Urinary crystal growth: effect of inhibitor mixtures

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Summary

1. The crystal growth inhibitory activity of mixtures of known inhibitors and of mixtures of known inhibitors with normal urine was determined in calcium oxalate monohydrate and hydroxyapatite seeded crystal growth systems.

2. The inhibitory activity of the mixtures was compared with the measured activity of the individual components of the mixtures. All mixtures had inhibitory activity equal to the sum of the activities of their components, with the exception of RNA/urine mixtures in the calcium oxalate monohydrate system.

3. RNA/urine mixtures had inhibitory activity toward calcium oxalate monohydrate crystal growth which was less than would be predicted from the activity of the RNA and of the urine which were added. This reduced inhibitory activity was shown to be due probably to hydrolysis of RNA by the ribonuclease activity normally present in urine.

4. The results of these experiments make it possible to determine quantitatively the contribution of various naturally occurring urinary crystal growth inhibitors to the total measured inhibition observed in urine.

Key words: calcium oxalate monohydrate, crystal growth inhibitors, hydroxyapatite, seeded crystal growth.

Introduction

Normal urine inhibits the growth of calcium oxalate [1] and hydroxyapatite crystals [2]. Inhibitors of calcium oxalate crystal growth normally present in human urine include pyrophosphate [3], citrate [4], glycosaminoglycans [5], RNA-like compounds [6] and specific acidic proteins [7]. Hydroxyapatite crystal growth inhibitors present in human urine include pyrophosphate [8], citrate [9], and magnesium [10].

Since naturally occurring inhibitors of crystal growth may help protect against stone disease, the nature of these inhibitors is of considerable interest. It may be possible to design therapy that increases excretion of specific inhibitory compounds, either those normally excreted or others. To accomplish this, it is necessary to be able to assess the contribution of the various inhibitors to the total inhibitory activity of urine. Compounds known to be inhibitors can be tested in assay systems in vitro, but the true situation in urine may be quite different. Urine contains multiple crystal growth inhibitors which may interact with one another either to increase or decrease the potency of an individual inhibitor. Different inhibitors may act at the same growth sites on the crystal surface and it is possible that certain inhibitors would be bound preferentially causing a blunting of the effect of other inhibitors. If this were the case, mixtures of inhibitors would show less activity than the sum of the activities of the individual components. It is also possible that urinary components which do not affect crystal growth themselves interact with crystal growth inhibitors either to reduce or potentiate the activity of the crystal growth inhibitors.

We have measured the inhibitory activity of mixtures of known inhibitors of calcium oxalate monohydrate and hydroxyapatite crystal growth to determine if inhibitory activity is additive. We have also studied mixtures of known inhibitors with normal urine to determine if urine contains inhibitors or other compounds which either
reduce or potentiate the activity of added inhibitors or if added inhibitors affect the inhibitory activity observed for the urine.

Materials and methods

Materials

Reagent-grade chemicals were used without further purification. Heparin (from porcine intestinal mucosa, sodium salt, grade I), ribonucleic acid (from yeast, type XI) and chondroitin sulphate (from whale and shark cartilage, sodium salt, grade III) were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.). Seed crystals of calcium oxalate monohydrate and hydroxyapatite were prepared as described previously. All solutions used in the hydroxyapatite seeded crystal growth system were prepared with carbonate-free water.

Calcium oxalate monohydrate seeded growth

Inhibitory activity toward calcium oxalate crystal growth was determined by a modification of a method described previously [1]. Control runs (no inhibitors) were performed along with each series of inhibitor runs by adding 2.0 ml of CaCl₂ (0.016 mol/l) to 65.0 ml of NaCl (0.148 mol/l), equilibrating to 37°C and adding slowly with stirring 3.0 ml of K₂C₃O₄ (0.01 mol/l). The pH was adjusted to 6.0 and seed slurry added (~0.2 ml) to give a growth rate of about 600 mol min⁻¹ 1⁻¹. Portions were removed at 0, 5, 15, 30 and 50 min, filtered through a 0.22 μm Metricel filter and analysed for calcium by atomic absorption. Rate constants were calculated as described previously [1]

Each inhibitor was assayed individually to determine its potency. The concentration needed to give a 50% reduction in crystal growth rate was defined as 1 inhibitor unit (I.u.) [1]. Inhibitors were added to the supersaturated calcium oxalate assay solution before the addition of seed crystals. At least three concentrations of each inhibitor were used to calculate the inhibitory activity by fitting to the Langmuir adsorption isotherm [13]. When mixtures of inhibitors were tested, the data from the single inhibitor runs were used to make mixtures in which each inhibitor theoretically contributed an equal amount of inhibition.

