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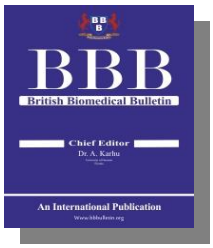

Original

Inhibition of Urolithogenic Calcium Phosphate Mineralization by Some Natural Acids in Aqueous and Urinary Milieu

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A B S T R A C T

Objective: Calcium phosphate forms one of the important constituents of urinary stones. Present objective is to Study the inhibition of mineralization of urolithogenic calcium phosphate by some natural acids. This study would lead to trace the role of such acids in prevention / prophylaxis of phosphate urolithiasis.
Methods: Inhibition of mineralization of urolithogenic calcium phosphate by some natural acids has been studied at a pH of 6.5 in aqueous and urinary milieu in an experimental model. Various natural acids studied were succinic, malic, citric, gluconic, aspartic or glutamic acid. Infrared spectra of the mineralized as well as sequestered (inhibited) phosphate, in the range of 4000-650 cm^{-1}, has been recorded and studied.
Results: Results revealed a moderate to good inhibition (12-75%) of phosphate by the natural acid inhibitors in aqueous medium. In urinary medium also moderate to good inhibition (16-94%) was observed. Infrared spectra suggested the precipitated phosphate to be calcium hydroxyapatite. Infrared spectra of the sequestered (inhibited) phosphate suggest it to be in a coordinated/complexed state.
Conclusion: Natural acids inhibit calcium phosphate mineralization by soluble complexation. Cycling of these acids through the urinary tract would be helpful in the prevention/ prophylaxis of phosphate urolithiasis.

Introduction

Calcium phosphate forms one of the important constituents of urinary stones[1-3]. It might mineralize in the urinary tract in various forms viz.,whitelockite [ß-$Ca_3(PO_4)_2$], brushite ($CaHPO_4.2H_2O$) calcium hydroxyapatite, [$Ca_{10}(PO_4)_6(OH)_2$].

Phosphate stone might form either singly or in mixture with some other constituents such as calcium oxalate monohydrate, calcium oxalate dihydrate, uric acid, ammonium urate, cystine etc.[4-8]

Inhibition of calcium phosphate mineralization by some physiologically nontoxic and naturally occurring acids would lead to prevention/prophylactic care of calcium phosphate urolithiasis. Chemodis solution of phosphate part of stone by such inhibitors would also lead to crumbling of stones into small pieces, which would be flushed out through urine[9-10].

With the above views in mind, we have presently studied the inhibition of mineralization of calcium phosphate by some natural acids viz., succinic, malic, citric, gluconic, asparatic or glutamic acid, at a pH of 6.5 in aqueous and urinary milieu.

Materials and methods

All chemical used were of AR quality.

Inhibition experiment in aqueous medium

Solution of calcium chloride (0.05M) and ammonium dihydrogen phosphate (0.03M) were prepared in distilled water. 50ml of ammonium dihydrogen phosphate solution (0.03M) was taken in a beaker. A weighed quantity of sodium salt of the natural acid viz., trisodium citrate, disodium malate, sodium malonate, sodium succinate, sodium aspartate, sodium glutamate or sodium gluconate were added to the solution of ammonium dihydrogen phosphate so that the concentration of the inhibitor (sodium salt of natural acid) became 0.05M in the solution.50ml of calcium chloride solution (0.05M) was added drop wise from a burette into the above solution (containing ammonium dihydrogen phosphate and the inhibitor). The pH of reaction mixture was maintained at 6.5 by the addition of small quantities of 0.05M sodium hydroxide solution. CO_2-free N_2 gas was bubbled through the reaction mixture to eliminate the possibility of formation of carbonate apatite. The whole experiment took about 40 minutes. At the end, the reaction mixture was digested on a hot water bath for 1h and then left over night (well covered) at room temperature. The precipitate was filtered off and washed with distilled water till the washing were free from ammonium salts. The precipitate was dried at 100^0C and preserved over fused calcium chloride. The weight of the precipitate was recorded.

