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Effects of trace metals on the inhibition of calcium oxalate crystallization

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Abstract The aim of this study was to examine the possible effects of some trace metals on the inhibition of calcium oxalate crystallization. A test of urinary lithogenic risk was used to follow the crystallization of calcium oxalate from artificial urine in the presence of several metal ions assayed in their physiological concentrations. Interactions of these metal ions with known inhibitors of such crystallization (phytate, pyrophosphate, citrate and chondroitin sulphate) were also investigated. None of the metals affected the inhibition of calcium oxalate crystallization at concentrations approximating those found in normal urine, with the exception of the Fe^{3+} ions. Interactions of Fe^{3+} with some urinary components produced both synergic (phytate and pyrophosphate) and negative (citrate) effects on preventing crystallization. These effects are explained in terms of the affinity of the inhibitors for the calcium oxalate crystal surface and their ability to form stable complexes in urine. Because of the minimal concentrations, we conclude that physiological concentrations of trace elements in urine have no significant influence on calcium oxalate crystallization. In this sense, ferric ions, which exhibit an intrinsic high inhibitory capacity of calcium oxalate crystallization at physiological concentrations, even increased by the concomitant presence of phytate and pyrophosphate, are probably unable to act as powerful inhibitors in the presence of physiological urinary concentrations of citrate, due to the formation of highly stable complexes in solution without inhibitory activity.

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Introduction

Urolithiasis is a process that results from a combination of factors in which the main phenomenon is the supersaturation of some compounds in urine that might crystallize forming solid concretions. This process is affected by the lack of crystallization inhibitors, the presence of crystallization promoters and some morphoanatomic factors [1]. A lithiasic episode may occur when the equilibrium between these factors is broken. Thus, crystallization inhibitors are critical for urolithiasis, being used for the preventive treatment of urolithiasis recurrences [2].

In contrast to the well known inhibitory activity of some urinary components such as citrate, magnesium, phytate, pyrophosphate and glycosaminoglycans [3–6], little attention has been paid to trace elements [7, 8]. On the one hand, trace elements are known to influence the external morphology of crystals, as well as to speed or retard the crystallization process [9–12]. Unfortunately, the predominantly unphysiologically high concentrations used in these studies do not permit any reliable conclusion on the in vivo effect of trace elements on stone formation [13]. On the other hand, some attempts to study the interaction of metal ions with some urolithiasis inhibitors, such as several bisphosphonates [14] and citrate, have been reported [15]. However, a great effort is required to ascertain the effects of trace metal interactions with the main known inhibitors of urolithiasis.

Several procedures have been developed to evaluate the crystallization properties of urine and to study the inhibitory capacity of given substances in urine [16, 17]. Recently, a very simple test (the urinary lithogenic risk test or ULR) to evaluate the capacity of a urine to

crystallize calcium salts has been presented [18]. The ULR test is based on the fact that if an unprotected, non-renewed surface (i.e. histologic paraffin or polyethylene) remains in contact with a urine, sooner or later the supersaturated substances contained in the urine will crystallize on it. Thus, the URL test represents a simple test to evaluate the capacity of urine to crystallize calcium salts. Its application for the evaluation of inhibition of calcium oxalate crystallization by trace metals is presented in this study. The advantages of this test are its ease of application to artificial and real urines, its proved efficacy on screening for increased risk of calcium oxalate crystallization in stone-formers, its rapidity and low-cost.

Within this context, the aim of this work is to systematically study the inhibitory activity of certain trace metals, both the individual effect and in the presence of the main inhibitors of calcium oxalate crystallization (i.e., phytate, pyrophosphate, citrate and glycosaminoglycans), taking into account the possible synergic or negative interactions on inhibition.

