was first studied. Slight inhibition was observed. At urate levels of 1 mM and 2 mM the inhibition was similar, 10.6% and 11.7% respectively. In contrast the inhibitor aprotinin (0.064 TIU) completely modified the activity of the enzyme [8].

Inhibition studies of urokinase were carried out with two synthetic tripeptidyl-p-nitroanalide substrated (Method B). With Chromozym UK as the substrate, no effect whatsoever was observed in the presence of 4 mM urate, and hardly any inhibition occurred with antipain or leupeptin. After an activity curve of urokinase (Sigma) was constructed with the substrate S-2444 (Fig. 6), the urate inhibition was investigated (Fig. 7).

Discussion

In the present study we have investigated the inhibition of the urokinase system. Initially, urokinase activity in urine was monitored to determine the stability of urinary inhibitor(s). To test the liability of this system, urinary assays were done in the presence and absence of a pH 3.9 acetate buffer at different temperatures (Table 1). It was earlier demonstrated that inhibition is overcome by acidification of urine [14]. Although the authors give no explanation for this effect, our results supported their finding. Increased activity was observed with acidification at different temperatures, which may be explained by the solubility of urinary urate. The monobasic urate salt has a pK_a value of 5.7. After acidification, pH 3.9, the acid urate is formed, which has a very low water solubility of 0.384 mmol/l [5]. Higher urokinase activity was therefore observed with acidification of urine because the putative inhibitor was removed by precipitation. Investigation showed that the inhibitor(s) was stable for at least 4 days at room temperature. The decrease in activity after 4 days may have been caused by enzyme denaturation. Because the aim of our study was to investigate urinary inhibition on the coupled urokinase/plasmin system, the urine was not acidified and the assays were done with fresh urine kept at room temperature.

The higher percentage inhibition in stone formers compared to non-stone formers may also have been due to an increase in urinary urate. This was supported by a positive correlation found between the total percentage inhibition of urokinase/plasmin and urinary urate concentrations (Fig. 3). This finding is in accordance with published data on urinary urate concentrations. Coe [3] demonstrated that most patients with renal stones exhibit higher urinary urate concentrations than healthy subjects. This work was followed up by studies of urate inhibition on coupled urokinase supplied by different companies (Table 4). Both the low and high molecular weight urokinase activities were modified in the urokinase/plasmin assays. Although it would have been desirable to use higher urate values in these inhibition studies, this was not possible: as soon as the urate concentration in the pH 7.3 stock solution, was increased, the urate precipitated. A coupled urokinase-plasmin system was used for the assays. To determine which enzyme was involved in the inhibition, both enzymes had to be tested separately for inhibitory effect. Although the same tripeptidyl-p-nitroanilide was used as the substrate in the plasmin assay as in the original urokinase/plasmin experiments, no inhibition occurred with urate. In contrast to this, urokinase acitivty was modified with urate (Fig. 7). However, inhibition was observed when S-2444 was used as the substrate, but not with the synthetic substrate Chromozym UK. The only explanation for this is that the native protein plasminogen was more specific than the synthetic compounds for urokinase. This is supported by Fassler et al. [6] who doubted the specificity of urokinase for Chromozym UK.

It can be concluded that the inhibiting effect of urine on the coupled urokinase/plasmin system is the result of urate inhibition of urokinase. The increase in inhibition obtained with the urine of stone formers may be the result of a higher urate concentration in their urine than in the urine of healthy subjects. This agrees with published data, namely that the urine of stone formers contains higher urate concentrations than the urine of normal subjects [3]. These conclusions would explain why allopurinol is administered to kidney stone patients. Allopurinol causes a decrease in urinary urate excretion by inhibiting the xanthine oxidase, which would cause a higher urinary urokinase activity. It therefore seems that the activity of urokinase plays a role in the complex process of stone formation. The importance of high urokinase activity in the prevention of stone formation has not yet been established and required further study.

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