

CALCIUM OXALATE CRYSTALLIZATION IN UNDILUTED URINE OF HEALTHY MALES: IN VITRO AND IN VIVO EFFECTS OF VARIOUS CITRATE COMPOUNDS

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Abstract

We evaluated the crystallization of calcium oxalate (CaOx) in undiluted urine, collected 3 hours after intake of a test meal by healthy males. In two experiments *in vitro*, either the total citrate concentration was increased (urine A) or the pH was elevated (urine B). In addition, in three clinical trials, the bioequivalence of orally taken potassium citrate (PC) and potassium-sodium citrate (PSC) (n = 9), and the dose-response effects of oral PC (n = 8) and oral calcium-sodium citrate (CSC; n = 8) were studied. Elevation of urinary citrate (urine A) decreased CaOx crystallization (nucleation, growth, agglomeration time), and increased the calcium and citrate content of the crystallized mass; elevation of urinary pH (urine B) also suppressed CaOx crystallization, the calculated molar ratio ionized citrate/ionized calcium at pH 7 was about twice the value observed at pH 5.5, and the ratio complexed citrate/complexed calcium was low. PC and PSC inhibited CaOx crystallization to a similar extent. CSC increased crystal growth but left agglomeration time unchanged. The urinary molar ratio (total calcium/total citrate) appeared to be directly related to the CaOx crystal growth rate.

We concluded that: (1) changes in urine pH, citrate, and calcium are reflected by CaOx crystallization; (2) free citrate and a calcium citrate complex (stoichiometry $\leq 1:1$) inhibit CaOx crystallization; (3) in individuals taking PC, PSC or CSC, the renal stone risk may be characterized using the presented CaOx crystallization test and the urinary calcium/citrate ratio.

Key Words: Calcium oxalate, crystallization parameters, urinary citrate and pH, urinary calcium, crystallization inhibition, calcium/citrate ratio.

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Introduction

Renal calcium-containing concretions represent a major health care and financial burden worldwide, and enormous efforts are made by investigators to scale down the incidence rates. The predominant component of renal calcium stones is calcium oxalate (CaOx), mostly in the mono- or dihydrate form.

CaOx crystal nucleation, growth and agglomeration in urine are believed to be controlled by a balance between the ambient physico-chemical supersaturation and inhibitors capable of acting on one or more of these crystallization steps. To imitate in the laboratory CaOx crystallization processes resembling those occurring under *in vivo* conditions, several test procedures have been developed [2, 29]. However, studies on crystallization inhibition in diluted urine may lead to false conclusions; for example, the inhibitory actions of small-molecular substances like citrate depend on their stoichiometry [14], and those of large-molecular substances like proteins depend on molecular configuration [18]. In contrast, crystallization tests which take into account that, at some site within the nephron a degree of supersaturation prevails that is sufficiently high to allow for precipitation, more realistically reflect the sequence of events assumed to occur in stone formation [25]. Evidence is increasing that among the crystallization steps (nucleation, growth, agglomeration of crystals) the time elapsing until crystals agglomerate to larger particles is a crucial, if not the most important; stones may develop owing to retention of particles at anatomically narrow intraluminal nephron sites [22]. We described a crystallization test that is applicable to undiluted urine [11, 32]. The test distinguishes non-stone-forming healthy males from untreated male stone patients [11]; but whether it is sensitive for crystallization changes introduced by citrate so far is insufficiently documented.

At present, citrate-containing preparations are marketed as alkali (potassium, sodium, and combinations of these) and alkaline earth (calcium, magnesium) salts, or mixtures of the former and the latter. Alkali citrates have gained widespread interest in the treatment of renal stones because it elevates urine pH, inhibits CaOx and calcium phosphate crystallization [41], correct a urinary citrate

deficiency common to stone patients [33, 34], and may halt stone formation in the long term [1, 19, 20]. Thus, alkali citrates are increasingly being used as anti-stone drugs. In contrast, calcium citrate is being considered as a treatment for osteoporosis [7], but it may carry the risk for urinary stone formation due to increasing calciuria [23, 24]. Also, while urinary alkalization drives deprotonation of citrate, probably resulting in CaOx crystallization inhibition by charged citrate [9, 13], the alkalization potential of calcium citrate may be weak. On the other hand, although the urinary pH may remain more or less unchanged by calcium citrate, a citruria-stimulating effect may be detectable. While under alkali citrate medication CaOx crystal agglomeration time was found to be directly related to the citrate concentration [21], it is unknown whether the amount of citrate appearing in urine in response to intake of calcium citrate also permits prolongation of the agglomeration time. Theoretically, the urinary calcium/citrate ratio could be a link between agglomeration time and urinary citrate concentration achieved with the various citrate compounds. Thus, the present work addresses questions on the state of CaOx crystallization seen with manipulating citrate in undiluted urine of males.

In the first part of the work, the effects of varying *in vitro* the citrate level or pH of undiluted urine on the morphology of CaOx crystals, and on calcium, oxalate and citrate in dissolved crystals were investigated. In the second part, the effects of orally taken citrate preparations, differing with respect to the cation composition, were studied in healthy males, emphasizing CaOx crystal growth, crystal agglomeration time, and the calcium/citrate ratio.

Materials and Methods

Chemicals and equipment

The chemicals used for analyses were of analytical grade (Fluka, Buchs, Switzerland), the citrate-containing preparations (see **Effects of *in vitro* variation of urinary citrate and pH** below) of the highest degree of purity available. Conventional laboratory facilities were used throughout, with the exception of specialized software (see **Analyses and calculations** below) and scanning electron microscopy (SEM) (ORTEX System 5000, EG&G ORTEC, Oak Ridge, TN, USA) equipped with energy-dispersive X-ray analysis (EDX) to probe the elements contained in crystals.

Urine collection

A total of 31 male volunteers, aged 21–40 years, were studied after an overnight 12–15 hour fasting period. They were stone-free, and were not suffering from any clinical disorder known to predispose to renal stones or osteoporosis. After voiding the bladder, at about 9:00 AM,

they took a standardized meal together with a 200–300 ml drink. The composition of the meal and the drink differed in the *in vitro* and *in vivo* studies, but both had a high calcium content (see below). Three hours after the meal the bladder was voided into a pre-warmed (37°C) beaker, the urine immediately filtered through a 4–7 µm pore size filter paper (type 595 1/2, diameter 125 mm, Schecicher and Schüll, Dassel, Germany) to remove gross particles, such as mucus, and then centrifuged at 3000 g, for 2 minutes, at room temperature. Thereafter, urine was further processed as detailed below.

