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The Effect Of Citrate, Ferric, And Aluminium Ions On The Crystallisation Of Phosphates At Different pH Values (6.5;7;8)

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ABSTRACT

The calcic phosphates represent a complex family. Their conditions of crystallisation and inhibition were studied at various pH values. Phosphate precipitates obtained from artificial urine in the pH range (6.5; 7; 8) were identified using FTIR and XRD. At pH = 6.5 brushite accompanied by carbonated amorphous phosphate complexes precipitated while at pH = 7, the coexistence of brushite, struvite and amorphous phosphate complexes carbonated were observed. On the other hand carbapatite and struvite were formed exclusively at pH = 8. The complete disappearance of brushite crystals was obtained after addition of 1mM of citrate ion at pH = 6.5 to artificial urine. Its effect on struvite was more important at pH=7 as at this pH value inhibition was complete. Total inhibition of brushite was only observed for a concentration of citrate ion of 4 mM. At lower concentrations inhibition was partial. At the same pH, pentahydrated octocalcic phosphates (POP) and amorphous carbonated calcium phosphates (ACCP) were formed as identified by FTIR spectroscopy. In the presence of aluminium ion, the inhibition of brushite growth and aggregation increased. In fact at a concentration of 0.05 mM the formation of bushite crystals was halted but ACC P persisted. The addition of up to a concentration of 1 mM of ion aluminium resulted in total inhibition and crystalline transformation of the ACCP into withlockite. On the other hand, the effect of ferric ion was less important than that of aluminium ion at pH = 6.5. However, inhibition was total with a concentration of 0.1 mM ferric ion where ACCP and POP were the only compounds formed. The total disappearance of the crystalline forms was attained with a ferric ion concentration of 0.5 mM, while amorphous carbonated calcium phosphate and the carbapatite precipitated. For the iron III ion, total disappearance of brushite was reached at 0.25 mM, only the ACCP and POP were present. The inhibition effect of citrate, aluminium, and ferric ions on the aggregation and size of struvite crystals was significant at pH=8 but inhibition and transformation of this crystalline species were not complete.

KEYWORDS Bushite; Struvite; pH effect; Crystallisation; Inhibition.

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INTRODUCTION

The calcic phosphates represent a complex family. Their survey is difficult because of the crystalline species diversity composing them and the relative ease of the crystalline transformation that may take place. Most the calcic phosphates is sensitive to the influence of the environment and particularly to mono or divalent ions of urine. These ions may move into crystal lattice and can alter the species under consideration. As a consequence, crystallization studies, more for the calcic phosphates than calcium oxalates, should grasp better the governing factors of crystallization[1].

In this work, we performed an *in vitro* crystallization study enabling the specification of kinetic and thermodynamic conditions of formation and growth of crystalline species. Different experimental procedures have been proposed using synthetic, diluted or natural supersaturated aqueous solutions of urine[1]. Crystallization can be triggered by adding, to reaction medium calcium, oxalates or phosphates, or by crystalline germination of the species under investigation. Crystallization can also take place, by changing the pH of substances having pH - dependent solubility[1,2].

We used inhibitors, which are chemical substances which prevent, slow down or reduce crystallization phases. They are substances capable of opposing, one or several steps of calcic salts lithogenesis (calcium oxalate, calcium phosphate), either by a specific mechanism of the crystal considered, or through a more general action mode. They act only in the metastable oversaturation zone between the solubility product Ksp and formation product Kf. They work by complexing promoting ions, or by fixing themselves to growth sites, or through poly anions complex formation[3,4]. Citrate ion is one of the most abundant inhibitors used in urine owing to its ability to complex calcium, hindering the formation calcium oxalate crystals. Moreover, it may react with aluminium, ferric and phosphate ions resulting in complexes with inhibiting capability against calcium phosphates crystallization[5]. Citrate ions are capable of reducing significantly *in vitro* hydroxyapatite crystallization. Several works have shown the capacity of citrate to form complexes or molecular associations with metal atoms or polyatomic ions acting against calcium phosphate growth. It has a specific effect on the number and aggregation of ammonia-magesium phosphates[6,7]. Inhibitory action is more significant on calcic phosphates than calcium oxalate[1,8,9].

