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Rapid method of measuring the inhibition of calcium-oxalate monohydrate growth in urine

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Summary. In order to have a rapid method of measuring the inhibition of calcium-oxalate monohydrate growth in freshly voided whole urine, a test system by Meyer and Smith that has originally been developed for diluted urine was modified. The crystallization processes were monitored by an ion-selective calcium electrode, which allowed determination of the half-life value of the decrease in calcium within 25 min. Even given the high inhibitory activity of whole urine, the test gave reliable results when a high seed concentration was used. Inhibition was expressed as the ratio between the half-lives of the calcium decrease obtained in the presence and in the absence of inhibitors. This approach allowed kinetic studies of individual inhibitors in model solutions. Furthermore, the measurements of inhibitors in urine could be performed before the chemical composition was determined.

Key words: Inhibitors – Calcium-oxalate monohydrate growth – Pyrophosphate – Citrate – Urine

Stone formation is generally attributed to crystallization processes in supersaturated urine, processes which are influenced by promoters and inhibitors. The state of urinary supersaturation can be calculated by computer programs, initially based on the calculation of 23 complexes and in the last version, of 103 complexes [1]. The physicochemical implication of inhibitors seems to be even more complicated. The number of known urinary inhibitors is increasing. However, their influence on stone formation is still ill-defined. Studies of the effect of individual inhibitors as well as of whole urine in crystallization tests are therefore mandatory for stone research.

Many sophisticated test systems have been developed, but only a part has been applied in clinical research. Most systems are time consuming and do not allow a larger series of analyses to be performed. Results are often not expressed in the terms generally accepted in physicochemistry and are therefore not directly comparable with results obtained by other systems [3]. Several methods and even the sophisticated ones [9, 10] do not tolerate the addition of more than 20% of urine. However, results obtained in diluted urine do not always reflect the conditions in whole urine [7]. Furthermore, inhibitor measurements should be performed in freshly voided urine. Substances to prevent bacterial growth may influence the crystallization processes. Freezing enhances the precipitation of the crystals that absorb inhibitors. Macromolecular inhibitors may be altered by dissolving of crystals or are lost by centrifugation, filtration and even sieving urine [8].

Meyer and Smith have developed a kinetic test to measure the inhibition of calcium-oxalate growth by natural urinary inhibitors and by diluted urine [3]. These authors induce crystallization with a low seed concentration of 0.0625 mg/ml and work with an equimolar calcium-oxalate ratio and an initially constant supersaturation that only tolerates a maximal urine addition of 12%. From periodic measurements of the calcium concentration in the filtered solution, a crystal growth constant is extrapolated. Apart from the nonphysiological calcium-oxalate ratio, the main drawback of the test system seems to be the fact that crystallization is already blocked by the low urine concentrations (97% inhibition by only 3% of urine [2]. Based on this test system, therefore, we can hardly come to a conclusion with respect to the situation in whole urine. In order to measure the urinary inhibitor capacity in native whole urine, we introduced the following modifications:

1. To overcome the high inhibition capacity of whole urine, the seed concentration was increased up to 1 mg/ml.
2. When working with urine, variable initial supersaturations or calcium and oxalate concentrations were used.
3. Crystallization was monitored by an ion-specific calcium electrode.
4. Results were expressed as the half-life values of the calcium decrease.

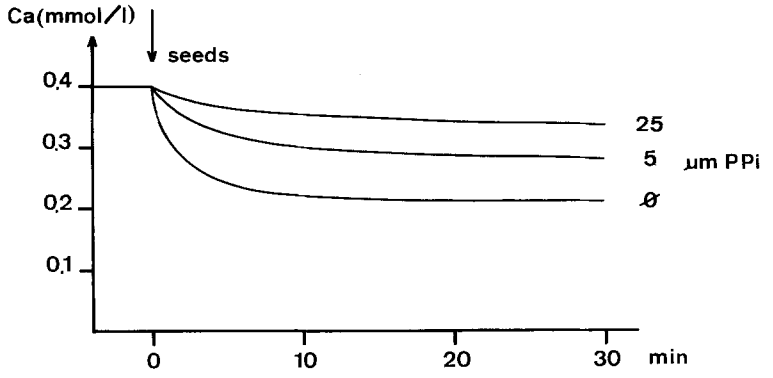

Fig. 1. Influence of pyrophosphate (PPi) on calcium oxalate monohydrate growth. Calcium plotted versus time in supersaturated and seeded calcium-oxalate solution (calcium, 0.4 mmol/l; oxalate, 0.4 mmol/l; calcium-oxalate monohydrate seeds, 1 mg/ml)

