Crystal growth and dissolution of brushite crystals by different concentration of citric acid solutions

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Calcium hydrogen phosphate dihydrate (CHPD) or brushite crystals are well-known urinary crystals and frequently found in urinary stones. The CHPD crystals were grown by the single diffusion gel growth technique in sodium metasilicate gel. The grown crystals were having platelet and star shape morphologies. The maximum growth was observed for the first five days, thereafter, the growth became almost steady. After achieving maximum growth of CHPD crystals, the aqueous solutions having different molar concentrations of citric acid were added into the supernatant solution containing calcium chloride. In the case of low concentrations of citric acid, the inhibition of the growth of CHPD crystals was observed, whereas for the higher concentrations of citric acid the dissolution of the grown crystals was observed. This indicates that the citric acid inhibits the growth of CHPD crystals in the lower concentrations and dissolves them in the higher concentrations. This supports the citrate inhibition theory of urinary calculi and crystals.

Keywords: Crystal growth, Brushite crystals, Citric acid, Calcium stones

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1 Introduction

Calcium stones are the most commonly occurring form of nephrolithiasis or urinary stones. Calcium oxalate stones are more common than calcium phosphate ones, but mixed calcium oxalate-calcium phosphates are the most common variety. Also, calcium phosphate is present in urinary calculi as either apatite \( \text{Ca}_10(\text{PO}_4)_6(\text{OH})_2 \) or brushite \( \text{CaHPO}_4 \cdot 2\text{H}_2\text{O} \). Brushite or calcium hydrogen phosphate dihydrate (CHPD) crystals have been grown and characterized by various researchers\(^{1-3}\).

The first attempt to dissolve calcium stones was made by Hellstron and Albright in 1930, using the combination of sodium citrate and citric acid. It was noted that sodium acted as a carrier. Citrate prevented crystallization by binding with calcium as well as citric acid brought the pH of the solution to 3.0-4.0. Citrate is the most important complexer of calcium in urine and reduces ionic calcium concentration\(^{4-8}\).

The growth of crystals by gel technique is well-known\(^{9,11}\), which can also be used as a simple technique to grow the urinary type crystals\(^{1-3,12}\) and study the various inhibitors \textit{in-vitro}\(^{13-16}\). Recently, the effect of herbal extracts such as, \textit{Tribulus terrestris} and \textit{Bergenia ligulata}, has been investigated by Joshi \textit{et al.}\(^{17}\) under \textit{in-vitro} conditions in the presence of artificial reference urine and human urine. Moreover, the growth inhibition study of brushite crystals in presence of tamarind solution and tartaric acid solutions have been reported by Joseph \textit{et al.}\(^{18}\).

2 Experimental Details

In the present investigation, CHPD crystals were grown by the single diffusion gel growth method, when crystals achieved the maximum growth the inhibitive effects of citric acid solutions having different concentrations were studied by adding them into the supernatant solution. Whereas, in the earlier studies different inhibitive solutions were added in the supernatant solutions right from the beginning of the growth of crystals and compared with the growth of crystals without inhibitor solutions\(^{8,14-16}\).

Glass test tubes of 2.5 cm diameter and 15 cm length were used for growing the crystals. Sodium metasilicate solution of specific gravity 1.06 was acidified by adding appropriate amount of orthophosphoric acid so that 4.0 and 5.0 pH could be obtained for the mixture, which was subsequently transferred into different test tubes. After gelation took place, 10 ml, 1 M, aqueous solution of calcium chloride was carefully poured on the set gels. Crystals were found to be growing very rapidly within two days from pouring the supernatant solutions.
Elongated platelet type and star shaped crystals were grown in the gel. The apparent length of growing crystals was measured by traveling microscope of 0.001 cm least count, at different time intervals. From the plot of apparent length of growing crystals versus time, it was found that on the fifth day of pouring supernatant solution on set gel the crystals achieved maximum length and, thereafter, they did not increase appreciably in length. In the present investigation, the molar concentrations 0.2, 0.4, 0.6, 0.8 and 1.0 M of aqueous solutions of citric acid were added in the same volume as the calcium chloride solution and their effect was studied on the growth of CHPD crystals, after acquiring the maximum length of CHPD crystals. For 4.0 pH only a few platelet type crystals without any presence of star type crystals were grown, on the other hand, for 5.0 pH many crystals having star and platelet type morphologies were observed.

3 Results and Discussion

Figure 1(a) is a plot of apparent length of growing crystal versus time for pH 4.0, in case of 10 ml calcium chloride containing supernatant solution without adding any citric acid solutions. From this plot, it can be seen that after five days of pouring the supernatant solution, the crystals almost acquire the maximum values of length. For the pH value of 4.0, citric acid solutions of different concentrations were added in equal amount to the calcium chloride

![Plot of apparent length of growing CHPD crystal versus days for supernatant solutions containing:](image)
containing solutions in the respective test tubes. Figure 1(b) is a plot of apparent length of growing crystal versus time for 10 ml, 0.2 M citric acid solution; it indicates a slight decrease in length of crystal after pouring citric acid. By adding 10 ml of 0.4 M citric acid solution into CaCl₂ solution, it was found that the growth rate decreased rapidly. The crystals dissolved faster than the previous case. This can be seen from Fig. 1(c). Whereas, Fig. 1(d) is a plot of apparent length of growing crystal versus time, for adding 10 ml, 0.6 M citric acid solution into the supernatant solution of CaCl₂; it shows that the growth decreases very rapidly. In case of adding 10 ml of 0.8 M and 1.0 M citric acid solutions into CaCl₂ solution, interestingly, crystals dissolved within two days and one day, respectively. This can be verified from the Figs 1 (e and f).