Calcium oxalate crystallization

The single step, small-scale test procedure was carried out in a series of up to 20 Eppendorf vials (Eppendorf GmbH, Hamburg, Germany), each containing 490 µl undiluted postprandial urine [11, 32]. The procedure is as follows: induction of CaOx nucleation by addition of 10 µl sodium oxalate of increasing concentration (0.2 to 1.5 mM), 30 minute incubation in an agitated water bath (37°C; 120 rpm), and light microscopic identification within 2 minutes of the tube in which crystals were unequivocally visible when urine was transferred to a counting chamber (type Neubauer hemocytometer, 0.1 mm depth, 0.0025 mm² area, Brand GmbH, Wertheim, Germany) kept at room temperature; the oxalate concentration in the tube preceding this so-called “critical” tube was considered the “tolerable oxalate” (synonymous metastable limit of CaOx solubility). The growth of CaOx crystals was stimulated by immediate addition of 10 µl 0.2 mM sodium oxalate to the “critical” tube, followed by another incubation period of 30 minute duration; thereafter, the mean diameter of the ten largest crystals visible was assessed using image analysis equipment connected to a personal computer (Global Lab Image, Data Translation, Bietigheim-Bissingen, Germany). Crystal agglomeration time was assessed at intervals of 10–15 minutes over a 90 minute period, beginning at nucleation, and was read on the first emergence of at least three aggregates per counting field (see above). In contrast to stone patients, CaOx nucleation in non-stone-forming healthy males regularly occurs at a total oxalate concentration of between 0.5 and 0.6 mM. The degree of solution depletion (evaluated by ¹⁴C-oxalic acid) at the stage of nucleation is negligible, and both crystal number and size (approx. 3 µm diameter) are small. During a post-nucleation incubation period of 90 minutes the crystal diameter rarely exceeds 8 µm, and the agglomeration time is regularly > 30 minutes (95% confidence interval 25–82). The apparent CaOx crystal growth rate is linear (range 0.05–0.17 µm/min), as several additional modes of calculation (exponential, reciprocal, multiplicative) failed to demonstrate a higher order of kinetics. A similar growth rate (0.1 µm/min) was observed in artificial urine [12, 15], and in a test system using undiluted whole urine [30]. The ratio of crystal

diameter at 30 minutes post-nucleation time-point and crystal agglomeration time was therefore taken as the apparent crystal growth rate.

Effects of *in vitro* variation of urinary citrate and pH

The rationale for these experiments was as follows. Although initial clinical trials revealed that oral intake of alkali citrate by healthy volunteers is followed by delayed nucleation, recognizable by higher tolerable oxalate (see above) in postprandial urine, it is still not clear whether citrate acts primarily through calcium complexation or direct inhibition of the clustering of molecular calcium oxalate. For example, the total oxalate concentration tolerated at the stage of nucleation does not discriminate non-stone-forming healthy controls from renal calcium stone patients [11], but discrimination was possible when the total crystal surface available, calculated by appropriate formulas, was taken into consideration [32]. This observation suggests that the superficial growth sites of CaOx particles, the diameter of which is below the detection limit of light microscopy (approx. 2 μm), are the major targets of citrate in crystallization inhibition. Furthermore, the concentration of free (triple-negatively charged) citrate is pH-dependent, and its inhibitory action is stronger than the one of protonated citrate species [9]. To elucidate the situation further, we studied the citrate content of the crystallized matter, after prior *in vitro* addition of citrate to urine, or after changing the pH of urine.

In practice, six participants were loaded with a calcium (1000 mg)- and carbohydrate (72 g)-rich synthetic meal of fixed composition. The meal was taken in the form of a suspension (in 300 ml distilled water). Due to its weak buffering capacity the meal decreases the postprandial urinary pH to values < 6.0 , and due to its high calcium and carbohydrate content the urinary phosphate concentration decreases {as a result of calcium suppression of parathyroid glands and release of insulin which is anti-phosphaturic [10, 35, 38]}. In this urinary environment the co-precipitation of calcium phosphate [5, 17] is only exceptionally seen. The individual urines were pooled, and two aliquots, urine A and B, were made. The pH of urine A was adjusted to 6.0 with 1 M potassium bicarbonate; aliquots of sufficient volume to perform replicates of the crystallization test (see below) were spiked with ammonium citrate to give citrate concentrations 1, 2 and 4 mM above the initial citrate concentration. In urine B, the total citrate concentration was adjusted to 2.5 mM with ammonium citrate; in one aliquot the original pH of 5.5 was left unchanged, in other aliquots potassium bicarbonate was added to give a pH of 6.0, 6.5 and 7.0, respectively.

The eight urine samples were further studied for calcium oxalate crystallization as outlined above using two 2 ml samples (instead of 0.5 ml as in the routine procedure). After 90 minutes post-nucleation incubation, one sample

was vacuum-suctioned through an 0.22 μm pore size filter (Nuclepore, Pleasanton, CA, USA), the filter rinsed twice with bidistilled water, air-dried, covered and carefully stored at a dry place, until SEM examination and EDX analysis using standard procedures. To assess the amounts of calcium, oxalate, and citrate associated with crystals formed, the other sample was centrifuged (3000 g, 10 minutes), the supernatant suctioned off, the crystal-containing sediment pooled and washed twice with bidistilled water, and thereafter dissolved in 1 ml 0.1 M HCl, for analysis of the three substances.

Oral citrate loads

Neutral potassium citrate (PC), potassium-sodium-hydrogen citrate (PSC), and calcium-sodium citrate (CSC) were used (all from Madaus, Cologne, Germany). CSC undergoes complete dissolution at *in vitro* pH < 4.0 ; its solubility in the stomach during fasting can be deduced from the fact that calcium has been shown to be effectively absorbed in the intestine (unpublished data); hence CSC is readily bioavailable.

Trial 1 comprised 9 participants, age 22-32 (mean 28) years, and was designed to show whether there is bioequivalence (with respect to inhibition of calcium oxalate crystallization; see below) of 40 milliequivalent citrate taken orally as PC and the same amount of PSC taken on a separate day (1 week apart). Both preparations were dissolved in 200 ml distilled water and taken together with a continental breakfast (2 sandwiches, butter, honey, 1 glass of milk), the total calcium content of this meal being approx. 400 mg.

The other two trials comprised 8 participants each, age 23-31 (mean 27), and were designed as dose-response studies of PC and CSC, respectively. Both preparations were taken together with the meal described in the above mentioned bioequivalence trial. The dosages (as milliequivalent citrate) for PC were 20, 40 and 60, for CSC 30, 60 and 90. The higher citrate dosage of CSC was chosen to compensate for the smaller alkali content of this preparation (10 milliequivalent versus 20 milliequivalent in PC), i.e., to minimize the possibility that CaOx crystallization was enhanced by the calcium moiety. CSC at the doses used in this trial supplies calcium in amounts of 400, 800, or 1200 mg, in agreement with accepted treatment regimens for osteoporosis [28].