Materials and methods

Reagents and solutions

Synthetic urine [19] was prepared immediately before use by mixing equal volumes of solutions A and B, both prepared with reagents of analytical reagent grade and deionised redistilled water, and adjusted to pH 5.5. Solution A contained 4.86 g/l Na_2SO_4, 1.02 g/l $MgSO_4{\cdot}7H_2O$, 4.65 g/l NH_4Cl, 12.2 g/l KCl and 2.24 g/l $Ca(NO_3)_2{\cdot}4H_2O$. Solution B contained 2.4 g/l $NaH_2PO_4{\cdot}2H_2O$, 3.0 g/l $Na_2HPO_4{\cdot}2H_2O$, 13.12 g/l NaCl and 0.075 g/l $Na_2C_2O_4$. The concentration of the different compounds in the synthetic urine solution was: Na^+ 171.7 mM, K^+ 81.3 mM, $NH_4{}^+$ 43.5 mM, Ca^{2+} 4.7 mM, Mg^{2+} 2.1 mM, Cl^- 237.0 mM, $SO_4{}^{2-}$ 20.1 mM, $PO_4{}^{3-}$ 16.1 mM and oxalate 0.28 mM.

Tetra-sodium pyrophosphate 10-hydrate (Panreac, Barcelona, Spain), myoinositol hexaphosphoric acid hexasodium salt from corn (Sigma, Steinheim, Germany), trisodium citrate dihydrate (Merck, Darmstadt, Germany), chondroitin sulphate A from bovine trachea (Sigma, Steinheim, Germany) and certified analytical standard solutions (1,000 mg/l) of assayed metals (J.T. Baker, Devented, Holland) were used as inhibitors.

Measurements of calcium oxalate crystallization

Figure 1 shows a diagram of the reaction unit employed. Before the experiment, 500 µl of an ethanolic 100 g/l solution of thymol (antiseptic action) was spread in each polypropylene container and the ethanol was evaporated. Then, 30 ml of artificial urine was placed in the polypropylene container and sealed, leaving the polyethylene tube in contact with the urine for 24 h at room temperature. Then, the urine was discarded and the polyethylene tube, bearing the calcium oxalate crystals on its surface, was carefully rinsed with water. Calcium oxalate was finally redissolved by introducing the tube into a vial containing 4 ml HCl 0.3 M, and calcium measured by ICP-OES (model Iris Intrepid II XLS from Thermo Electron, USA).

Inhibitory effects of various compounds

To study the activity of some inhibitors on calcium oxalate crystallization and to evaluate the effect of their interactions, the amount of calcium precipitated from artificial urine containing the target inhibitors was determined by the ULR test. Both the inhibitory effects on calcium oxalate crystallization of several metal ions in the physiological concentration ranges shown in Table 1 and the effect of binary mixtures of these metal ions in the presence of phytate (0.2 mg/l), citrate (80 mg/l), pyrophosphate (1.5 mg/l) or chondroitin sulphate (4.5 mg/l) were evaluated by the addition of appropriate volumes of concentrated stock solutions of these substances to the artificial urine just before the ULR test. Phytate, citrate, pyrophosphate and chondroitin sulphate were used in these experiments in concentrations below those of normal human urine because utilization of physiological concentrations leads to very high inhibition values, thus masking the lower effect of the metals under investigation at trace levels. If needed, such conditions can be modified to achieve normal urine concentrations after considering the inhibition results obtained.

The percentage of inhibition of calcium oxalate crystallization was determined for each experiment by comparison of the amount of calcium precipitated in each condition with that of the assay in the absence of inhibitors (see equation). To evaluate the reproducibility of the obtained results, each experiment was repeated three times.

$$\%\ \text{Inhibition} = 100\left(1 - \frac{\mu\ \text{g Ca formed in presence of inhibitor}}{\mu\ \text{g Ca formed in absence of inhibitor}}\right)$$

When the effect of citrate was studied, we took into account the decrease of free calcium in artificial urine because of the formation of calcium-citrate soluble

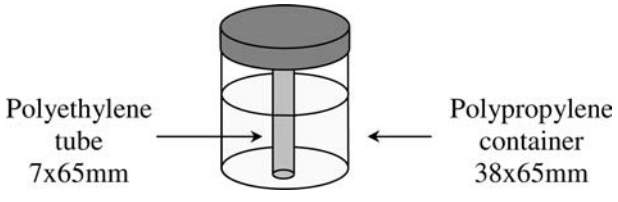

Fig. 1 The reaction unit of the ULR test

Table 1 Effect of individual trace metals on the inhibition of calcium oxalate crystallization. Representative ranges of these trace metals for normal urines are given for comparison [20, 21]

