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The Direct Measurement of Inhibitory Capacity to Crystal Growth of Calcium Oxalate in Undiluted Urine and in Other Inhibitor Containing Solutions

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Summary. This paper describes a simple method to measure the capacity of undiluted urine and of other inhibitor containing solutions (PPi and EHDP) to protect a given mass of calcium oxalate crystals from growth. The method has also been used to determine relative urinary saturation with respect to calcium oxalate. It is based on titration with oxalate and measures the critical changes of concentration necessary to reach saturation or to induce crystal growth. From these changes inhibitory capacity as well as the level of urinary saturation can be calculated in terms of differences of concentration products. The use of the methods at present available to measure urinary oxalate are thereby avoided. In order to compare the results from different urines without the need for cumbersome calculations of activity products we have introduced a saturation-inhibition ratio.

Key words: Inhibitors - Urinary saturation - Oxalate - Calcium stones - Pyrophosphate - Diphosphonate.

INTRODUCTION

Urinary calcium stone formation seems to be the result of crystallisation processes occurring in urine which is supersaturated with stone forming minerals. On the one hand these crystallisation processes are enhanced by the degree of supersaturation and by the presence of nucleators. On the other hand they are opposed by the action of crystallisation inhibitors (4). Multiple disorders may initiate calcium stone formation by disturbing the equilibrium between promoting and protecting factors (1). Such a disequilibrium has been measured by the determination of a saturation-inhibition index in calcium oxalate stone formers and some correlation was found between its level and the incidence of stone recurrence (8). Unfortunately this procedure is too complicated to be carried out in the routine clinical laboratory.

Exact determination of urinary saturation requires the measurement of 9 important ligands and computer analysis of 22 complexes (7). An easier approach is to compare concentration products of stone forming minerals before and after equilibration with the solid phase in excess (6). The problems relating to the measurement of crystallisation inhibitors have been discussed elsewhere (2). Most of the current test systems will not tolerate the addition of urine in concentrations of more than 3%. They can hardly reflect physiological conditions because the correlation between concentration and inhibitory activity can be dissimilar in different inhibitors. Furthermore the effect of substances with weak inhibitory activity may be lost by high dilution. The lack of a satisfactory and clinically practical method for the measurement of oxalic acid in urine is another handicap in calcium stone research. This paper describes a simple method to study crystallisation conditions for calcium oxalate in undiluted urine and control solutions without the need to measure oxalic acid.

MATERIAL AND METHODS

1. Chemicals

All chemicals were of analytical grade. Ethane-1-hydroxy-1,1-diphosphonate (EHDP) was obtained from Procter and Gamble Comp., Cincinnati, Ohio, USA, sodium cacodylate and sodium azide from Fluka AG., Buchs SG, Switzerland and all remaining chemicals from Merck, W-Germany.

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2. Solutions

A basic solution of 0.15 M sodium chloride buffered with 5 mM sodium cacodylate at pH 6.0, containing 0.02% NaN_3 as preservative, was prepared. With this basic solution all the following solutions were made.

a) Test solutions (A) containing 1.22 mM $CaCl_2$ and either pyrophosphate in concentrations ranging from 0.5 - 5.0 x 10^{-5} M or EHDP in concentrations from 2.5 - 10 x 10^{-6} M.

b) Sodium oxalate solutions (B) with concentrations ranging from 0 - 61 mM.

c) Calcium oxalate crystal suspension (C). 2 suspensions of calcium oxalate monohydrate crystals, one containing 2.04 mg/ml, the other containing 500 mg/ml were made up in measuring cylinders. An homogeneous suspension was achieved by vigorous stirring and the suspensions were then equilibrated on a midget shaker for 2 hours.

3. Urine

All the portions of a 24 hour period were frozen immediately after voiding. For reconstituting a 24 hour urine the portions were thawed and equal fractions of the voided volumes were mixed and NaN_3 was added as preservative in a final concentration of 0.02%. The urine was alkalinized to pH 6.7 with NaOH to dissolve precipitated urate and centrifuged. The calcium salts in the sediment were dissolved by incubation with 2 N HCl (1/100 of the reconstituted urine volume) until microscopy had shown that the crystals had disappeared. The sediment was then added to the supernatant while the pH was maintained between 6.0 and 6.7). Finally the urine was adjusted to pH 6.0 and recentrifuged for 10 minutes at 2500 rpm to eliminate mucus and cells.