Analyses and calculations

All chemical analyses followed established procedures, among others calcium (by atomic absorption spectroscopy), citrate (enzymatically), and oxalate (by high performance ion chromatography); for details, see ref. [11, 32, 33]. The ionized and complexed calcium, magnesium, citrate, and the CaOx supersaturation (ΔG) in pool urine B were derived from the EQUIL-2 software [44]. In the clinical trials, the significance ($p \leq 0.05$) of differences between the

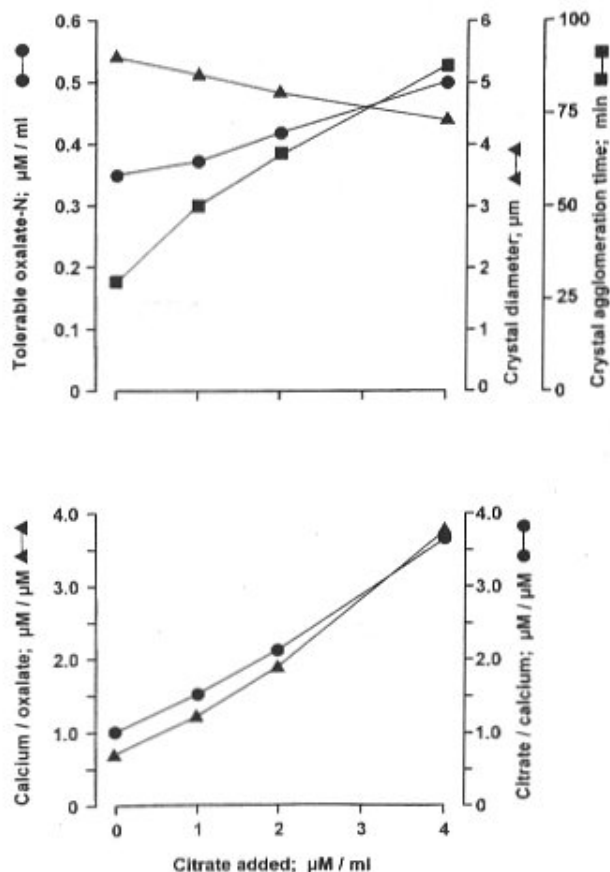


Figure 1. CaOx crystallization steps in pool urine A, pH 6.0, without (0) and with addition of ammonium citrate (1, 2 and 4 µMol per ml urine). **Upper part:** three parameters as read from the test procedure. **Lower part:** Ratios of substances after dissolution of crystals. Each point represents the mean value of triplicate determinations. For further details, see **Materials and Methods**.

control and post-citrate load groups were examined using appropriate statistics software.

Results and Comments

CaOx crystallization on *in vitro* variation of citrate in pool urine A (Fig. 1, Table 1)

At pH 6.0, increasing the total citrate concentration evoked a marked change of crystallization. At a total citrate concentration of > 5 mM [endogenous citrate (1.6 mM) plus added citrate (4 mM); see Table 1] tolerable oxalate concentration (see above) and crystal aggregation time rose above the control value (no citrate added) by about 37 and 200%, respectively, while the crystal diameter decreased to 78% of the control value (Fig. 1, upper part); the

accompanying growth rate was 0.05 µm/min in the citrate-spiked (> 5 mM citrate) versus 0.2 µm/min in the unspiked urine. Using light microscopy, no calcium phosphate was detectable, although at pH 6 the acidic calcium phosphate (brushite) could theoretically have developed. The predominant crystal type was the energetically less stable dihydrated calcium oxalate, but to some extent the energetically more favorable monohydrate form was also present in the unspiked urine. This crystal morphology was confirmed by SEM examination of crystallized particles harvested from the 0.22 µm pore size filter, and the absence of a phosphorus peak was confirmed by EDX analysis (data not shown). Analysis of calcium, oxalate, and citrate in the filtrate and in the dissolved crystals showed the following: with increasing urinary citrate concentration there was an increase of citrate, but to a lesser degree also of oxalate and calcium in the filtrate; in contrast, oxalate and calcium directly associated with the crystals decreased dramatically (oxalate by 93%, calcium by 70%), while citrate increased (by 15%), as compared with the control value (Table 1). The underlying molar calcium/oxalate and citrate/calcium ratio in the solubilized crystals rose almost in parallel (Fig. 1, lower part). We interpret this spectrum of changes to mean that in the presence of excess citrate the 1:1 stoichiometry of crystallized CaOx was lost in favor of a calcium-containing citrate compound, stoichiometry < 3:2, presumably monovalent calcium citrate (CaCit⁻). On the basis of theoretical considerations of citrate adsorption to CaOx crystals previous workers postulated that at pH 6.0 and above not only trivalent citrate, but also other citrate species, including CaCit⁻ should inhibit CaOx crystallization [6, 13]. The relative increase in calcium over oxalate, and in citrate over calcium, in crystallized CaOx (Fig. 1, lower part) are strong arguments against the theory that urinary citrate acts exclusively as a calcium chelator. Collectively, the findings show that once CaOx crystals are formed in urine, citrate, even in the presence of unchanged pH, can associate with them and thereby diminish their growth and agglomeration, and that the effect is impressively demonstrable at high citrate concentrations, preferably 5–6 mM (approx. 1000 mg/litre), which are still within the normal range in humans. In accord with this observation is a report by other authors of citrate inhibition of CaOx crystallization in whole urine [3], and also one of our previous studies [36]; in this latter report the long-term treatment of renal stone patients with alkali citrate successfully prevented new stones, but re-examination in the laboratory showed that urinary pH had returned to pre-treatment baseline values, while urinary citrate was high.

CaOx crystallization on *in vitro* variation of pH in pool urine B (Fig. 2, Table 2)

The selected pH range was 5.5–7.0. The morphology of crystals on SEM revealed a predominance of CaOx

Table 1. Citrate concentrations of pool urine A*, pH 6.0, and additional data on calcium, oxalate, and citrate of filtrate and crystals. For further information, see **Materials and Methods**. Values are means of duplicate determinations.

Citrate		Filtrate			Crystals		
Endogenous (mM)	Added (mM)	Calcium (mM)	Oxalate (mM)	Citrate (mM)	Calcium (mM)	Oxalate (mM)	Citrate (mM)
1.6	0	2.333	0.543	1.491	0.093	0.121	0.089
1.6	1	2.340	0.608	2.465	0.068	0.056	0.099
1.6	2	2.343	0.626	3.389	0.046	0.031	0.095
1.6	4	2.410	0.662	5.017	0.028	0.008	0.102

*concentration (mM) of major constituents: sodium (150), potassium (70), calcium (2.5), chloride (100), inorganic phosphate (8), sulfate (15), oxalate (0.30), citrate (1.60).