The filtrate, after removing the above precipitate, was subjected to crystallization by evaporating to a small volume. The crystals, so obtained, were filtered off from the mother liquor and dried at 110^0C in an air oven and preserved over fused Calcium chloride.

Simultaneous blank/ control experiment i.e., without adding any inhibitor was also performed to ascertain the inhibition efficiency of the inhibitors.

Percentage efficiency of inhibition by the inhibitor was calculated by using the formula:

$$\% \text{ inhibition} = \frac{(\text{Weight of ppt. In blank set} - \text{weight of ppt. In experimental set}) \times 100}{\text{Weight of ppt. In blank set}}$$

Experimental set = with natural acid as inhibitor.

Blank set = without inhibitor.

Infrared spectra

Infrared spectra of the precipitates as well as of the crystals isolated from the corresponding filtrates were recorded in the range of 4000-500cm^{-1} in KBr phase

Experiments in urinary medium

Urine sample of a male, age 35yrs was collected in a plastic container. A 24hour urine output was collected and a bit of camphor was added as preservative. It was filtered through whattman filter paper. It was kept in the refrigerator and was used out in minimum possible time after collection.

0.05M solution of calcium chloride was prepared in the urine sample (as a solvent) Slight precipitation was there. This solution was next treated with dilute acetic acid to get a clear solution. The solution of 0.03M ammonium dihydrogen phosphate was also prepared in the urine. The inhibition experiments with and without inhibitors were preformed exactly as that in the case of aqueous medium, maintaining the pH at 6.5.

The inhibition efficiency of the natural acids towards inhibition of mineralization of calcium phosphate in the urinary medium were also found out in the same manner as that in aqueous medium.

Results and Discussion

Inhibition of calcium phosphate mineralization by the natural acids in aqueous and urinary milieu are recorded in Table-1and Table-2, respectively.

In aqueous medium, the inhibitors have shown a moderate to good inhibition (12-75%) of calcium phosphate mineralization. Succinate showed the lowest (12.5%) and citrate showed the highest (75%) inhibition. In urinary medium, also the inhibition efficiency exhibited by the natural acids towards calcium phosphate mineralization has been moderate to good (16-94%).In urinary medium, Glutamate showed the least (16%) and malate showed the highest (94%) inhibition. It looks that solute load increased by the urinary medium did not much reflect in the suppression of inhibition as compared to that in water. Rather there has been much increase in the inhibitory power of malate as well as gluconate in urinary media as compared to their inhibition efficiencies in aqueous medium. This might be due to the fact that urine itself is known to posses some in-built inhibitors[11-15], which also might have inhibited mineralization in addition to that by the externally added inhibitor (natural acid).

A comparative study of the net phosphate precipitation in the absence of any inhibitor (blank set) in aqueous and urinary medium also suggest that urine itself have some in- built inhibitors, which have resulted in a decreased blank set precipitation in urinary medium (180mg) compared to that in aqueous medium (240mg).

The present experiments were conducted at a pH of 6.5. This is the usual urinary pH. The pH of urine is ideally around 5.6. However, as urinary pH increases, the insoluble salts (particularly the calcium salts) start precipitating out,making the person susceptible to stone formation. We attempted to find the phosphate precipitation at a pH of 6.5. Around this pH, the most stable / insoluble solid phase of calcium phosphate is likely to be calcium hydroxyapatite[16]. In fact, the phosphate in the urinary stones mostly occurs as calcium hydroxyapatite. Infrared study of the precipitates have also corroborated with this observation.