The urines were first assayed for calcium and sodium concentrations and the crystal growth assay solutions adjusted to compensate for the calcium and sodium added by the addition of urine. The amount of oxalate contributed by normal urine was small enough that no compensation was needed. The inhibitory activity of each urine was measured as above and mixtures of urine and individual inhibitors tested.

All solutions were unbuffered and the pH was continuously monitored and adjusted to remain between 5.9 and 6.1. Calculations were performed as described previously by linear regression analysis [1].

Hydroxyapatite seeded crystal growth

Hydroxyapatite seeded crystal growth experiments were performed by a modification of a method described previously [12,14]. Control runs (no inhibitors) were performed by adding 4.86 ml of CaCl₂ (0.028 mol/l) and 4.88 ml of KH₂PO₄ (0.018 mol/l) to 65 ml of NaCl (0.15 mol/l), equilibrating at 37°C and adding NaOH (0.086 mol/l) to adjust the pH to 7.4. A 0.1 ml portion of hydroxyapatite seed slurry (~20 mg/ml) was added and the reaction vessel set in a pH-stat (Metrohm, Combitrator-3D). The volume of NaOH (0.086 mol/l) added to keep the pH constant was recorded for 60 min. Rate constants were calculated from the amount of base added and the known stoichiometry of the precipitation reaction [12].

Inhibitors were assayed as described above for the calcium oxalate monohydrate system. The urines used were first assayed for calcium and phosphate concentrations and the crystal growth solutions adjusted if necessary. Complexes of citrate with calcium [15] and magnesium with phosphate [16] were compensated for in all experiments to maintain constant initial supersaturation when different amounts of the inhibitor were added.

Results

Calcium oxalate monohydrate seeded growth

The inhibitory activity of urine, heparin (Hep), chondroitin sulphate (CDS), pyrophosphate (P₂O₅) and ribonucleic acid (RNA) is shown in Table 1. The glycosaminoglycans and RNA are likely a mixture of different molecular-weight species. A molecular weight of 20 000 was assumed for heparin, 50 000 for chondroitin sulphate and 140 000 for RNA. The inhibitory potency of pyrophosphate is pH dependent in this system [17] and the value presented is for pH 6.0, the pH of the assay system. These numbers represent average values obtained over a 3 year period. The exact concentration needed to obtain 1 I.u. varies slightly for heparin, chondroitin
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### TABLE 1. Calcium oxalate monohydrate crystal growth inhibitors

Results are means ± SEM; numbers of runs are shown in parentheses. For abbreviations see the text.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>I.u./l</th>
<th>Concentration for 1 I.u. (μmol/l)</th>
<th>Theoretical I.u. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine (frozen)</td>
<td>(11)</td>
<td>34 ± 4</td>
<td></td>
</tr>
<tr>
<td>RNA</td>
<td>(38)</td>
<td>0.0032 ± 0.0002</td>
<td></td>
</tr>
<tr>
<td>Hep</td>
<td>(29)</td>
<td>0.0063 ± 0.0006</td>
<td></td>
</tr>
<tr>
<td>CDS</td>
<td>(9)</td>
<td>4.8 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>P2O7</td>
<td>(20)</td>
<td>14.0 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>RNA + Hep</td>
<td>(6)</td>
<td>105 ± 7</td>
<td></td>
</tr>
<tr>
<td>RNA + P2O7</td>
<td>(5)</td>
<td>111 ± 14</td>
<td></td>
</tr>
<tr>
<td>Hep + P2O7</td>
<td>(6)</td>
<td>110 ± 4</td>
<td></td>
</tr>
<tr>
<td>RNA + Hep + P2O7</td>
<td>(7)</td>
<td>105 ± 12</td>
<td></td>
</tr>
<tr>
<td>Hep + urine (frozen)</td>
<td>(6)</td>
<td>103 ± 9</td>
<td></td>
</tr>
<tr>
<td>P2O7 + urine (frozen)</td>
<td>(6)</td>
<td>103 ± 12</td>
<td></td>
</tr>
<tr>
<td>RNA + urine (frozen)(13)</td>
<td></td>
<td>66 ± 4*</td>
<td></td>
</tr>
<tr>
<td>RNA + urine (fresh)</td>
<td>(10)</td>
<td>82 ± 1*</td>
<td></td>
</tr>
<tr>
<td>CDS + urine (fresh)</td>
<td>(6)</td>
<td>107 ± 12</td>
<td></td>
</tr>
</tbody>
</table>