4. Measurement of Crystal Growth

a) Calcium oxalate crystals 0.02 mg/ml. 1.0 ml aliquots of either solution A or of 24 hour urine (prepared as above) were supersaturated with respect to calcium oxalate in 5 ml polystyrol test tubes by adding 0.01 ml of solution B. Crystal growth was induced by the addition of 0.01 ml of the well mixed 2.04 mg/ml calcium oxalate crystal suspension. The samples were incubated on a midget shaker and after 90 minutes the crystals were removed by centrifuging the samples at 12000 rpm for 3 minutes. The calcium concentrations in the supernatants of the samples and in blanks (containing 1.0 ml of test solution A or urine and instead of solutions B and C 0.02 ml of the basic solution) were then determined on a Corning Calcium Analyzer, Model 940.

b) Calcium oxalate crystals 5 mg/ml. The test was performed in the same manner as described above but with volumes of 2.0 ml of solution A or urine, 0.01 ml of solution B and 0.02 ml of the crystal suspension (500 mg/ml). After incubation for 24 hours the crystals were removed by twofold centrifugation.

5. Calculations

Using the numerous calcium measurements in both the blanks and the samples the Ca_t values were statistically evaluated for significant changes from Ca_i by the formula: $\bar{x}\ Ca_i \pm 2\ SE \neq \bar{x}\ Ca_t \pm 2\ SE$ (Ca_i = calcium concentration of the blank, Ca_t = calcium concentration in the supernatant after incubation). At least 3 consecutive Ca_t measurements which were significantly different from Ca_i were plotted on the Y-axis against their corresponding oxalate additions on the X-axis. From this the linear regression line was calculated. The critical change of oxalate concentration (= $\Delta 0x$) at which no change in the initial calcium concentration had occurred after incubation, was calculated by plotting the $\bar{x}\ Ca_i$-value on the regression line (Figs. 1 and 2). With the critical $\Delta 0x$-value for 5 mg/ml crystals (=$\Delta 0x_5$) the degree of urinary saturation was calculated by the formula: $Ca_i\ (-\Delta 0x_5)$. This represents the difference between the actual calcium x oxalate product and the one after saturation with 5 mg/ml crystals. A positive value expresses supersaturation, a negative value undersaturation. The inhibitory activity was defined as the maximum supersaturation at which the growth of crystal mass of 0.02 mg/ml was prevented and was expressed by the formula: $(\Delta 0x_{0.02} - \Delta 0x_5)\ Ca_i$; in which $\Delta\ 0x_{0.02}$ represents the critical oxalate value after the incubation with 0.02 mg/ml crystals. To compare the results obtained from different urines a saturation-inhibition ratio was established by the formula: $-\Delta 0x_5 (\Delta 0x_{0.02} - \Delta 0x_5)^{-1}$.

RESULTS

The measured final calcium concentrations plotted against the concentration of oxalate added to the test system prior to incubation showed a linear relation in test solutions (Fig. 1a and b) and in urine (Fig. 2). This linear function allows an exact determination of the critical oxalate addition at which no measurable change in the test system occurs. Equilibration of solutions containing high concentrations of Inorganic Pyrophosphate (PPi) and EHDP, and of solutions without inhibitors with the addition of 5 mg/ml calcium oxalate crystals showed that a high crystal concentration is able to eliminate the influence of inhibitors (Fig. 1a) which is in agreement with other observations (6). This procedure was therefore used to determine the point of saturation

