Evaluation of a Routine Method for Determination of Calcium Oxalate Crystal Growth Inhibition in Diluted Urine Samples

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We have evaluated a routine method for determination of the inhibition of calcium oxalate crystal growth in diluted urine samples. A crystallization inhibition index is calculated from the decrease in $[^{14}\text{C}]$oxalate concentration in a metastable solution of calcium chloride and sodium oxalate after addition of seed crystals of calcium oxalate. The coefficient of variation between different determinations on the same sample is <1.5%, and there is good correspondence between indices obtained by this method and by a more laborious older method. We also studied the influence of different urinary constituents on the crystal growth rate in the system; whereas there was significant inhibition with citrate, chondrin sulfate, and pyrophosphate, no effects were observed with ordinary urinary concentrations of calcium, magnesium, and oxalate. The mean inhibition index was lower in a group of male patients with calcium oxalate stone disease than in a control group of normal men.

Additiona l Keyphrase: radioassay

In urine supersaturated with respect to calcium oxalate, there is an increased risk for formation of calcium oxalate crystals with subsequent development of urinary concrements. Robertson et al. (1) demonstrated that patients with recurrent calcium stone disease could be distinguished on a biochemical basis, by means of a saturation-inhibition index.

Factors thought to be important for the formation of calcium oxalate concrements are calcium, magnesium, oxalate, citrate, and inhibitors of crystallization (2-4). Whereas most clinical laboratories can analyze for calcium, magnesium, oxalate, and citrate, analysis of the crystallization inhibitory activity of urine may present considerable difficulties. Even where the equipment is available, the methods described are often complicated and time consuming, so that the analyses can be performed only on a very limited number of samples. As emphasized by Boyce and Resnick (5), it is therefore necessary to develop simple but accurate standardized methods for the assessment of stone formers. With the increasing number of patients on different forms of stone-prophylactic therapy, it is important to have methods that will allow a biochemical evaluation of the urine.

We present a method for estimating the inhibition of calcium oxalate growth in diluted urine. The method, an adaptation of the method described by Robertson et al. (6), was designed to fulfill the requirements of a rapid routine method that is applicable to large numbers of urine samples.

Materials and Methods

Preparation of Urine

Twenty-four-hour urine specimens, collected with no preservative, in plastic bottles, were promptly brought to the laboratory and, if not analyzed without delay, a suitable aliquot of the thoroughly mixed specimen was taken and frozen.

Before analysis each sample was warmed to 37 °C for at least 30 min, carefully mixed, diluted with 0.15 mol/L sodium chloride so as to contain 5 μmol of creatinine per milliliter, then filtered through a 0.22-μm filter (GS; Millipore Corp., Bedford, MA 01730). Excessive bacterial contamination was excluded by means of a Uriglox-test (Kabi Diagnostica, Stockholm, Sweden).

Usual Method

The composition of the system used is essentially as described by Robertson et al. (6), where the growth of calcium oxalate crystals was monitored in 50 mL of a metastable solution consisting of, per liter, 1 mmol of calcium chloride, 0.2 mmol of sodium oxalate, and 10 mmol of sodium cacodylate buffer adjusted to pH 6.0. A constant ion strength was maintained by addition of sodium chloride to the system to give a concentration of 0.15 mol/L. $[^{14}\text{C}]$Oxalate was added to give a final isotope concentration in the crystallization system of 5 μCi/L.

We prepared a suspension of calcium oxalate monohydrate seed crystals by adding 100 mg of the crystals (Merck, A.G.) to 100 mL of distilled and Millipore-filtered water. A homogeneous suspension was obtained by ultrasonication. The crystal suspension was carefully mixed at 37 °C and stored at this temperature for at least 24 h before being added to the crystallization system.

Crystal growth was monitored by determining the amount of $[^{14}\text{C}]$oxalate remaining in solution at different intervals during the first 2 h after the seed crystals were added to the system. The solution was kept in a water bath at 37 °C with continuous magnetic stirring. Samples were withdrawn at 30, 60, 90, and 120 min, passed through Millipore filters, and measured for radioactivity.

Figure 1A illustrates the rate of crystal growth in the crystallization system, with and without addition of urine, as reflected in the decreased concentration of $[^{14}\text{C}]$oxalate in solution.

Rapid Method

A metastable solution as described above was prepared immediately before use and filtered through a 0.22-μm filter.