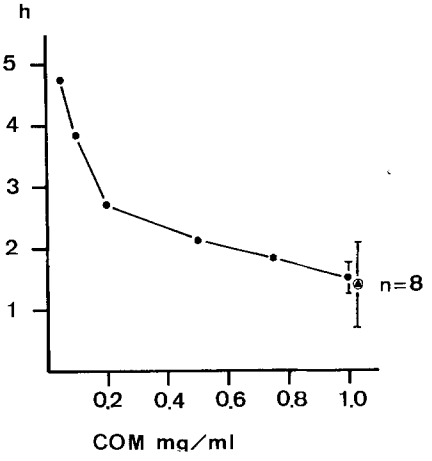

Fig. 2. Influence of seed concentration on half-life (h) of calcium decrease. (●) = h calculated, (▲) = graphically determined, $\bar{x} \pm 2$ SE, initial calcium and initial oxalate concentration 0.4 mmol/l each

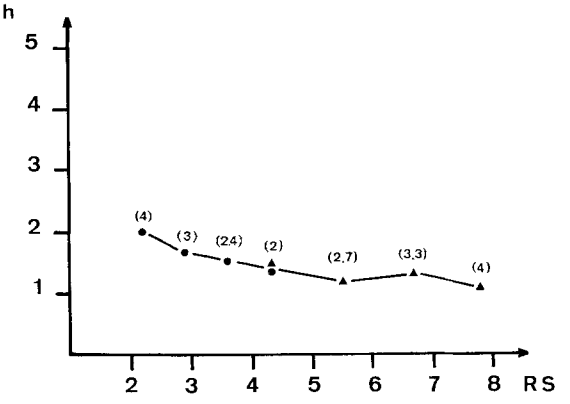

Fig. 3. Influence of relative supersaturation (RS) on the half-life (h) of calcium decrease. Calcium-oxalate monohydrate seeds, 1 mg/ml. (●) = Constant initial calcium, 0.6 mmol/l, and variable oxalate, 0.15–0.3 mmol/l; (▲) = constant initial oxalate, 0.3 mmol/l, and variable calcium, 0.6–1.2 mmol/l; (number) = calcium/oxalate ratio

Materials and methods

Crystallization experiments were performed in model solutions and urine metastably supersaturated with respect to calcium oxalate monohydrate. The model solutions were prepared by mixing 2 stock solutions containing twice the final calcium or oxalate concentration desired in 150 mmol/l NaCl, buffered with sodium cacodylate to pH 6.0. In order to work with a constant urinary inhibitor composition, experiments were always performed with the same pooled 24 h urine of a healthy person. Repeated chemical analyses showed the following urinary concentrations: calcium 2.3 mmol/l, oxalate 0.15 mmol/l, citrate 0.91 mmol/l, and pyrophosphate 0.028 mmol/l. Urinary calcium and oxalate concentrations were adjusted to the desired values by adding calcium chloride and sodium oxalate from stock solutions of 100 mmol/l each.

Crystal seeds were prepared in a stock solution of 150 mmol/l NaCl, buffered with sodium cacodylate to pH 6.0 and containing 100 mg/ml of commercially available calcium-oxalate monohydrate crystals (Merck). The crystals were stirred and aged for at least 1 week at 37°C.

The crystallization experiments were performed in a double-walled thermostated glass chamber under constant magnetic stirring, at 37°C and at pH 6.0. Calcium activity was monitored by an ion-specific electrode (Metrohm EA 302 Calcium) with an Ag/AgCl reference electrode and a pH meter (Metrohm E 603) connected to a penrecorder (Philipps PM 8251).

The stability of supersaturation was proved by the constant calcium activity during a period of at least 30 min in nonseeded urine and model solutions. Crystallization was initiated by the addition of seed crystals and the decrease of calcium activity was recorded as a function of time. The growth curves were evaluated by determining the half-life (h) of the calcium decrease. Two methods were used to obtain this half-life value, which was defined as the intersection between the growth curve and 50% of the calcium decrease after 20 min: (a) h was graphically determined by designing this intersection in the original curve of the penrecorder and (b) h was calculated as the intersection of the linear regression of calcium decrease observed within the first 2 min and half the value of the calcium decrease after 20 min. Comparison of the two methods showed that the mathematical approach had less variability (Fig. 2). Therefore, the latter procedure was used in the following experiments. Relative supersaturation was calculated, for calcium-oxalate monohydrate with the computer program EQUIL II [11].

Results

The typical curves of calcium decrease as a function of time obtained after seeding are shown in Fig. 1. Inhibitors like pyrophosphate seemed to influence this curve in two ways, namely: (a) they decreased growth rates and (b) stabilized the solution at higher calcium and oxalate concentrations or supersaturation levels, respectively.