Table 2. pH of pool urine B*, and additional data derived from EQUIL-2. RSP-CaOx: relative supersaturation product, expressed as free energy (ΔG); $\text{Cit}^3\text{-}/\text{Ca}^{2+}$: ionized citrate/ionized calcium; $\text{Cit-C}/\text{Ca-C}$: complexed citrate/complexed calcium.

pH**	RSP-CaOx; (ΔG)	Calcium (mM)		Citrate (mM) $\text{Cit}^3\text{-}/\text{Ca}^{2+}$		Cit-C/Ca-C (mM/mM)	
		Ionized	Complexed	Ionized	Complexed	(mM/mM)	(mM/mM)
5.5	2.43	1.51	0.99	0.27	2.23	0.179	2.252
6.0	2.37	1.38	1.21	0.35	2.15	0.254	1.777
6.5	2.32	1.29	1.21	0.40	2.10	0.310	1.736
7.0	2.27	1.23	1.27	0.43	2.07	0.350	1.630

*for concentration of major constituents see Table 1, except that total citrate concentration was brought to 2.5 mM by addition of ammonium citrate;

**adjusted by addition of potassium bicarbonate.

monohydrate over CaOx dihydrate at pH 5.5, but this situation was reversed with increasing pH: at pH 7.0 not only was there predominance of CaOx dihydrate, but also crystal number and diameter were dramatically reduced (Fig. 2). Accordingly, the crystallization test revealed higher tolerable oxalate and agglomeration time but smaller crystal diameter at pH 7.0 versus pH 5.5. Again, more or less crystallized calcium phosphate was not recognizable by optical means, and EDX analysis failed to identify a phosphorus peak (Fig. 3). Obviously, CaOx crystallization inhibition was effectively driven by the increasing pH alone. Thus, elevation of urinary pH is a powerful means of reducing CaOx crystallization, despite the coexistence of unchanged urinary total citrate concentration. Previous investigators of possible interdependencies of pH and CaOx supersaturation in urine found that they varied largely independently [16]. In support of this view would be the present observation that the decline in CaOx supersaturation at pH 7 was only 6.5% (Table 2), but the decrease in CaOx crystallization in these urines was marked. When explaining similar findings in whole urine, other investigators ascribed

them to interference of calcium phosphate crystallization, which per se may have lowered the ambient calcium concentration [4, 40] (see below).

The calculations using EQUIL (Table 2) indicate that the pH-dependent changes in crystallization included an increase in ionised citrate (approx. 59%) and a markedly smaller increase in complexed calcium (approx. 28%), while complexed citrate and ionised calcium declined moderately (approx. 7 and 18%, respectively). These data argue against the possibility that the strongly reduced CaOx crystallization exclusively reflects the declining CaOx supersaturation, introduced by solution depletion during the post-nucleation incubation period. It should be further noted that the accompanying magnesium data (obtained from EQUIL; not shown) were similar to those for calcium (Table 2), which is consistent with the fact that the complexes magnesium citrate and calcium citrate share similar stability constants [42].

Formation of the magnesium complex should therefore have reduced the overall inhibitory potential of citrate, a possibility that cannot be ruled out. Magnesium is under

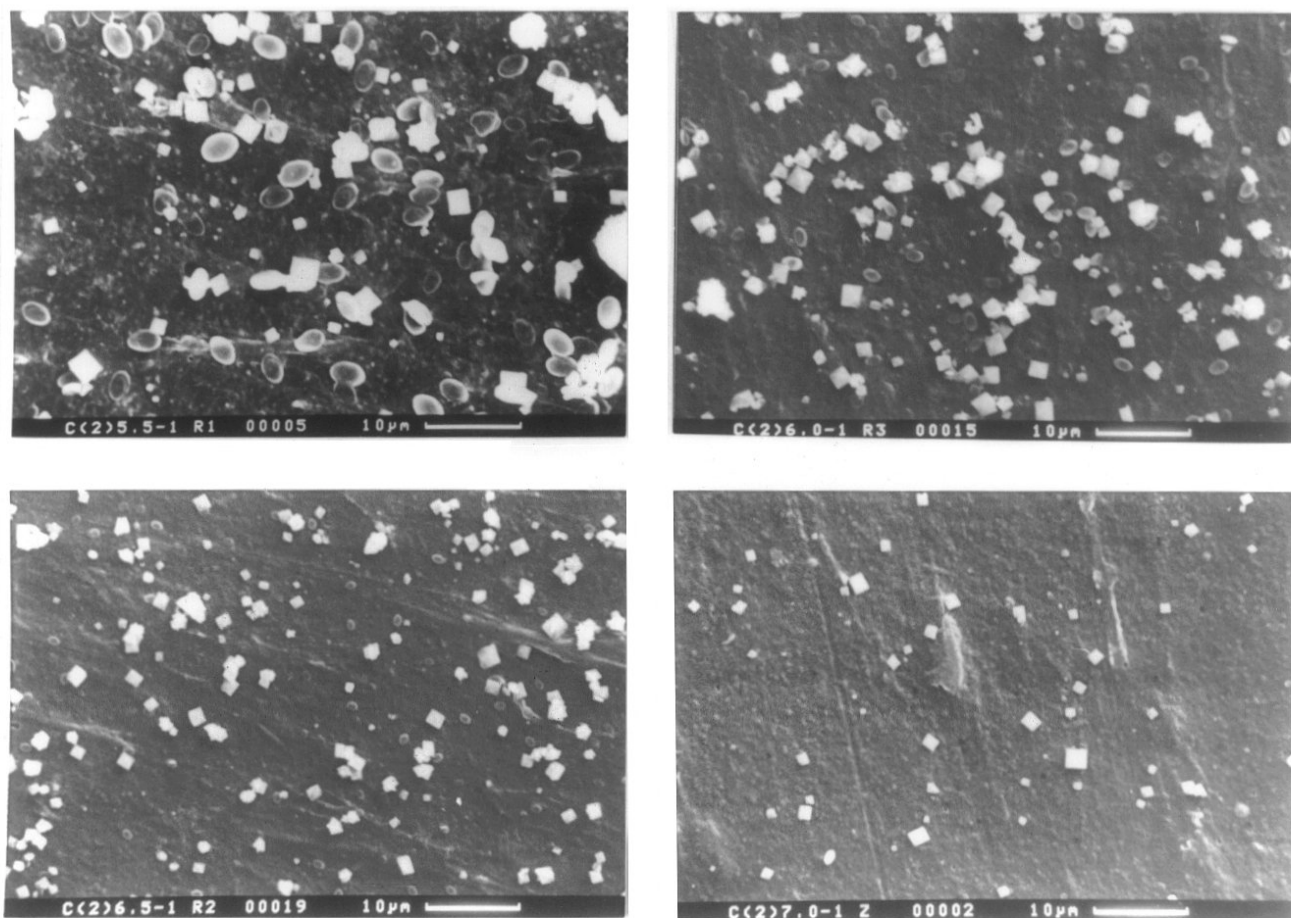


Figure 2. Scanning electron micrograph of crystals harvested from the four preparations of pool urine B (total citrate concentration 2.5 mM; see Table 2), after 90 minutes incubation following nucleation. The pH was adjusted to 5.5, 6.0 (upper left and right), 6.5, 7.0 (lower left and right). For details see **Materials and Methods**.