Metal ion	Zn^{2+}	Fe^{3+}	Cu^{2+}	Sn^{2+}	Pb^{2+}	Al^{3+}	Ni^{2+}	Cd^{2+}	Mn^{2+}
Representative range (μg/l)	0–767	0–170	0–55	0–35	0–4	0–34	0–12	0–1	0–2
Assayed range (μg/l)	0–2,000	0–250	0–100	0–50	0–50	0–50	0–50	0–50	0–50
Maximum inhibition observed	6 ± 3	55 ± 4	3 ± 2	2 ± 2	3 ± 2	4 ± 2	0 ± 1	1 ± 1	2 ± 1

complexes. Thus, a corresponding supplement of calcium was added by controlling the free calcium concentration using a calcium-selective electrode, in order to attain the same calcium oxalate supersaturation value that is found in the absence of citrate. A decrease in the supersaturation would produce a decrease in the crystallization rate that can not be attributed to inhibitory effects. Due to the low levels of phytate, pyrophosphate or chondroitin sulphate, the decrease in the free calcium concentration was negligible, making the addition of a calcium supplement unnecessary.

Study of the solid phase formed

Solids formed on the surface of polyethylene tubes were also studied by scanning electron microscopy (model JSM-6300 from JEOL, Japan) in order to determine the composition of crystals occurring in in vitro assays. Such a determination was carried out both by morphological characterization and elemental composition by using X-ray energy dispersive analysis.

Results

In vitro studies were performed in synthetic urine, using crystallization conditions that avoid calcium phosphate precipitation (pH = 5.5) with the aim of examining the formation of calcium oxalate crystals exclusively. In Fig. 2, an image of the crystals obtained in vitro with the model described above shows the formation of well developed polygonal crystals of calcium oxalate monohydrate, as previously found [5, 19]. X-ray energy dispersive analysis also supports this finding by providing the corresponding signal for calcium and the absence of the signal for phosphorus (no calcium phosphate formation).

Results in Table 1 indicate that none of the metals affect the inhibition of calcium oxalate crystallization at concentrations approximating those found in normal urine, with the exception of the Fe^{3+} ions. Furthermore, the effect of some other substances such as phytate, citrate, chondroitin sulphate and pyrophosphate on the inhibition of calcium oxalate crystallization was not significantly influenced (neither increased nor decreased) by the assayed concentrations of metal ions, again with the exception of the Fe^{3+} ions. The effects of binary mixtures of the aforementioned crystallization inhibitors of calcium oxalate crystallization together with Fe^{3+} ions are shown in Figs. 3, 4, 5 and 6. As can be seen, both phytate + Fe (Fig. 3) and pyrophosphate + Fe (Fig. 4) mixtures manifested significant synergic effects, whereas citrate + Fe mixtures (Fig. 5) show important negative effects on inhibition of calcium oxalate crystallization. Finally, the combination of chondroitin sulphate with Fe^{3+} ions (Fig. 6) shows additive interactions.

Discussion

The essential trace elements As, Cr, Co, Cu, F, Fe, I, Mn, Mo, Ni, Pb, Se, Si, Sn, V and Zn must be present in the body in minimal concentrations to guarantee specific functions, such as enzyme reactions, electronic transfer, redox reactions, etc. Although their presence in urinary stones has been demonstrated [22, 23], whether this is simply a result of external deposition from urine or whether the presence of excess amounts of elements such as iron, zinc and copper in a local environment may become an initiating factor in the process of rapid stone precipitation [24] remain unknown. However, it seems clear that if a particular trace metal has an effect on