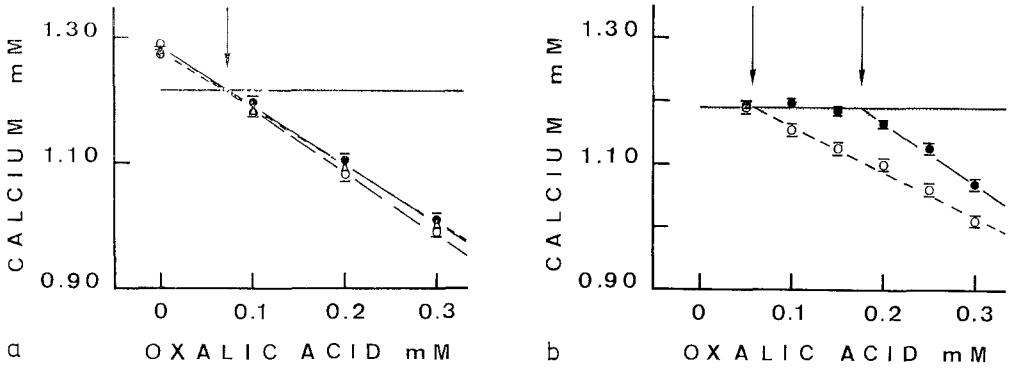

Fig. 1. a The final calcium concentrations ($\bar{x} \pm 2$ SE) in the supernatant after 24 hours of incubation with 5 mg/ml calcium oxalate crystals are plotted against the initial oxalate additions of the test system. The critical oxalate addition (⟶) at which no significant change of the initial calcium concentration (┈┈ = $\bar{x} \pm 2$ SE) occurs shows no difference between solution with 2.5×10^{-5} M PPi (●), 10^{-5} M EHDP (Δ) and without an inhibitor (O) (n = 7). b The final calcium concentrations ($\bar{x} \pm 2$ SE) in the supernatant after 90 minutes of incubation with 0.02 mg/ml calcium oxalate crystals are plotted against the initial oxalate additions to the test system. The maximum oxalate addition (⟶) at which no significant change of the initial calcium concentration ┈┈ = $\bar{x} \pm 2$ SE) occurs is more than twice elevated in a solution containing 2.5×10^{-6} M EHDP (●) compared to the inhibitor free control (O) (n = 17)

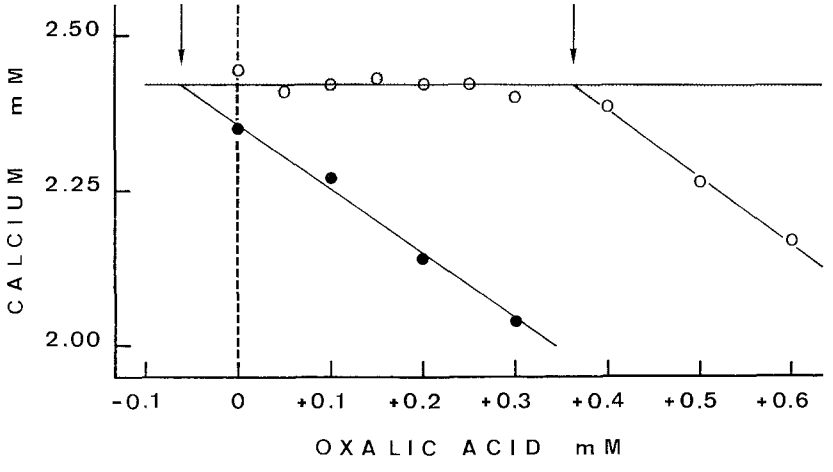

Fig. 2. Final calcium concentration in urine of a healthy man after 24 hours of incubation with 5 mg/ml calcium oxalate (●) and after 90 minutes of incubation with 0.02 mg/ml crystals (O) are plotted against initial oxalate additions. Other details see Fig. 1. After equilibration with 5 mg/ml calcium oxalate a negative value for the critical oxalate (⟶) indicating supersaturation can be extrapolated. The great difference between the critical oxalate values for 5 mg/ml and 0.02 mg/ml calcium oxalate shows a high inhibitory capacity of urine with respect to crystal growth

with respect to calcium oxalate. In urine which is generally supersaturated with respect to calcium oxalate the critical oxalate values are negative and have to be calculated by extrapolation (Fig. 2).

Incubation of test solutions with crystals in low concentration (0.02 mg/ml) revealed that in the presence of crystallisation inhibitors the critical oxalate addition and thus the critical calcium x oxalate product necessary to initiate a measurable crystal growth were elevated far above the corresponding values for saturation (Fig. 1). A similar inhibitory effect was found in urine (Fig. 2). Physiological concentrations of PPi are able to prevent crystal growth up to 6 times the concentration product found in solutions without inhibitors (Fig. 3). The inhibitory effect of PPi reaches a maximum with a concentration in excess of 2.5×10^{-5} M. When the incubation time was prolonged from 90 minutes to 24 hours all critical calcium x oxalate products which were above the value for spontaneous nucleation measured after 24 hours of incubation markedly decreased. This effect was probably due to an increase of crystal mass in the system by spontaneous nucleation. All calcium x oxalate products necessary for crystal growth during 90 minutes of incubation were below the corresponding value for spontaneous nucleation. Under these condi-