discussion as inhibitor of CaOx crystallization [39], but the net effect on the latter of a change in the magnesium status in the present experiment remains elusive. The possibility of interference by struvite, the nucleation of which starts at a pH of around 7, can be ruled out by the absence of a magnesium peak in EDX analysis (Fig. 3). Collectively, the EQUIL data, together with the markedly attenuated CaOx crystallization seen at pH 7, should primarily reflect the excess of ionised citrate over ionised calcium. This spectrum of observations is reminiscent of an etiologically poorly characterized subgroup of renal calcium stone patients that present with low-normal urinary citrate and high-normal urinary pH, but still only moderate metabolic activity (unpublished observations).

CaOx crystallization after oral citrate

Rationale: The CaOx crystallization test with different citrate compounds was based on the following considerations. Variations in the degree of urinary citrate and

pH can be observed in response to oral citrate; however, the latter depends on the cationic composition (alkali versus alkaline earth). It was therefore assumed that the calcium, oxalate, and citrate content of the crystallized matter follows the rules recognizable in the above described *in vitro* experiments. The usefulness of sodium citrate as a CaOx crystallization inhibitor has been doubted, because after its intake the proneness of urine to form stones may remain largely unchanged [27, 31] due to the known calciuric actions of sodium [43]. Thus, generation of a renal stone risk either by high oral sodium or calcium, or both, may be real [23, 24]. In contrast, the stone risk should be less when calcium is taken in the form of citrate, because citrate is thought to inhibit steps of crystallization. This inhibitory effect should then be even more pronounced with a calcium citrate preparation also containing alkali, because alkali would increase both urinary pH and citrate, and thereby augment CaOx crystallization inhibition.

Bioequivalence of PC and PSC: Table 3 demonstrates

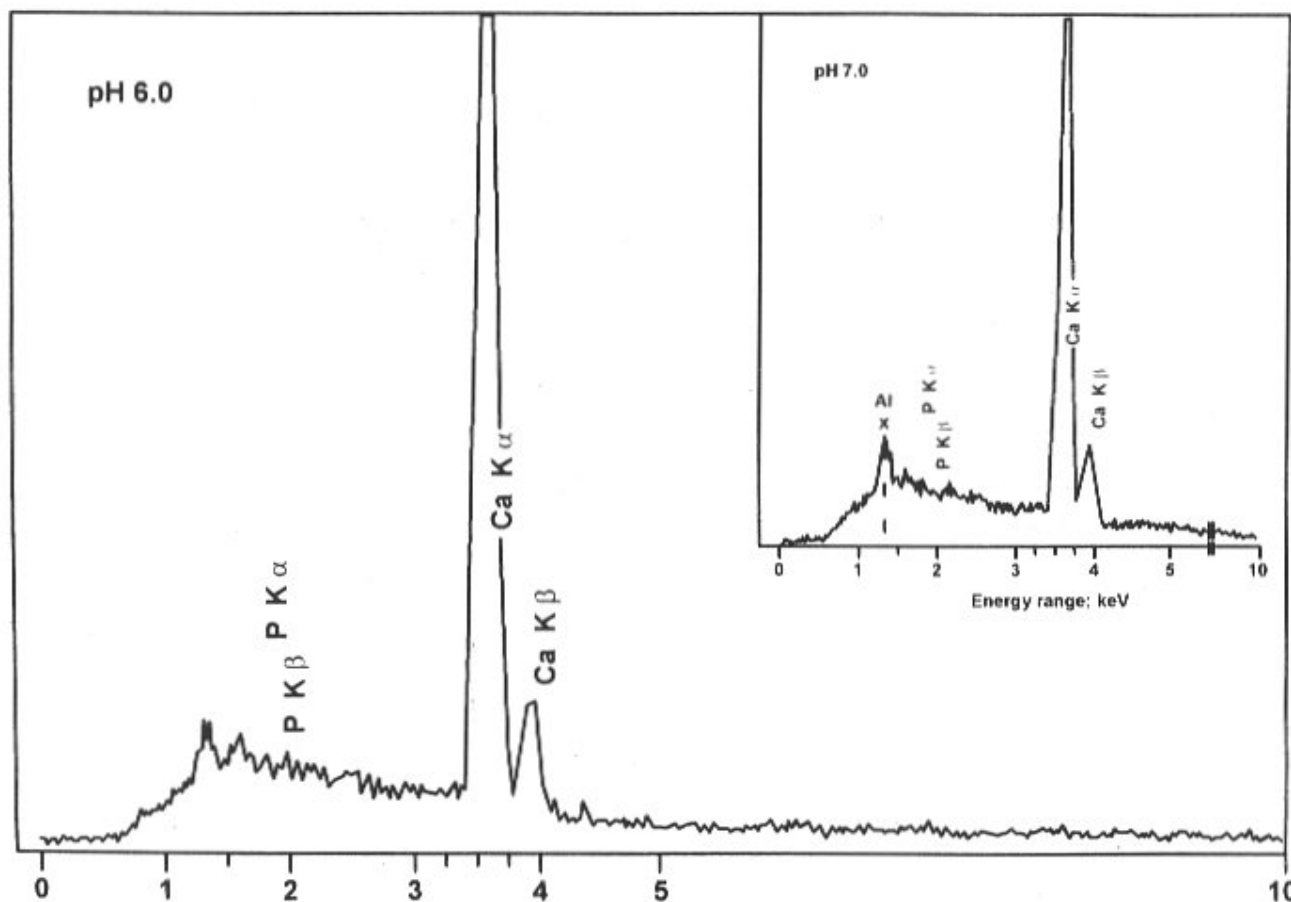


Figure 3. EDX analysis of elements contained in the crystals developed in pool urine B, at pH 6.5 and (inset) 7.0. Note the absence of a phosphorus peak. X: Aluminum (Al) contamination.

that in comparison with the effects of the control load (breakfast alone) both preparations elevate pH and citrate almost equally and significantly, and significantly reduce the calcium/citrate ratio; in contrast, the reduction of the crystal growth rate is only of borderline significance ($p < 0.10$), and agglomeration time as well as other CaOx crystallization test parameters, although uniformly signalling calcium oxalate inhibition, were not always significantly changed versus the control load. In other words, the widely used alkali citrates, taken by healthy males at commonly recommended dosages, achieved the desired increase in citrate and pH; the concentration of calcium, lower with PC than with the control or PSC load, was outweighed by the smaller rise in citrate with PC versus PSC, and thereby resulted in a roughly equal reduction of the calcium/citrate ratio. The question arises as to whether this ratio is in fact a superior marker of the state of CaOx crystallization under oral citrate, since it obviously reflects the combined changes of urinary acid-base (citrate included) and calcium status. Further answers were provided by the effects of PC and

CSC (see below).