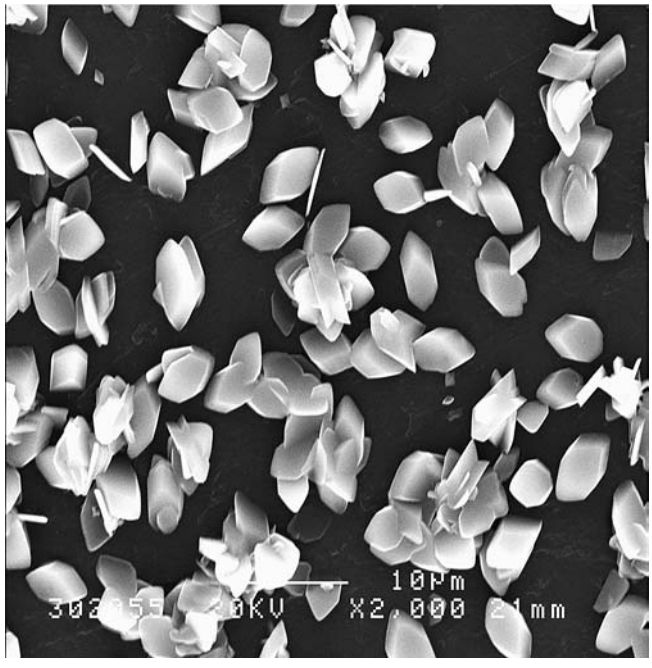

Fig. 2 Scanning electron micrograph of well-developed polygonal crystals of calcium oxalate monohydrate formed in vitro on the surface of polyethylene tubes of the reaction unit (ULR test)

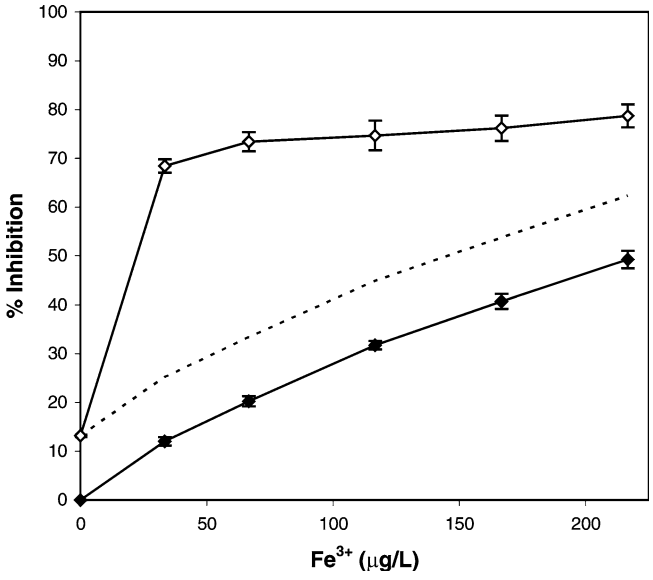

Fig. 3 Effects of Fe^{3+} + phytate mixtures on the crystallization of calcium oxalate. Percent inhibition ± SD in the presence of different concentrations of Fe^{3+} and absence of phytate (*filled circles*) or the presence of phytate 0.2 mg/l (*empty circles*). The *dashed line* is the line that would be obtained if only additive effects of inhibition were produced