EXPERIMENTAL

We chose the classical model for the study of phosphate crystallization because of its simplicity and satisfactory reproducibility. This model includes the study of crystallization without inhibitor and with it, in order to assess the inhibiting capacity of any chemical species used. Two solutions of following composition were mixed:

A : 11.02 g/l $Na_2SO_4 \cdot 10H_2O$, 1.46 g/l $MgSO_4 \cdot 7H_2O$, 4.64 g/l NH_4Cl, 12.13 g/l KCl et 0.24 g/l Ca^{2+} and

B : 2.65 g/l $NaH_2PO_4 \cdot 2H_2O$, 18.82 g/l $Na_2HPO_4 \cdot 12H_2O$, 13.05 g/l NaCl, 1 g/l $Na_3C_6H_5O_7 \cdot 2H_2O$ et 0.05 g/l $C_2O_4^{2-}$. The solution in $C_2O_4^{2-}$ is prepared from oxalic acid 0.05 g.

The precipitation of the solid phase of phosphates from artificial urine at different initial pH values (pH = 6.5; 7 and 8) was the object of our investigation. Artificial urine is prepared by mixing and stirring two equal volumes of 250 ml of solutions A and B at constant temperature (37°C) in capped vessels to give final artificial urine at pH = 7. The pH of solution B was adjusted to required value by adding either HCl or NaOH as appropriate.

Mixture agitation was maintained to prevent sedimentation. The crystal size development was monitored by polarized microscopy at different time intervals by proceeding as follows: Sample drops were examined every five minutes by polarising optical microscopy. Crystals were identified with x 40 magnifying lens. After crystallization time, the mixture was filtered, the recovered dried precipitates were analysed by FTIR spectroscopy and X-rays diffraction technique.

RESULTS

Study of the phosphate crystallization without inhibitors

Since crystallisable phosphatic species are pH dependent, we studied their precipitation from artificial urine at different pH values. The crystallization

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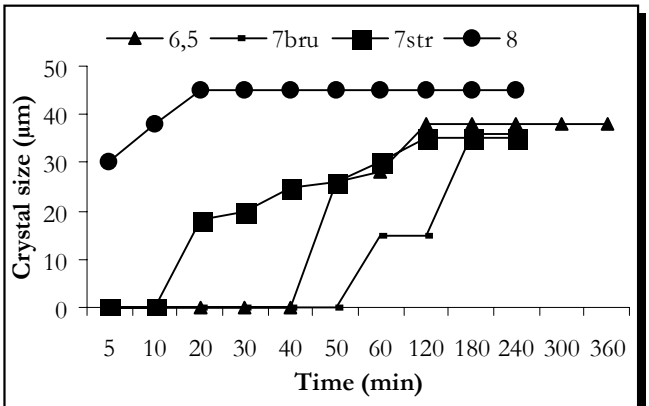

Figure 1: Development of struvite and brushite crystal size as function of time at different pH values

of phosphates at pH = 6.5 in the absence of inhibitor, led to the formation of brushite and granulations identified by polarized light microscopy as the amorphous carbonated calcium phosphates (ACCP), which finally were transformed into brushite after 6 hours (see Figure 2).

At pH = 7, both brushite and struvite are present and granulations were identified as being amorphous carbonated calcium phosphates (ACCP) (see Figure 3). The results showed that at pH = 8, crystals and granulations are respectively carbapatite and struvite (see Figure 4).

The development of the size of struvite crystals (str) and brushite (bru) at different pH values is summarized in figure 1.