Dose-response effects of CSC and PC: Table 4 demonstrates that CSC at dosages delivering 30, 60, or 90 milliequivalent citrate, and PC at dosages delivering 20, 40, or 60 milliequivalent citrate, achieve a clearly different urine composition. PC induced a dose-dependent increase of pH, citrate, tolerable oxalate, and agglomeration time, decreased the calcium/citrate ratio, crystal diameter, and crystal growth rate, but left oxalate statistically unchanged. The impressive rise in agglomeration time under PC is further illustrated in Figure 4, upper part. CSC increased calcium, which was expected; there was also a tendency for an increase of citrate, calcium/citrate ratio, crystal diameter, crystal growth rate, and oxalate, while tolerable oxalate tended to low values. CSC left crystal agglomeration time unchanged, however, and this was further illustrated in Figure 4 (lower part). The combined data seemed to suggest that the rather disparate development of urine pH and substance concentration under PC or CSC are best reflected by the calcium/citrate ratio and, to a certain extent, also the

Table 3. Effects of potassium citrate (PC) and potassium-sodium citrate (PSC) on pH, substances, and CaOx crystallization parameters in postprandial urine of 9 male volunteers participating in the clinical trial 1, designed to evaluate the bioequivalence of the two substances. A continental breakfast was taken alone (B) or, on a day one week apart, together with 40 milliequivalent citrate of the respective preparation. Data are mean values (SE). For further details see text.

Variables	B		B + PC		B + PSC	
pH	5.83	(0.18)	6.87	(0.06) ^a	6.50	(0.10) ^a
Oxalate (mM)	0.069	(0.015)	0.078	(0.015)	0.119	(0.034)
Calcium (mM)	1.39	(0.39)	0.90	(0.10)	1.42	(0.23)
Citrate (mM)	0.72	(0.08)	1.27	(0.15) ^a	1.42	(0.25) ^a
Calcium/Citrate (mM/mM)	2.0	(0.6)	1.0	(0.2) ^a	1.1	(0.3) ^a
Tolerable oxalate* (mM)	0.68	(0.10)	1.33	(0.16) ^a	1.03	(0.19)
Crystal diameter** (μm)	4.8	(0.1)	4.6	(0.2)	4.4	(0.1) ^a
Agglomeration time (min)	55	(8)	71	(14)	66	(6)
Crystal growth rate*** (μm/min)	0.10	(0.02)	0.08	(0.03)	0.07	(0.01)

^a $p \leq 0.05$ versus B, according to one-way ANOVA and LSD (least square differences) test

*at nucleation;

**crystal diameter observed at time-point 30 minutes after nucleation;

***expressed as ratio of crystal diameter and agglomeration time.

crystal growth rate, but not by agglomeration time alone. We interpret this to mean that calcium supplementation of daily food in fact may be able to generate a risk for kidney stones, unless calcium is supplied as calcium citrate and a sufficiently high alkali content of the preparation, thereby counterbalancing the threat of increasing CaOx crystal growth. In a preliminary communication the latter idea was further pursued, using a preparation with a higher alkali but similar calcium content [37]. The information now available in our laboratory on the calcium/citrate ratio and the crystal growth rate has been tentatively incorporated in Figure 5; it is apparent that the higher the alkali moiety of a given citrate preparation, calcium citrate included, the lower will be the calcium/citrate ratio and the CaOx crystal growth rate. A combined assessment of the calcium/citrate ratio, crystal growth, and agglomeration time may therefore permit deeper insight into urinary chemistry, supersaturation products, and CaOx crystallization, than any of these parameters alone.

Concluding Remarks and Outlook

CaOx crystallization studies as carried out in undiluted or even whole urine from non-stone-forming humans suffer from the disadvantage that they cannot mirror the complexity of CaOx stone formation, which is assumed to take place in the various parts of the nephron, where actual solute concentrations deviate by factors from those in urine. Within this limitation, the present and previous [11, 32] work on CaOx crystallization in undiluted urine, collected under strictly controlled conditions, gives further

insight. The absence of particulated calcium phosphate, even at pH 7, from the urine of healthy individuals allows to discount heterogeneous nucleation as event interfering with visible CaOx crystallization. Therefore, the described simple test procedure is specific for CaOx and obviates the need for extensive equipment. The predominant development at $\text{pH} < 6$ of CaOx monohydrate rather than the dihydrate form may indicate that urine composition, not the test characteristics, dictates crystal morphology [26].

The urine of healthy volunteers in the clinical trials should give a realistic picture of the effects of oral citrate(s); indicative of this is the delay in crystal agglomeration time as well as the low calcium/citrate ratio, both seen with oral PC (Figs. 4 and 5). Interestingly, the present method of assessing CaOx crystallization was able to show that CaOx crystal growth also in renal calcium stone patients was virtually normalized by PC taken over several months [11]. When the so-called “constant composition technique” was applied to the same urine samples, it was not found to be superior in detecting CaOx crystal growth inhibition [11], suggesting that both methods are equally sensitive to the PC-induced beneficial alterations of urine composition. To further enlighten the situation in stone patients, research on the response of other test parameters to acute loads with various citrates is currently in progress.