Experiments with a variety of seed concentrations in model solutions revealed that the seed concentration was another important factor which influenced the half-life of

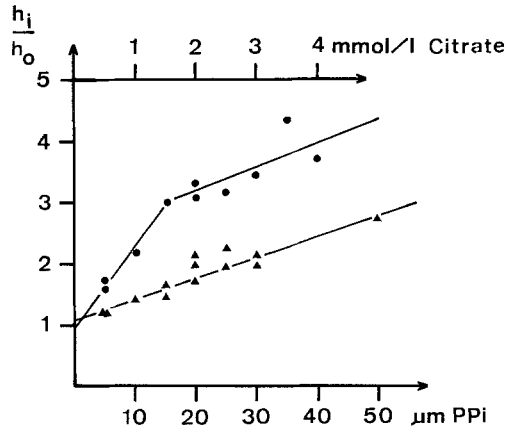

Fig. 4. Dose-response curves of the inhibition (h_i/h_0) of calcium oxalate monohydrate growth by physiological concentrations of citrate (●) and of pyrophosphate (▲) (other test conditions as in Fig. 1)

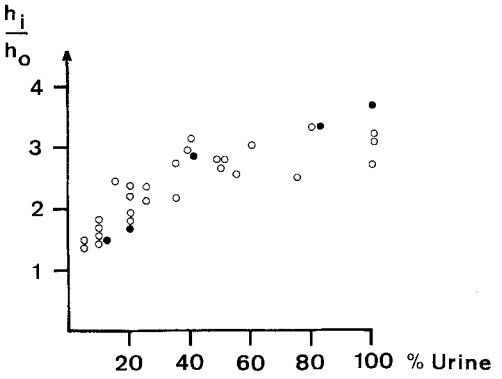

Fig. 5. Dose-response curve of the inhibition (h_i/h_0) of calcium-oxalate monohydrate growth by 5–100% of urine. Urine portions seeded with 1 mg/ml calcium-oxalate monohydrate, (●) at constant calcium, 1.4 mmol/l, and oxalate, 0.35 mmol/l; (○) at variable calcium, 0.4–1.2 mmol/l, and oxalate, 0.24–0.4 mmol/l

calcium decrease (Fig. 2). At high seed concentrations, this influence diminished, and the end of the crystal growth was rapidly reached. Therefore, further experiments were performed with a crystal concentration of 1 mg/ml.

The influence of the initial calcium and oxalate concentration in the test system was studied in the two series of experiments shown in Fig. 3. The half-life value of the calcium decrease slightly diminished with increasing relative supersaturation and was not influenced by the calcium/oxalate ratios being tested.

Since half-life (h) was not only dependent on the inhibitors but also on the seed concentration, supersaturation and probably other factors not examined in our study, inhibition was expressed as a ratio (h_i/h_0), which was calculated from h obtained in the presence (h_i) and in the absence (h_0) of inhibitors. Figure 4 shows the typical plots of h_i/h_0 versus citrate and pyrophosphate in the concentrations found in the urine of stone formers and healthy controls. The two substances proved to have an important inhibitor activity, high physiological citrate concentrations being more active than high pyrophosphate concentrations. The dose-response curve revealed a straight line for pyrophosphate and had a double slope for citrate.

The inhibitory effect of 5 to 100% of the test urine with variable as well as fixed calcium and oxalate concentrations is shown in Fig. 5. Again, a characteristic dose response curve was found, which was almost independent of the initial calcium and oxalate concentrations.

Discussion

The growth of calcium-oxalate crystals is influenced by several factors that must be taken into account for interpretation of the results of crystallization experiments. These factors comprise supersaturation, calcium-oxalate ratio, concentration and quality of seed crystals, temperature, pH, stirring rates, and inhibitors [5, 12]. In order to get comparable results of inhibitor measurements, the other factors must be strictly controlled. In our test system, temperature, pH, and stirring rates were kept constant and the quality of seeds was controlled by the application of commercially available crystals always originating from the same stock. The effect of supersaturation could partially be cancelled out by the determination of a half-life value (h) of calcium decrease (Fig. 3). The variability of h due to the concentration of seed crystals could be minimized by using high crystal concentrations (Fig. 2). This might also be helpful in examining freshly voided urine with crystalluria.

Although big crystal masses may neutralize inhibitors [4], the seed concentration of 1 mg/ml proved to be suitable for measuring the inhibitory effects of citrate and pyrophosphate in urinary concentrations, as shown in Fig. 4. Plotting inhibition (h_i/h_0) versus pyrophosphate concentration revealed a straight line, which is typical for inhibitors that are mainly active by absorption at the growing sites of crystals [6]. Analyses of the dose response curve of citrate revealed two slopes with a kink at about 1.5 mmol/l citrate. Interpretation of this curve requires further evaluation.

The measurements of urinary oxalate is still cumbersome and the ideal method has not yet been found. The aim of this study was to develop a rapid test to check the inhibitor activity in urine of yet unknown chemical composition. Therefore, studies in urine were performed with variable oxalate and calcium concentrations. A plot of the inhibition measured in 30 experiments and expressed as h_i/h_0 shows that the initial oxalate and calcium values had almost no influence on the results (Fig. 5). No differences could be found between tests performed at an initially constant calcium and oxalate concentration compared to those with variable calcium and oxalate values. The method presented may therefore also be suitable for the measurement of inhibition in freshly voided urine with a yet unknown oxalate concentration. Using an ion-

selective calcium electrode, h_i can be obtained within 25 min. To calculate inhibition as h_i/h_0, h_0 can be determined either by normograms, as shown in Fig. 3, or by a second experiment in a model solution with the same calcium activity and oxalate concentration as measured in the urine. The method is now being evaluated by a number of experiments performed with freshly voided urine of stone formers and healthy controls.

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