The size of brushite crystals was more significant at moderately acidic pH values. The size of struvite crystals at pH = 8 confirmed that alkaline environment is favourable to the struvite formation. The induction times for brushite crystals at pH 6.5 and 7 were respectively of 50 and 60 minutes. It can be noted that at pH = 6.5, artificial urine was more favourable to thermodynamically stable brushite formation at this pH[1,10]. The formation of brushite after 60 minutes, at pH = 7, is in agreement with the high stability of brushite at moderately acidic pH rather than at neutral pH. Induction time for the two solutions at pH = 7.5 and 8 tends toward zero, due to the fact that at high pH struvite crystallization is favoured. Struvite crystals appeared immediately, even when adding the B solution. The results seem to agree with those obtained in the synthesis of the hydroxyapatite, octocalcic phosphates, withlockite,

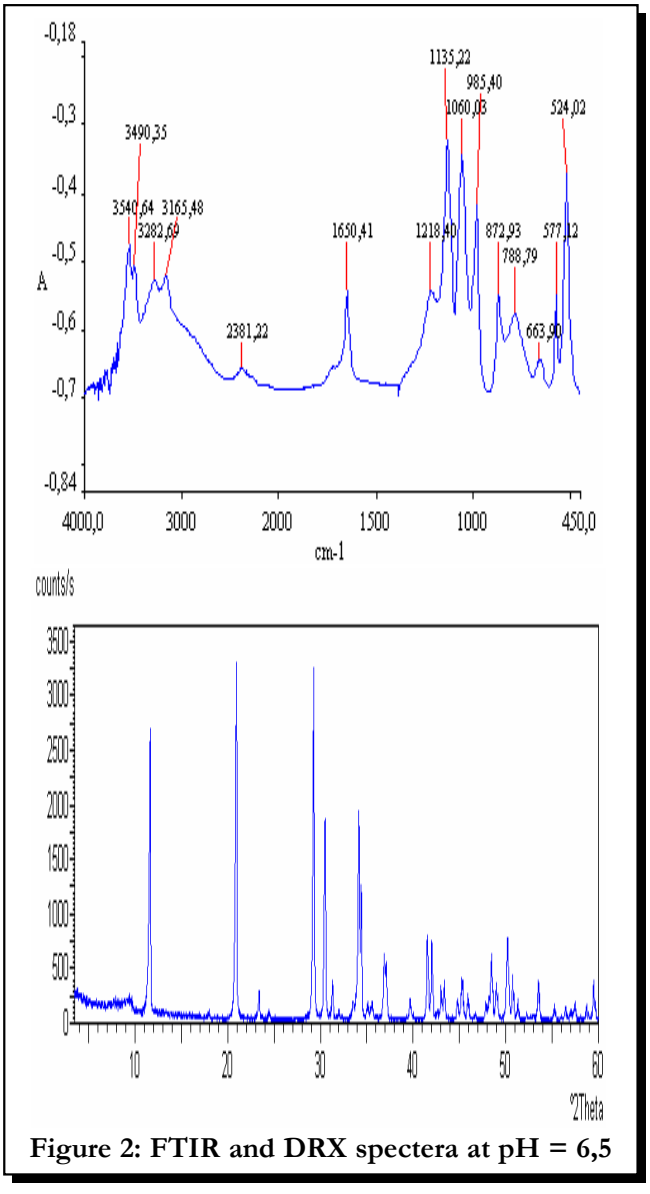

Figure 2: FTIR and DRX spectera at pH = 6,5

carbapatite, brushite and the amorphous carbonated calcium phosphates (ACCP)[1,10,11]. In fact, each calcium phosphate has a given molar precipitation concentration and pH dependent solubility product. The usual precipitation range of the calcic phosphates in urine is higher than pH = 6[12]. In urine, as in aqueous solution, when phosphocalcic oversaturation is important (pH=7), apatites with a solubility product Ksp in the order of $10^{-55.96}$ are the first species formed. Then comes struvite and finally brushite with solubility product equal to $10^{-13.04}$ and $10^{-6.73}$ respectively[12].