The finding of increasing citrate and decreasing calcium in dissolved CaOx crystals, developed in a citrate-rich urine at pH 6, is new; more specific investigations should help to define whether apart from free citrate more citrate species are able to inhibit CaOx crystallization. Thus, CaOx

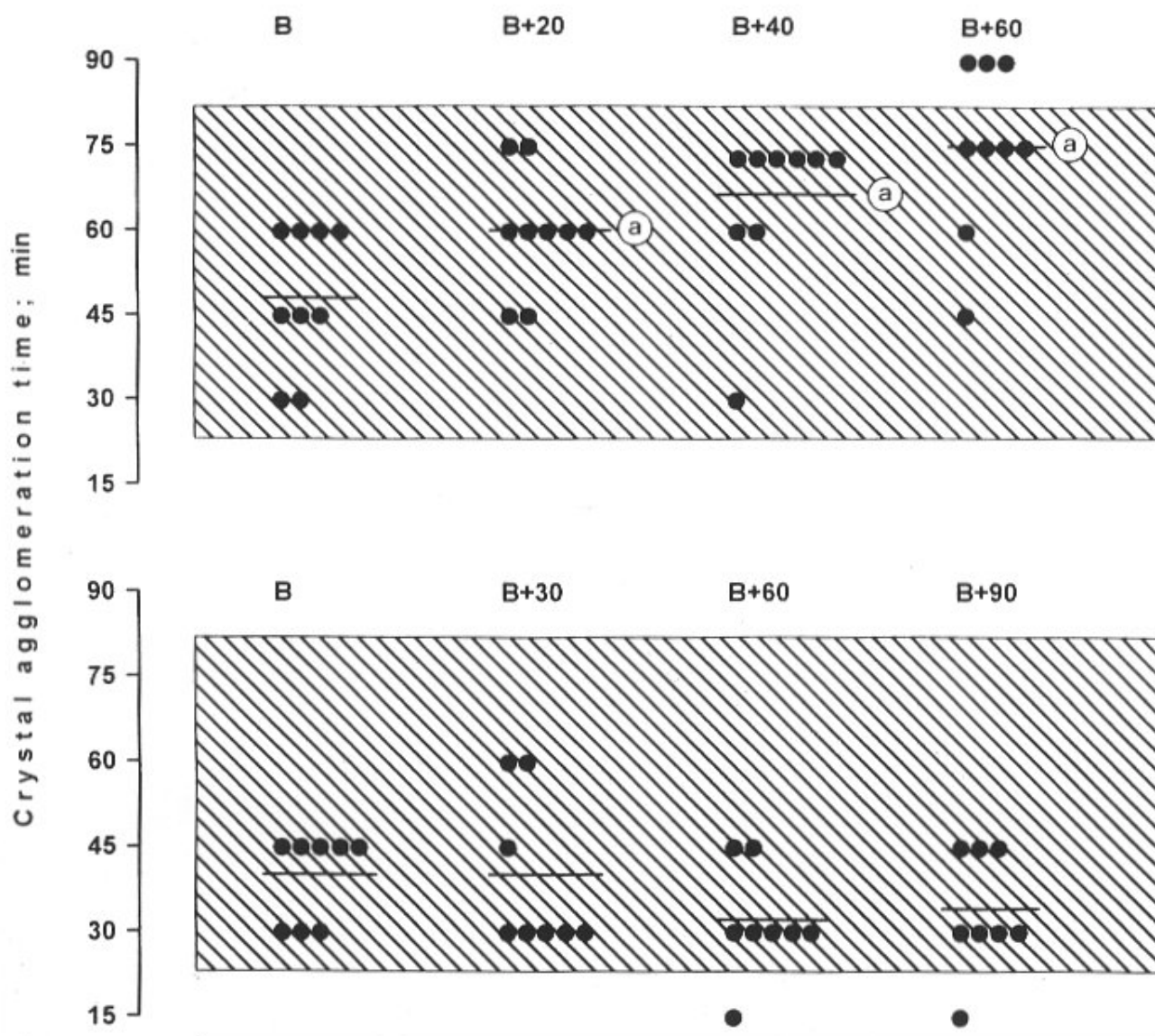


Figure 4. Agglomeration time of calcium oxalate crystals after oral intake of three doses citrate. **Upper part:** B: breakfast alone; B + 20, B + 40, B + 60 mean breakfast supplemented with the respective number of milliequivalent citrate delivered as PC. **Lower part:** B: breakfast alone; B + 30, B + 60, B + 90 mean breakfast supplemented with the respective number of milliequivalent citrate delivered with CSC. —: mean values. Hatched area: 95% confidence range of untreated non-stone-forming healthy male volunteers (n = 32; unpublished data). a: $p \leq 0.05$ versus B (ANOVA, LSD test). For further information, see Table 4 and text.

crystallization parameters, the urinary calcium/citrate ratio, and analysis of crystals should become major objectives of studies on the effect of alkali, calcium, and citrate containing preparations inasmuch as they are part of treatment regimens: in renal calcium stone disease the possible anti-stone effect of a high calcium diet [8] or of calcium supplementation awaits clarification, in osteoporosis there

is a need to protect patients from renal stone formation.

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Table 4. Effects of potassium citrate (PC) and calcium-sodium citrate (CSC) on pH, substances, and CaOx crystallization parameters in postprandial urine of male volunteers (PC: n = 8; CSC: n = 8) participating in the clinical trials 2 and 3, designed to evaluate the risk for stone formation with increasing CSC versus increasing PC. A continental breakfast was taken alone (B) or, on a day one week apart, together with one of three doses PC or CSC (B + 1, B + 2, B + 3): PC - 20, 40, 60 milliequivalent potassium and bases; CSC - 20, 40, 60 milliequivalent calcium plus 10, 20, 30 milliequivalent sodium, corresponding with 30, 60, 90 milliequivalent bases. Data are mean values (SE). For further details see Table 3, and text.

Variables	Test substance	Loads							
		B		B + 1		B + 2		B + 3	
pH	PC	6.06	(0.16)	6.70	(0.11) ^a	7.06	(0.10) ^a	7.18	(0.10) ^a
pH	CSC	6.55	(0.17)	6.19	(0.15) ^a	6.73	(0.27)	6.69	(0.22)
Oxalate (mM)	PC	0.076	(0.010)	0.080	(0.009)	0.083	(0.012)	0.081	(0.010)
Oxalate (mM)	CSC	0.051	(0.005)	0.066	(0.014)	0.091	(0.051)	0.068	(0.029)
Calcium (mM)	PC	1.41	(0.30)	1.20	(0.23)	1.11	(0.17)	1.15	(0.13)
Calcium (mM)	CSC	1.34	(0.16)	2.39	(0.49) ^a	2.91	(1.09)	2.59	(0.77)
Citrate (mM)	PC	0.80	(0.08)	1.17	(0.17) ^a	1.74	(0.20) ^a	2.39	(0.28) ^a
Citrate (mM)	CSC	0.66	(0.13)	1.14	(0.26)	1.05	(0.30)	1.08	(0.26)
Calcium/Citrate (mM/mM)	PC	2.0	(0.6)	1.0	(0.2) ^a	0.7	(0.2) ^a	0.5	(0.1) ^a
Calcium/Citrate (mM/mM)	CSC	2.6	(0.6)	2.8	(0.7)	3.3	(0.7) ^a	2.6	(0.5)
Tolerable oxalate (mM)	PC	0.61	(0.06)	1.13	(0.15) ^a	1.97	(0.48) ^a	1.89	(0.32) ^a
Tolerable oxalate (mM)	CSC	0.51	(0.03)	0.40	(0.05)	0.52	(0.06)	0.50	(0.04)
Crystal diameter (µm)	PC	4.6	(0.2)	4.5	(0.3)	4.3	(0.1) ^a	4.2	(0.05) ^a
Crystal diameter (µm)	CSC	5.6	(0.4)	8.9	(0.6) ^a	7.3	(0.4) ^a	7.7	(0.6) ^a
Agglomeration time (min)	PC	48	(4)	60	(4) ^a	67	(5) ^a	75	(5) ^a
Agglomeration time (min)	CSC	39	(3)	39	(5)	32	(3)	34	(4)
Crystal growth rate (µm/min)	PC	0.11	(0.02)	0.08	(0.00)	0.07	(0.01)	0.06	(0.01) ^a
Crystal growth rate (µm/min)	CSC	0.09	(0.01)	0.15	(0.02)	0.16	(0.03)	0.16	(0.05)