Same types of results are available for 5.0 pH. It also indicates that as the molar concentration of citric acid increases, the crystals dissolve rapidly. For 5.0 pH the length of crystals is less than that of for 4.0 pH, this is because of harder gel and smaller pore size so that the diffusion process becomes slow.

Hypocitraturia, a low amount of citrate in urine, is considered to be an important risk factor for urinary stone formation. Urinary citrate is considered as an important inhibitor of the crystallization of stone forming calcium salts. The mean normal urinary citrate excretion is 640 mg/24 hr. However, the hypocitraturia is, generally, considered when the citrate excretion is less than 320 mg/24 hr. But the severe hypocitraturia is when the citrate excretion is of less than 100 mg/24 hr. The normal urine citric acid level is within 320 to 1240 mg/24 hr. There are many reasons for increase or decrease of urinary citric acid levels. The urine citric acid level may decrease with acidosis, severe muscular activity, hypoparathyroidism, diabetes and chronic renal failure. On the other hand, urine citric acid levels may increase with a high carbohydrate diet, astrogen therapy and vitamin D.

In the pathophysiology, the excretion of citrate in urine is a function of filtration re-absorption, peritubular transport and synthesis by renal tubular cell. The proximal tubule reabsorbs most 70-90% of filtered citrate and as a result the citrate secretion is negligible. Altogether, the acid-base status plays the most significant role in the citrate excretion. Alkalosis enhances citrate excretion, whereas acidosis decreases it. In acidosis, increased citrate utilization by the mitochondria in the tricarboxylic acid cycle occurs. This results in lower intracellular levels of citrate facilitating citrate re-absorption and hence reducing citrate excretion. Citrate excretion is impaired by acidosis, hypokalemia, high animal protein diet and urinary tract infection.

Citrates play predominant roles in the mechanisms of urinary calculi formation. First of all citrate binds with calcium ions in the urine and reduces calcium ion activity, consequently, the lowering of the urinary supersaturation of calcium phosphate and calcium oxalate. Second, citrate has a direct inhibitory effect on the crystallization and precipitation of salts. Citrate also decreases the calcium oxalate aggregation inhibitory activity of urine macromolecules (Tamm-Horsfall protein) and also expected to reduce urinary osteopontin, which is an important component of the protein matrix of urinary calculi. Apart from these, urinary citrate excretion also increases urinary pH, which is a factor in uric acid crystallization and uric acid stone formation.

Citrate inhibits nucleation and growth steps of calcium oxalate monohydrate (COM) as well as CHPD² crystallization. The citrate inhibition suggests that, sodium citrate, citric acid and other citrate compounds are acting as alkalinization agents indicated for systemic metabolic acidosis (renal tubular acidosis), while urinary alkalinization or hypocitraturia contains di-sodium citrate. Pak et al.⁵ reported successful management of uric acid nephrolithiasis with potassium citrate. Potassium citrate reduces urinary saturation of calcium salts by complexing calcium and reducing ionic calcium concentration. Pak²⁰ preferred potassium citrate because it appears to decrease urinary calcium excretion at least transiently. On the other hand, sodium citrate does not lower urinary calcium excretion; perhaps as the result of the increased sodium load associated with therapy²¹. Sodium-potassium citrate was used by Hofbauer²², this study did not give any conclusive results on patients. No prospective randomize study has confirmed the superiority of potassium citrate over sodium citrate in prevention of stone recurrence as Menon et al.²³ have noted down. This suggests that as sodium citrate and potassium citrate due to their alkalinization effect have inhibition effect, it is worth to study the effect of citric acid on the growth inhibition of CHPD crystals. This has leaded the present authors to verify the growth inhibition of CHPD crystal in-vitro condition.
Recently, it has been observed by Joshi and Joshi\(^8\) that the supernatant solutions, for the growth of CHPD crystals, containing citric acid, lemon juice, lemon juice and artificial reference urine (ARU) and lemon juice and natural urine in addition to calcium chloride solutions exhibited various degrees of inhibitions. In their study, they poured supernatant solutions containing different inhibitors in various test tubes and compared the results with the pure calcium chloride containing supernatant solution. They proved citrate inhibition\(^7\) for CHPD crystals as well as they also proved that the highest inhibition was in the case of lemon juice, which contains citric acid, plus natural urine as natural urine contains some natural inhibitors. The present investigation provides further proof of citrate inhibition of calcium containing urinary crystals, such as CHPD crystals, \textit{in-vitro} condition. The higher concentrations of citric acid in the supernatant solutions dissolved the grown crystals very rapidly by forming soluble salt calcium citrate, whereas in the case of lower concentrations of citric acid, that is less than 0.2 M concentrations, the inhibition to the growth of CHPD crystals was observed. These results may be useful to investigate the effect of citric acid on the dissolution and inhibition of urinary calculi. Though the level of mean citric acid excretion is very low in human body, this \textit{in-vitro} study may help to understand the inhibition of growth by raising citrate concentration in urine in patients who are prone to stone formations.

In conclusion, citric acid is found to be inhibiting the growth of CHPD crystals in the lower concentrations of less than 0.2 M and dissolving CHPD crystals for the higher concentrations of citric acid. As the concentration of citric acid is increased, the dissolution of CHPD crystals becomes more rapid.

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