Study of phosphate crystallization in the presence of inhibitors

We followed the same experimental procedure

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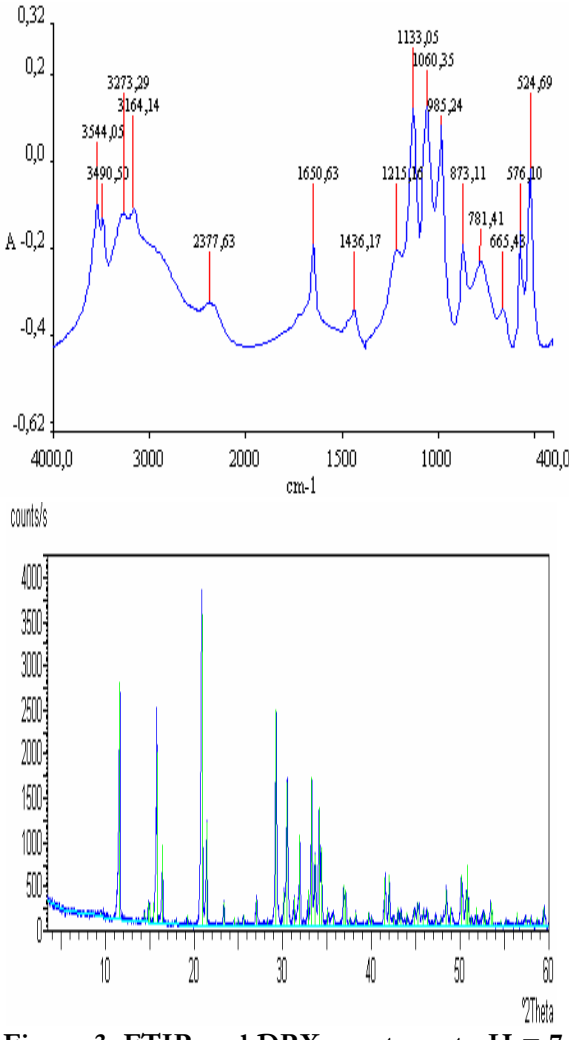