Infrared studies

Infrared studies of the precipitates of the inhibition experiments

The ideal symmetry of tribasic phosphate ion in free or undistorted state is tetrahedral, a member of Td point group. It generally shows four IR absorption modes, namely, asymmetric P-O stretch (ν_3) symmetrc P-O stretch (ν_1) and two O-P-O

bending modes (ν_2 and ν_4). In the ideal symmetry conditions, ν_3 and ν_4 are clearly seen. In a non equivalent force field around the PO_4^{3-}, however, there occurs distortion from the tetrahedral symmetry. In the case of ionic phosphate, the totally symmetric P-O stretching mode (ν_1) is Raman active. In the case of coordinated phosphates, however, this band (ν_1) has been found to become IR active[17] and show at 970cm^{-1}. Presently, the infrared spectra of precipitated phosphate in all of our experimental sets, as well as control sets exhibited the ν_1 band at 929cm^{-1} .The asymmetric P-O stretching band mostly showed either as a single band at 1215cm^{-1} or as a doublet at 1030cm^{-1} and 1215cm^{-1}. The O-P-O bending mode was found mostly at rather higher position, at around 660cm^{-1}. The position and nature of bands suggest that the phosphate precipitated out is mostly the calcium hydroxyapatite. The spectra of pure calcium hydroxyapatite also shows the phosphate bands at about the same positions[18-19]. In the spectra of the precipitates, strong band at 3000 cm^{-1}with some additional weak bands at ~ 2400 cm^{-1} were also observed. These bands may be assigned to the OH groups of the apatite, which are probably hydrogen bonded[18.]

Infrared studies of crystals isolated from the filtrates of the inhibition experiments

The filtrates of the inhibition experiments invariably contain the inhibited phosphate as well as the inhibitor (natural acid). There is every likely hood of soluble interaction between the inhibitor and the inhibited phosphate. Presently, the infrared spectra of isolated crystals (from the filtrates) showed two bands of medium intensity at ~1050-1100cm^{-1} and 1125-1200cm^{-1}. These bands may be assigned to the asymmetric P-O stretch(ν_3).The ν_1 band is exhibited as a weak band at ~990 cm^{-1}. The ν_4 (O-P-O bending mode) was mostly found split into two, showing at around 550 and 600 cm^{-1}. Rather high position of ν_1, coupled with the split of ν_3 and ν_4 bands into two, suggest some sort of coordinated nature of the inhibited phosphate in the solution[17]. It seems the inhibitor(natural acid) inhibits the precipitation of calcium phosphate by sequestering/solubilizing it by the formation of mixed ligand calcium complex of phosphate and natural acid ligands.

Conclusion

Our present study suggest that the natural acids are moderate to good inhibitors of mineralization of urinary stone forming calcium phosphate mineral at a pH of 6.5. The mechanism of inhibition is by soluble complexation of the calcium phosphate mineral by the natural acids (inhibitors). All the acids that we have worked with are naturally occurring and are also physiologically important. Their cycling through the urinary tract would be helpful in the prophylaxis of phosphate urolithiasis. In case of mixed urinary calculi also if the phosphate portion is dissolved out by the inhibitors, the stone would loose cementation, upto some extent. The stone would then become susceptible to crumbling into small pieces, which would eventually be flushed out through urine**.**

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Table 1: Inhibition of calcium phosphate mineralization in aqueous medium at pH 6.5.

Inhibitor	Wt. of calcium phosphate precipitate (mg)	% Inhibition (as compared to blank)
Blank/Control (water)	240	--------
Succinate	210	12.50
Malate	090	62.50
Citrate	060	75.00
Gluconate	180	25.00
Aspartate	150	20.83
Glutamate	190	29.16

Table 1: Inhibition of calcium phosphate mineralization in urinary milieu at pH 6.5.

Inhibitor	Wt. of calcium phosphate precipitate (mg)	% Inhibition (as compared to blank)
Blank/Control (urine)	180	-------
Succinate	160	11.11
Malate	010	94.44
Citrate	050	72.22
Gluconate	085	52.77
Aspartate	120	33.33
Glutamate	150	16.66