* Different from 100%, P < 0.05.

### TABLE 2. Hydroxyapatite crystal growth inhibitors

Results are means ± SEM; numbers of runs are shown in parentheses. For abbreviations see the text.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>I.u./l</th>
<th>Concentration for 1 I.u. (μmol/l)</th>
<th>Theoretical I.u. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine (frozen)</td>
<td>(6)</td>
<td>25 ± 3</td>
<td></td>
</tr>
<tr>
<td>P2O7</td>
<td>(13)</td>
<td>1.04 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>Cit</td>
<td>(12)</td>
<td>12.3 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>Mg</td>
<td>(10)</td>
<td>170 ± 9</td>
<td></td>
</tr>
<tr>
<td>Mg + Cit</td>
<td>(6)</td>
<td>106 ± 3</td>
<td></td>
</tr>
<tr>
<td>P2O7 + Cit</td>
<td>(6)</td>
<td>113 ± 5</td>
<td></td>
</tr>
<tr>
<td>P2O7 + Mg</td>
<td>(7)</td>
<td>103 ± 5</td>
<td></td>
</tr>
<tr>
<td>Mg + Cit + P2O7</td>
<td>(8)</td>
<td>109 ± 9</td>
<td></td>
</tr>
<tr>
<td>Mg + urine (frozen)</td>
<td>(6)</td>
<td>109 ± 5</td>
<td></td>
</tr>
<tr>
<td>P2O7 + urine (frozen)</td>
<td>(5)</td>
<td>101 ± 3</td>
<td></td>
</tr>
<tr>
<td>Cit + urine (frozen)</td>
<td>(6)</td>
<td>94 ± 5</td>
<td></td>
</tr>
</tbody>
</table>

sulphate and RNA with supplier and batch. In these experiments with mixtures of inhibitors, the actual measured inhibitory activity of each component was used in the calculations. Each mixture was assayed in at least two separate series of three or four different concentrations each. Mixtures were prepared so that each component contributed an equal amount of inhibition to obtain maximum sensitivity to non-additive effects and to simplify data analysis. The results are expressed as percentages of the theoretical sum of the activities of the components added, based on their individual activities determined under similar conditions. The results are presented as means ± SEM. Heparin plus urine and pyrophosphate plus urine showed additivity of inhibitory activity. The mixture of RNA and urine (stored frozen) showed a reduced activity compared with the sum of the RNA and urine activity. These experiments were repeated on a fresh urine pool to eliminate any artifacts that may have been introduced by the use of frozen samples of urine. Again with fresh urine a reduction in activity was observed. Experiments were also carried out with chondroitin sulphate mixed with fresh urine and the inhibitory activity was additive. Since normal urine contains ribonuclease activity [18, 19], the lower activity of the urine/RNA mixtures could be due to destruction of RNA by urinary ribonuclease during the course of the inhibitor assay. We tested this hypothesis by incubating urine/RNA mixtures under the same conditions as the inhibitor assay, but without seed crystals. Portions were removed periodically and 10% (v/v) perchloric acid added to precipitate any intact RNA [20]. The solutions were decanted and A260 read. Absorbance increased with time, demonstrating that RNA was being hydrolysed in the present study. This indicates that RNA could have been hydrolysed during the course of the inhibitor assay and inhibitory activity lost [6].
Hydroxyapatite seeded growth

The inhibitory activity for urine, pyrophosphate (P$_2$O$_5$), citrate (Cit) and magnesium (Mg) is shown in Table 2. None of the mixtures tested have inhibitory activity different from the theoretical sum of their components.