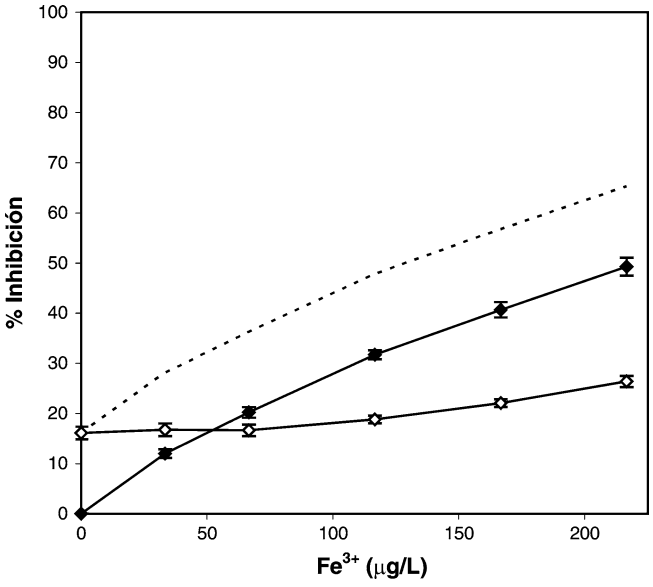

Fig. 5 Effects of Fe^{3+} + citrate mixtures on the crystallization of calcium oxalate. Percent inhibition ± SD in presence of different concentrations of Fe^{3+} and absence of citrate (*filled circles*) or presence of citrate 80 mg/l (*empty circles*). The *dashed line* is the line that would be obtained if only additive effects of inhibition were produced

crystallization of a urinary stone component, it must necessarily act at the surface of the crystals since the concentration of trace metals in urine is too small to affect the lattice ions in solution, being thus trapped and perhaps concentrated within the lattice of the crystals. The presence of Fe^{3+} in calculi is further explained by its adsorption on calcium oxalate crystals [25]. The ability of Fe^{3+} ions to establish highly stable chemical interactions with oxalate ions on the surface of calcium oxalate crystals, thus disturbing their development, explains its inhibitory effects on calcium oxalate crystallization. Fe^{3+} ions are, of all the metal ions

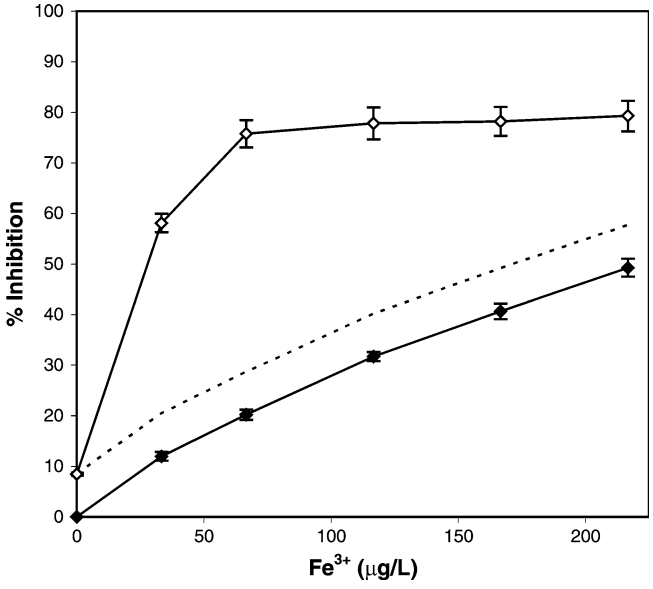

Fig. 4 Effects of Fe^{3+} + pyrophosphate mixtures on the crystallization of calcium oxalate. Percent inhibition ± SD in presence of different concentrations of Fe^{3+} and absence of pyrophosphate (*filled circles*) or presence of pyrophosphate 1.5 mg/l (*empty circles*). The *dashed line* is the line that would be obtained if only additive effects of inhibition were produced