Figure 3: FTIR and DRX spectera at pH = 7

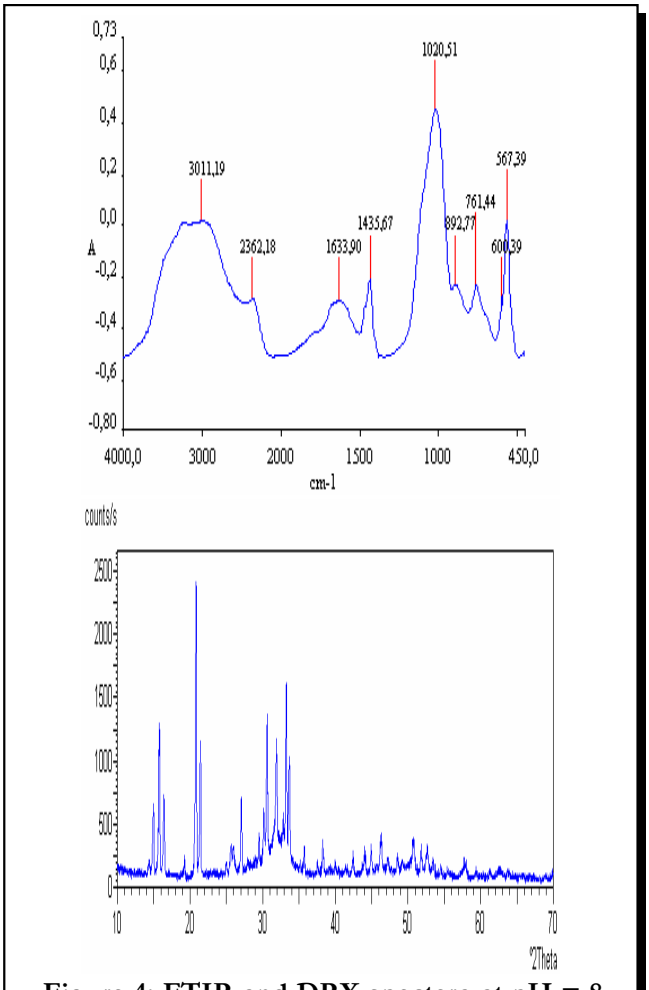

Figure 4: FTIR and DRX spectera at pH = 8

for the study of crystallization in the presence of inhibitors. In order to assess the inhibiting potential of substances for phosphates and understand the mechanisms of action of these inhibitors on phosphate crystallization steps (nucleation, growth, aggregation), we tested three inhibitors: citrate, aluminium, and ferric ions. The same procedure as above (absence of inhibitor) was followed. However, we added citrate amounts corresponding to the physiological concen trations to one of the two (A and B) solutions before mixing, at the same temperature 37°C. The plots of crystal size development as a function time follow the pattern as in the case without inhibitors. The same parameters (size and number of crystals, time of crystallization) were also investigated. Thus, knowing the size of crystals which are formed in the absence of inhibitor, it is easy to compare and to assess the role of the three inhibitors investigated.

A series of experiments corresponding to the physiological concentrations of 1, 2, 3, 4, 5 and 6 mM of citrate ion, 0.05, 0.07, 0.09, 0.1, 0.5, 0.8 and 1 mM of Al (III) ion and Fe (III) ion were carried out in order to cover the physiological excretion range. Equivalent amounts of sodium citrate have been added to solution B while those of Al (III) and Fe (III) were added to solution A. At the end of any experiment, the pH value was measured, because the crystallogenesis in these conditions is pH-dependent. To ensure reproducibility the results were obtained in triplicate, with the coefficient of variance (CV<10%). A six -hour test period was chosen for a pH = 6.5 and a four-hour one for the pH = 7 and 8[6].

The follow-up of the crystal size development by polarized light microscopy was carried out at time intervals of 5, 10, 20, 30 minutes up to 4 or 6 hours according to the pH of formation of crystals.

Afterwards, the collected precipitates were

TABLE 1: Precipitate composition

		pH=6.5			pH=7			pH=8		
		MO	**IRTF**	**RX**	**MO**	**IRTF**	**RX**	**MO**	**IRTF**	**RX**
Sans inhibiteur		Bru PACC	Bru PACC	Bru	Bru Str PACC	Bru Str PACC	Bru Str	Str CA	CA Str	Str
Citrate (mM)	1	Bru PACC	PACC bru	Bru	Bru PACC	Bru PACC	Bru	Str CA	Str CA	Str
	2	CA, OCP, PACC	CA, OCP, PACC	-	PACC Bru	PACC Bru	Bru	Str CA	Str CA	Str
	3	OCP PACC	OCP PACC	-	PACC Bru	PACC Bru	Bru	CA Str	CA Str	Str
	4	OCP PACC	OCP PACC	-	OCP	OCP	-	Str CA	Str CA	Str
	5	OCP PACC	OCP PACC	-	OCP	OCP	-	Str CA	Str CA	Str
	6	OCP Bru	OCP PACC	-	OCP	OCP	-	Str CA	Str CA	Str
Aluminium (mM)	0.05	PACC	PACC	-	PACC Bru	PACC Bru	Bru	Str CA	Str CA	Str CA
	0.1	PACC	PACC	-	PACC Bru	PACC Bru	Bru	Str CA	Str CA	Str CA
	0.5	PACC	PACC	-	PACC CA	PACC CA	-	Str CA	Str CA	Str CA
	0.8	With PACC	With PACC	-	PACC CA	PACC CA	-	Str CA	Str CA	Str CA
	1	With PACC	With PACC	-	PACC CA	PACC CA	-	Str CA	Str CA	Str CA
FerIII (mM)	0.05	Bru PACC	Bru PACC	Bru	Bru PACC	Bru PACC	Bru	CA Str	CA Str	Str
	0.1	PACC OCP	PACC OCP	-	Bru PACC	Bru PACC	Bru	CA Str	CA Str	Str
	0.25	PACC OCP	PACC OCP	-	PACC POP	PACC POP	-	CA Str	CA Str	Str
	0.5	PACC OCP	PACC OCP	-	PACC OCP	PACC OCP	-	CA Str	CA Str	Str

analysed by FTIR spectroscopy and XRD diffraction to determine the structure of the components formed or possibly extinct.