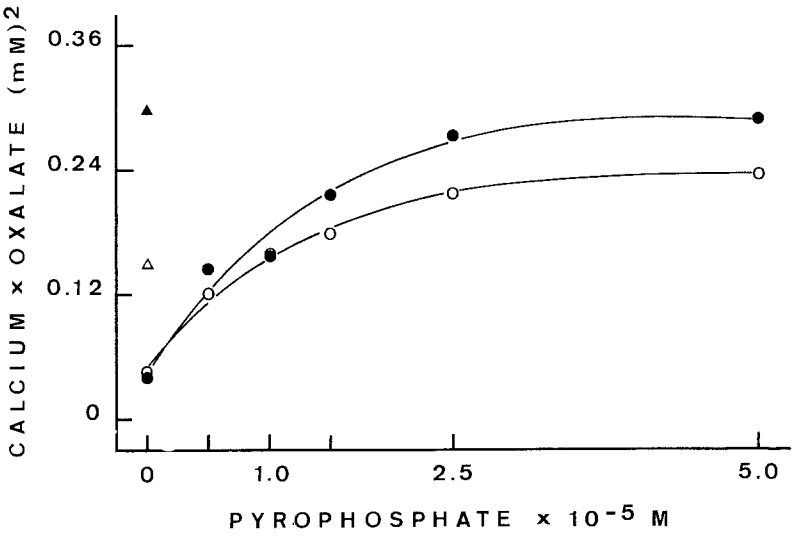

Fig. 3. Influence of $0 - 5.0 \times 10^{-5}$ M PPi on the maximum calcium x oxalate concentration product at which no measurable crystal growth of 0.02 mg/ml calcium oxalate takes place during 90 minutes (●) and 24 hours (O) of incubation. The minimum concentration products necessary for spontaneous precipitation of calcium oxalate in solutions without inhibitors are indicated by (▲) for 90 minutes and by (Δ) for 24 hours of incubation

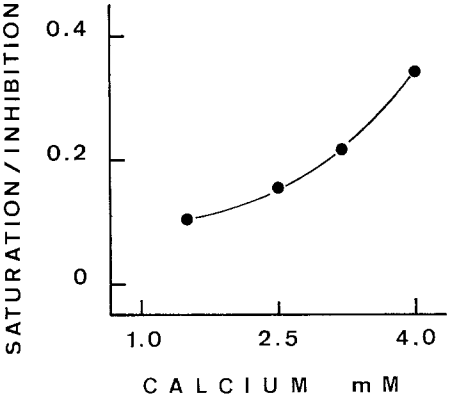

Fig. 4. Influence of the calcium concentration on the saturation-inhibition ratio in urine of a healthy man. Before performing the test increasing amounts of $CaCl_2$ were given to a urine with a low calcium concentration

tions the test system seems to measure preferentially heterogenous nucleation and crystal growth. The reproducibility of the method was evaluated with a small dose of EHDP which is, unlike physiological inhibitors, an extremely stable substance. In 17 runs performed on 5 different days (Fig. 1b) a coefficient of variation of 4.5% was observed.

In a preliminary study urine from healthy men showed a saturation-inhibition ratio of $\bar{x} = 0.19 \pm 0.03$ SE, (n = 10). The effect of increasing urinary calcium known to be one of the most important stone predisposing factors, was tested by the addition of $CaCl_2$ to a urine with an initially very low calcium content. A marked increase of the saturation-inhibition ratio was found (Fig. 4).

DISCUSSION

Since non-stone formers also sometimes show crystalluria, urinary crystallisation inhibitors must be unable to prevent crystal formation at high urinary supersaturation. A physiologically more relevant effect seems to be their potent inhibition of crystal growth (2), so that the tendency for large crystals to be trapped in the renal tubules with the consequence of stone formation is avoided (3). The method described in this paper measures the capacity of undiluted urine and other inhibitory solutions to protect a given crystal mass of calcium oxalate from growth. The level of urinary saturation with respect to calcium oxalate, another important factor in stone formation (8), is also determined. By titration with oxalic acid the critical changes of oxalate concentration necessary to reach saturation or to induce crystal growth are measured. From the critical change necessary to reach equilibration with the solid phase in excess, the initial level of saturation can be expressed as the difference between actual calcium x oxalate product and that product at saturation without knowing the initial oxalate concentration. Inhibitory activity also can be expressed without recourse to problematic analysis of urinary oxalate as a difference between the critical calcium x oxalate products for inhibition and for saturation. This value represents the maximum supersaturation above which a measurable crystal growth begins.

However, crystallisation processes are controlled by the thermodynamic state of supersaturation, which is calculated from differences of ionic activities but not ionic concentrations (5). Results from different urines expressed in terms of concentration products can hardly be compared, because ionic activity depends not only on ionic concentration but also on the effect of various

complexors and of ionic strength. The influence of these latter two factors is most difficult to determine (7). Measurement of activity products by different methods and the determination of concentration products show great variations (6). However, whatever method is used, a fairly constant ratio is found between the actual state of saturation and the value obtained after equilibration with a solid phase. With respect to calcium oxalate this observation was valid for urines with a calcium concentration below 5 mM and an oxalate concentration below 0.5 mM. Under these conditions the use of a ratio seems in part to eliminate two sources of error. One concerns activity products and is mainly due to the lack of an established method for their determination. The other relates to the neglection of chelating phenomena and ionic strength in evaluating concentration products. According to the rules of proportionality the same correcting effect must occur when the saturation-inhibition ratio is calculated from the difference of concentration products as described in this paper. The possible clinical importance of this ratio in estimating the risk of stone formation and in controlling the efficiency of metaphylaxis is now under investigation.

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