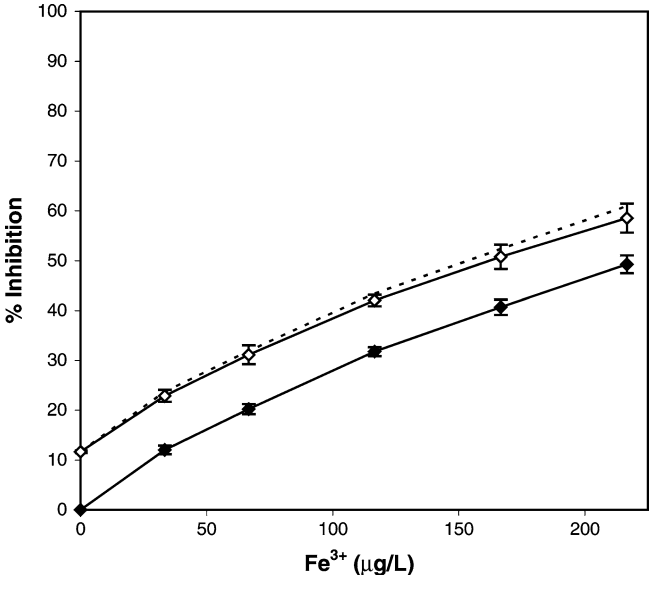

Fig. 6 Effects of Fe^{3+} + chondroitin sulphate mixtures on the crystallization of calcium oxalate. Percent inhibition ± SD in presence of different concentrations of Fe^{3+} and absence of chondroitin sulphate (*filled circles*) or presence of chondroitin sulphate 4.5 mg/l (*empty circles*). The *dashed line* is the line that would be obtained if only additive effects of inhibition were produced

assayed in this work, those which more stably bind oxalate ions [26], probably explaining why the other metal ions do not show significant inhibitory effects on calcium oxalate crystallization at physiological concentrations. Inhibitory properties previously reported for other metals ions, such as Zn^{2+} [9], Al^{3+} [9, 15] or Cu^{2+} [13], were not confirmed by this study, and are probably due to unphysiologically high concentrations used in those studies.

It is even more interesting to note how the interactions between Fe^{3+} ions and the calcium oxalate surface can be modulated by the action of common urinary components, inducing important changes in its inhibitory properties. The important synergic effects on inhibition exhibited by phytate + Fe^{3+} and pyrophosphate + Fe^{3+} are likely to occur via anchored mixed ligand complexes of Fe^{3+} or anchored binuclear complexes of phytate and pyrophosphate, which could cause a greater blocking effect of the active sites on the crystal surface than the individual components. Some stable mixed ligand complexes of Fe^{3+} with carboxylate and phosphate groups have been reported [27]. Thus, analogous complexes with oxalate and phytate or pyrophosphate, anchored in the calcium oxalate crystal surface, could explain the aforementioned synergic effects. Furthermore, the possible formation of binuclear complexes of phytate or pyrophosphate with Fe^{3+} ions and calcium, anchored by the latter to the calcium oxalate crystal surface, can also contribute to hindering crystal growth. A similar mechanism of action has been reported for the potentiation of bisphosphonate activity by Sn [14], in which any of the two phosphonate groups of the bisphosphonate molecule can act as a separate unidentate ligand, with one group binding the Sn^{4+} ion and the other binding a calcium on the crystal surface.

Apart from synergic effects with phytate and pyrophosphate, negative effects on inhibition by Fe^{3+} ions in the presence of citrate have also been observed. At high ratios of citrate to metal ions, Fe^{3+} is known to form highly stable low molecular weight complexes without the inhibitory properties of calcium oxalate crystallization [15]. Such complexes are likely to avoid the interaction of ferric ions with the calcium oxalate surface by displacing the Fe^{3+} ions from this surface, counteracting its inhibitory effect and resulting in the aforementioned negative effects of the Fe^{3+}-citrate interaction on calcium oxalate crystallization.

Conclusions

Renal lithiasis is known to be a multifactorial disease in which inhibitory crystallization deficit plays a major role together with supersaturation levels of different urinary salts, promoters of crystallization and diverse phenomena of crystal retention in the urinary tract. The inhibitory capacity of a given urinary compound can not only be related to its concentration, since the abundant likely interactions of this compound with other components in urine (complex formation, precipitation, chemisorption on a crystal surface, displacement from crystal surface) are known to significantly change its ability to disturb the development of a specific insoluble salt, producing either negative or synergic effects. In this sense, ferric ions, which exhibit a high intrinsic inhibitory capacity for calcium oxalate crystallization at physiological concentrations, even increased by the concomitant presence of phytate and pyrophosphate, are probably unable to act as a powerful inhibitor in the presence of physiological concentrations of citrate, due to the formation of highly stable complexes in solution without inhibitory activity.

Because of the minimal concentrations, we conclude that physiological concentrations of trace elements in urine have no significant influence on calcium oxalate crystallization.

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