To 100 mL of this solution was added 1 mL of a solution containing 0.5 μCi of sodium $[^{14}\text{C}]$oxalate (The Radiochemical Centre, Amersham, England); 5 mL of this mixture was placed in a plastic tube. To each such tube was added either 100 μL of 0.15 mmol/L sodium chloride (control sample) or 100 μL of a urine solution, prepared as described above. Then 200 μL of the 1 g/L suspension of seed crystals was added to each tube. The radioactivity in each tube was thus 25 nCi.

The tubes were closed with tight-fitting caps and placed horizontally in a shaking apparatus (E. Bühler, Model SM-B-1) for exactly 2 h at 37 °C. The samples were then either centrifuged for 10 min at 2500 rpm or filtered through 0.22-μm filters.

From each supernate or filtered solution, 500 μL was taken for determination of radioactivity with a liquid scintillation...
Fig. 1. Decrease in $[^{14}C]$oxalate concentrations in systems containing urine (O) and 0.15 mol/L sodium chloride (□): Usual method (A) and rapid method (B)

spectrometer. A blank sample containing 5 mL of the metastable solution with $[^{14}C]$oxalate and 300 μL of 0.15 mol/L sodium chloride was prepared for use in determining the zero-time isotope concentration.

Urine and control samples were all run in duplicate.

Effect on Rate of Crystal Growth

To study the effect of different urinary constituents on the crystallization process, we added 100 μL of the following solutions to 5 mL of the crystallization medium: calcium chloride (5 and 10 mmol/L), sodium oxalate (0.2 and 0.4 mmol/L), magnesium chloride (0.1 to 3 mmol/L), sodium pyrophosphate (2 to 50 μmol/L), and chondroitin sulfate (0.1 to 50 mg/L). Furthermore, we tested the effects of acid by adding 100 μL of hydrochloric acid (0.01, 0.1, and 0.5 mol/L) to give final concentrations of 0.2, 1.9, and 9.4 mmol/L in the crystallization system.

Effect of Storage on Inhibiting Activity

Urine was stored at room temperature and then at −20 °C for different intervals during the first 32 h or stored at −4 °C for a week. Specimens of urine that had been frozen immediately after collection were also analyzed after storage for different intervals.

Isotope Exchange

Equilibrated solutions of calcium oxalate, $[^{14}C]$oxalate, and calcium oxalate crystals and equilibrated solutions of calcium chloride and calcium $[^{14}C]$oxalate crystals were analyzed with respect to exchange of $[^{14}C]$oxalate.

Calculations

As previously described (7–10), the growth of calcium oxalate crystals follows a second-order differential equation:

$$-dOx/dt = k(Ox - Ox_{∞})$$

where $Ox_{∞}$ is the concentration of oxalate remaining in solution at infinity, corresponding to the solubility of calcium oxalate in the system used.

Integration gives the following equation:

$$(Ox - Ox_{∞})^{-1} = k \cdot t + A$$

where A is a constant of integration.

Giving Ox the value 100 at $t = 0$ (the starting point of crystal growth), one calculates the rate constant as follows, where $Ox_t$ is the percentage of oxalate in solution at time $t$:

$$k \cdot t = [1/(Ox_t - Ox_{∞})] - [1/(100 - Ox_{∞})]$$

From the control values ($C_1$ and $C_2$) in Figure 1B the percentage of isotope remaining in solution at infinity ($Ox_{∞}$) was calculated as follows:

$$Ox_{∞} = (200C_2 - 100C_1 - C_1C_2)/(100 - 2C_1 + C_2)$$

After which the rate constant for the control curve ($k_c$) was obtained ($t$ expressed in hours):

$$k_c \cdot t = (100 - C_1)/[(C_1 - Ox_{∞})(100 - Ox_{∞})]$$

Fig. 2. Comparison of inhibition indices obtained by the rapid method (A) and the usual method (B)

Each index was calculated as the mean of four different determinations during the first 2 h
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<td>Mean 0.65 (SD 0.01)</td>
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By means of the results from equations 4 and 5, the rate constant for the urine curve (U) was calculated accordingly:

\[ k_u \cdot t = \frac{(100 - U_t)}{(U_t - O_{u})(100 - O_{u})} \]  

(6)

Finally, the inhibition index (I) was determined as previously described (9):

\[ I = 1 - \left( \frac{k_u}{k_0} \right) \]  

(7)

Inhibition Index in Stone-Formers

The inhibition index was measured in 24-h urines collected from 169 men patients with urolithiasis and 98 normal men without known stone-disease.

**Results**

In Figure 2 we compare inhibition indices obtained by use of the rapid method with the inhibition index based on four different determinations during the first 2 h with the usual method. The agreement between the two methods was generally good, although some values are divergent.