Inhibition at pH = 6.5

The results show that the addition of 1mM of citrate ion to the mixture not only decreases brushite crystal size from 26 to 5 μm but delays appearance time (from 50 to 60 min). The FTIR spectra of the precipitates were investigated in order to confirm inhibition presence. At pH = 6.5, citrate ion concen tration of 1 mM remained insufficient to induce total inhibition of the brushite in accordance with results obtained by polarized light microscopy. For a concentration in citrate ion higher or equal to 2 mM, no crystals were observed but granulations persisted. Negative polarization of granulations indicated the presence of amorphous carbonated calcium phosphates. The addition of 0.05 mM aluminium ion resulted in complete disappearance of brushite but granulations alone remained.

On the other hand, the addition of 0.05 mM of ferric ion did not result in total inhibition. The extent of inhibition increased with ferric ion concentration, until the value of 1 mM, where brushite crystals disappeared completely, but the granulations remained. The FTIR spectrum confirmed this inhibition through the absence of characteristic peaks of the brushite. The characteristic bands of pentahydrated octocalcic phosphate (POP) and of the amorphous calcium carbonated phosphate (ACCP) replaced those assigned to brushite.

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Inhibition at pH = 6.5, showed that citrate ion was a very good inhibitor of crystalline phosphocalcics. Growth inhibition and aggregation of brushite in the presence of aluminium ion was significant nearly for all concentrations. This highlights the effect of aluminium ion on phases formation of brushite at pH = 6.5. These results are in agreement with literature data concerning action of aluminium ion on germination and crystal growth of calcium phosphates[8].

The addition of aluminium ions results in the disappearance of the brushite characteristic band at a concentration of 0.05 mM, but ACCP bands persisted. On the other hand, ferric ion addition resulted in total disappearance of the characteristic bend of brushite at a concentration of 0.1 mM as analysed by FTIR spectroscopy. Phases that were present are POP and ACCP. The results are reported in the TABLE 1.

Inhibition at pH = 7

At pH = 7, the effects of citrate, aluminium and ferric ions are remarkable. Total inhibition of struvite was observed starting from an added concentration of 1mM of citrate. This shows that citrate is a good inhibitor for struvite at neutral pH. Brushite crystals size decreased in the presence of citrate ion until a concentration of 4 mM where brushite disappearance was complete inhibiting the remaining granulations. A reduction of 30.5% was obtained for 1 mM concentration whereas for 3 mM concentration this attained 86.1%. The granulations formed were identified by negative polarization as being the CA (carbapatite) and ACCP (amorphous carbonated calcium phosphate).

A slowing down of brushite formation time was observed as a function of citrate ion concentration. This was 180 minutes for a 3 mM concentration. It ranged from 60 minutes in the absence of inhibitor to 180 minutes in its presence. For a 4 mM concentration, citrate inhibited completely brushite formation. The FTIR spectrum showed total inhibition of struvite through the absence of the characteristic peaks but, ACCP predominance was observed followed by brushite.

However, in this concentration total inhibition of brushite was not achieved. Granulations persisted throughout reaction time. Delayed brushite formation favoured granulations which were transformed into brushite resulting in a lesser amount at the end of the experiment.

Optical microscopy showed that for a 4 mM citrate ion concentration, the two crystalline species, brushite and struvite disappeared completely. The presence of granulations, suggested the presence of apatite. FTIR spectrum confirmed total disappearance of struvite and brushite and proved the inhibition of possible transformation of apatite into brushite or struvite. The only constituent present is the pentahydrated octocalcic phosphate (POP) with some withlockite. To simulate the physiological conditions of citrate ion concentrations, we also used a 6 mM concentration similar results were obtained. For aluminium ion, adding 0.1 mM to urine was enough to inhibit brushite crystals completely. However, total disappearance was observed at 0.5 mM concentration. Again ACCP and carbapatite remained. For ferric ion, total disappearance of brushite was achieved for a 0.25 mM concentration, with ACCP and POP remaining (see TABLE 1).