Discussion

There has been much interest in recent years in the compounds responsible for the inhibitory effect of urine on crystal growth [21]. The insights gained from the identification of naturally occurring urinary inhibitors of calcium oxalate monohydrate and hydroxyapatite crystal growth may have important implications for the investigation of the genesis and treatment of urinary stones. Most studies involve isolation of urinary components and then testing these compounds for inhibitory activity. Compounds isolated from urine that are potent inhibitors may be present in urine in such small quantities that they contribute little to the total inhibitory activity. To calculate the contribution of an isolated urinary component two assumptions must be made; first, that the urine in the test system acts the same as in the urinary tract, and secondly that the test system exhibits additivity toward mixtures of compounds. The first assumption is difficult to test, since most inhibitor assay systems test a diluted urine [22]. Dilution of urine is necessary in most cases since the concentration of inhibitors in urine is generally high enough to slow crystal growth to rates which make kinetic measurements difficult or impossible. It may be necessary to work with diluted urine, realizing that the situation in the body may be different. We have tested the second assumption in two seeded crystal growth systems.

The studies presented here demonstrate that, with the exception of RNA/urine mixtures, inhibitory activity is additive in the seeded crystal growth systems used. Our preliminary experiments with perchloric acid precipitation of intact RNA after incubation of RNA/urine mixtures suggest that the reduction in inhibitory activity of RNA/urine mixtures may be due to destruction of added RNA by urinary ribonuclease. We cannot rule out the possibility that RNA/urine mixtures do not show additivity of inhibition, but, since all other mixtures of RNA and inhibitors do show additivity, it seems unlikely that RNA/urine mixtures are non-additive. Under the conditions of our inhibitor assay system, RNA-like compounds naturally occurring in urine are not destroyed by urine ribonuclease probably because they are somewhat degraded already [23] and are not as reactive toward the naturally occurring ribonuclease. However, under appropriate conditions, RNA-like compounds in urine can be hydrolysed by added ribonuclease causing a reduction in the observed calcium oxalate monohydrate crystal growth inhibition of the urine [23, 26].

It is possible to draw some preliminary conclusions based on the results of the present study. The frozen urine used for the mixed-inhibitor study was analysed for pyrophosphate, citrate and magnesium. Inhibitor activity due to pyrophosphate accounted for 36% of the measured hydroxyapatite inhibitor activity of the urine. Citrate accounted for 2% and magnesium accounted for 7% of the hydroxyapatite inhibitor activity in this urine. Pyrophosphate has previously been reported to be a major urinary inhibitor in dilute urine, but of minor importance in whole urine [21]. In the calcium oxalate monohydrate system, pyrophosphate appears to contribute only a small amount to the inhibition of the urine. Since it is unlikely that the glycosaminoglycans and RNA commercially available are identical with those found in urine, the contribution of these compounds to the total inhibition cannot be assessed without characterizing them in urine. It is important to stress that our results apply only to diluted urine, as measured in the seeded crystal growth systems and that it may not be possible to extrapolate the results obtained to whole urine or to other inhibitor assay systems. Although inhibitors of crystal aggregation may play a role in kidney stone formation [21, 24, 25], the assays used in our work observe only effects on crystal growth.

The formation of stones within the urinary tract is dependent on multiple factors including nucleation, growth, aggregation, dissolution and retention [4, 24]. Since urinary stones are generally crystalline masses [26], any compound which retards or prevents crystal growth has obvious importance. The results presented here make it possible for the first time to assess quantitatively the importance of known and unknown urinary compounds as inhibitors of calcium oxalate monohydrate and hydroxyapatite crystal growth.

Acknowledgments

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References