Multiple determinations of the inhibition index of two urine samples were performed and, as is evident from Table 1, the inhibition index for the two urine samples ranged from 0.63 to 0.66 to 0.68, respectively. This corresponds to a coefficient of variation of less than 1.5% in both series.

In Figure 3 we compare radioactivity measured after centrifugation or after Millipore filtration. There was a good correspondence (r = 0.89).

Addition of 100 µL of 0.2 or 0.4 mmol/L sodium oxalate or of 5 or 10 mmol/L calcium chloride did not measurably increase crystal growth rate, nor was there any notable effect on the inhibition when magnesium chloride was added to give final concentrations as high as 60 µmol/L.

The effects of sodium pyrophosphate, chondroitin sulfate, and citrate are shown in Figure 4. Sodium pyrophosphate in a final concentration of 19 µmol/L completely inhibited crystal growth.

Addition of HCl to a final concentration of 0.19 to 1.9 mmol/L did not affect the crystal growth, whereas 9.4 mmol/L stopped it.

Storage of urine at room temperature resulted in a slight decrease in the inhibition index, so slight as to be negligible during the first 24–32 h. When the urine was stored frozen, the inhibition index remained unchanged for several weeks.

Bacterial contamination significantly increased values for the inhibition index.

Figure 5 illustrates the cumulative frequency distribution curves of inhibition indices for normal men and men who are stone formers. The mean inhibition indices were 0.56 (SD 0.06) and 0.54 (SD 0.08), respectively; the difference is statistically significant (p <0.05).

**Discussion**

There have been several reports of decreased inhibition of calcium oxalate crystal growth (11–14) and of calcium oxalate crystal growth and aggregation (1) in urine from patients with urolithiasis as compared with normal subjects. In evaluating and treating patients with calcium oxalate stone disease, it...
appears to be of fundamental importance to get information on the inhibiting properties of the urine. Several methods have been described for doing so. Some techniques are designed to measure the inhibition in untreated urine samples (11, 15), in which the result is a combined effect of the supersaturation of the urine and the inhibiting activity. For the study of only the effects of crystallization inhibitors, systems involving diluted urine have been described, the dilutions usually used being 20-fold or greater. In the method described by Robertson et al. (6) crystal growth and aggregation were measured simultaneously, whereas other methods (7, 9, 14, 16) were developed for determining only crystal growth rate. Most of these methods are complicated and time consuming and often require expensive special equipment, which considerably limits their usefulness in the routine clinical laboratory.

With the rapid, routine method we describe, 24-h urine is diluted to contain a fixed concentration of creatinine (5 mmol/L), because it has previously been demonstrated (9, 16) that inhibiting activity and urinary creatinine are correlated. Correcting for this correlation also makes it easier to compare the inhibition index with other information on urine composition (3) and with the inhibitory activity as measured on other occasions in urines of various native concentrations.

This initial dilution of a urine specimen to a fixed creatinine concentration and the subsequent dilution in the crystallization medium result in a 75- to 100-fold dilution of the original urine. As is evident from our results for addition of calcium and oxalate to the system, it is unnecessary to correct for these additions to the system with the urine sample when the concentrations of these substances in the samples are normal or slightly above normal, as is usually found in stone formers.

The crystals formed in the system can be separated either by filtration through Millipore filters or by centrifugation. Although the correspondence between the two methods was good, slightly lower values for the inhibition index are obtained when centrifugation is used, probably attributable to turbulence in the tube. We therefore prefer the use of Millipore filtration, believing that it gives more nearly accurate results.

To avoid loss of inhibiting activity in the sample, we recommend that urine samples to be used for inhibition analysis be frozen in small aliquots, because repeated thawing and freezing may be deleterious to the inhibitory components. Furthermore, the urine should be frozen as soon as possible after the collection is completed. It might well be possible to analyze the inhibitory activity in untreated urine samples, treated in the same way as the 24-h urine.

The inhibition indices were estimated for stone-forming and normal men, and, as shown in Figure 5, the difference between the two groups is small but statistically significant. This differs slightly from our previously presented results (4) where the difference between stone formers and normal subjects did not reach statistical significance. In both stone-formers and normal subjects, there was a wide range of values; with a rapid method for assessing the inhibiting activity we believe that risk of stone-formation by an individual patient can be more accurately evaluated.

It would be desirable to obtain information on the inhibitory activity in untreated urine samples (17), but as yet no such method is available. In any case, such methods will probably be inconvenient for routine purposes.

The present method is simple, rapid, and standardized to be useful in the routine biochemical investigation of patients with urolithiasis. It requires a minimum of laboratory equipment.

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References