Inhibition at pH = 8

It was observed that in general nucleation occurs quickly at all concentrations although some progressive reduction of crystals size was observed with citrate, aluminium and ferric ions concentration.

The effect of citrate on struvite at pH = 8 was not as significant as at pH = 7 for a reaction time of 4 hours. Crystal size reached a minimal value of 23 mm for a concentration of citrate ion of 6mM, hence a reduction of 48.9% with respect to the value without inhibitor. FTIR spectrum for the same concentration showed the presence of carbapatite as being the major component with some struvite, confirming the optical microscopy results.

The effect of citrate ion at pH = 8 resulted in more important granulations at the end of the experiment. Struvite crystal size and number diminished with respect to crystallization without inhibitors. It may be suggested that citrate ion interferes with growth and aggregation of crystals. Moreover, crystal size decreased with aluminium ions concentration. After the addition of 1mM aluminium ion concentration, the size was reduced by 16 % in comparison with results in the absence of inhibitor. FTIR spectrum

for a high concentration shows the persistence of carbapatite and the struvite. The effect of the ferric ions on the struvite size is less significant at pH = 8 than pH = 7. One notes a reduction of 23.3% after the addition a concentration of 0.75 mm of ferric ions. The results are reported in the TABLE 1.

The latter did not cause the disappearance of the bands characteristic of carbapatite and struvite. FTIR Spectrum confirms the observations by polarized light microscopy.

CONCLUSION

Phosphate compounds encountered in urine can be dangerous and the use of inhibitors to prevent, slow down or reduce crystallization phases might be very helpful. In this investigation, citrate ion proved to be a good small molecular weight inhibitor. Its effect increases with solution pH but it is more efficient in less acidic or neutral urine than in alkaline one.

The addition of citrate ions to phosphates stabilizes granulations and may retard for a certain time crystalline transformation. For many authors brushite may change into apatite which is the most stable phase thermodynamically.

The results showed that the addition of 1 mM of citrate ions at pH=6.5 resulted in 10 minute delay of bushite formation and size reduction of 65.8%. Inhibition was total with 2 mM citrate ion concentration but POP persisted through. Citrate provoked a crystalline transformation at pH=6.5, of ACCP and POP at all concentrations, since the POP doesn't exist in the absence of inhibitor. The POP amount was minor after the addition of 6 mM of citrate ion concentration.

In the presence of aluminium ion brushite growth and aggregation inhibition was significant even for small concentrations. Adding of 0.05 mM inhibited the formation brushite. This powerful effect of aluminium on brushite phase formation at pH = 6.5. The results seem in agreement literature data on the effect of aluminium on calcium phosphates crystallization, and growth. The working action of aluminium ions seems to be complexation and surfacic adsorption. Addition of a maximum concentration of 1 mM showed the total inhibition and crystal transformation of ACCP into withlockite.

On the other hand, the effect of ferric ions was less important than the aluminum ions at pH = 6.5. Inhibition was total at a concentration of 0.1 mM of Fe(III). The amorphous carbonate complex phosphate and the POP were the only constituents persisting. FTIR spectra showed the remaining of only amorphous species ACCP at 0.75 mM concentration addition. The effect of citrate ion on brushite was less important at pH=7, than pH=6.5. Crystal disappearance of brushite was observed for 3 mM of citrate ion. On the other hand, total disappearance of struvite for a concentration of 1 mM of citrate ion at pH=7, showing a greater effect at this pH than at ph = 8. Whatever the pH, the granulation level of P.O.P or ACCP or carbapatite was significant at higher concentrations. The inhibiting power of citrate ion was more significant at pH=8 on aggregation and crystal size, but total inhibition of crystalline species was not completely achieved.

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