^ap ≤ 0.05 versus B, according to one-way ANOVA and LSD (least square differences) test.

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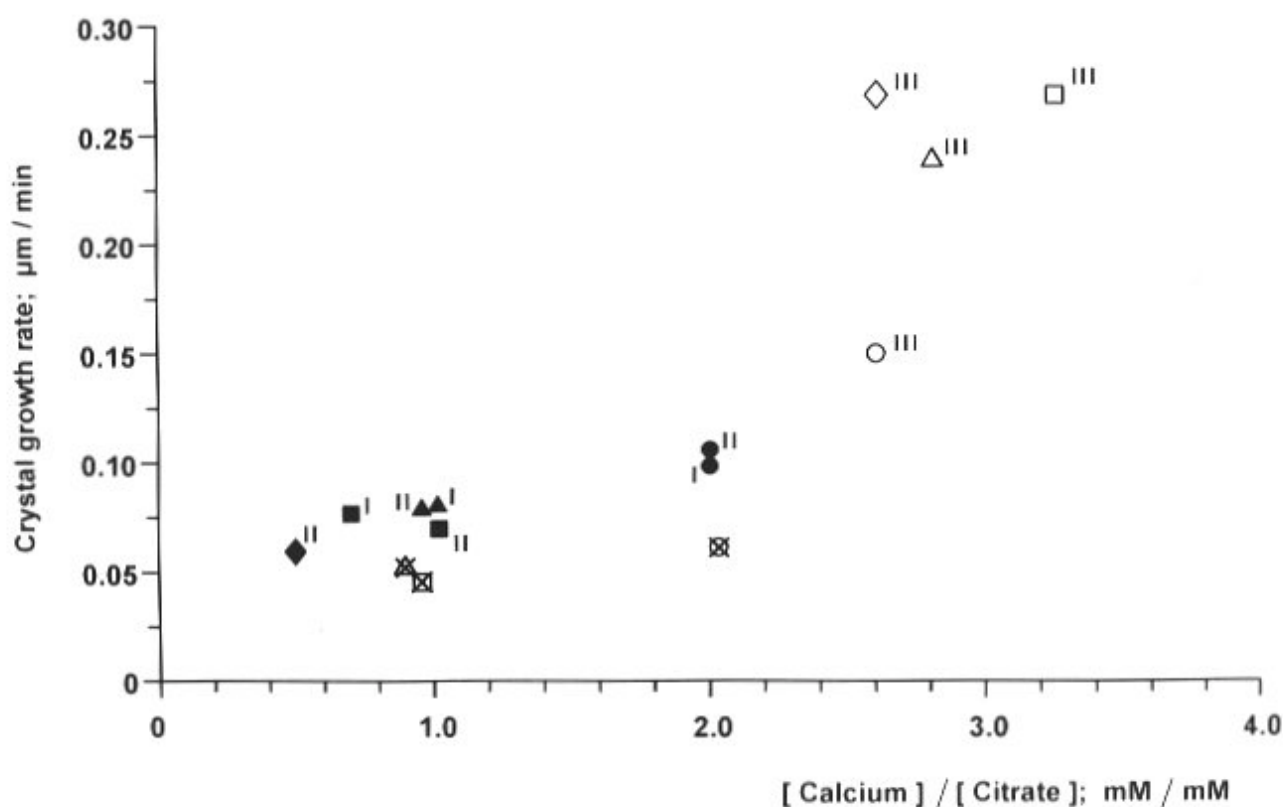


Figure 5. Plot of the mean molar concentration ratio urinary total calcium/total citrate in relation to the mean crystal growth rate, as observed in the three clinical trials in healthy volunteers.

The symbols for trial 1 (bioequivalence of citrate given with PC versus citrate given with CSC) are: I breakfast alone (I); S breakfast + 40 milliequivalent PC (I); ■ breakfast + 40 milliequivalent PC (I).

The symbols for trials 2 and 3 [comparison of citrate given with PC (II) versus citrate given with CSC (III)] are:

I, m breakfast alone

s, Δ breakfast alone + 20 or 30 milliequivalent citrate

□, ■ “+ 40 or 60 milliequivalent citrate ◆, ◇ “+ 60 or 90 milliequivalent citrate

(in all cases: PC: filled symbols; CSC: open symbols)

For illustration, the crossed open symbols represent data from unpublished work on an independent calcium and sodium containing citrate; it should be noted that a superior alkalinizing effect due to higher sodium moiety results into low values of both calcium/citrate and crystal growth rate.

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Discussion with Reviewers

J.D. Sallis: Are there any limitations in employing the test in respect to patient selection?

Authors: Principally no, but there are some limitations with respect to actual urinary pH. In the present work no stone

patients were investigated, but we have considerable experience with stone patients showing no urinary tract infection (infected urine shows pH 7-8). Over the years we studied numerous patients forming stones with documented calcium content, be it as calcium oxalate, calcium phosphate, or mixtures of these (idiopathic calcium urolithiasis). In general, these patients develop a urinary pH around 6. In addition, there exists a subset of patients with permanently low urinary pH (< 5.5) in both fasting and postprandial urine, frequently showing a high degree of uric acid supersaturation, forming either idiopathic uric acid stones, or mixtures of this with calcium concretions. All these patients can be readily studied. Our knowledge is limited with respect to the small subset of stone patients without urinary tract infection but yet showing fasting urinary pH around 7 or slightly above, mostly due to impaired tubular functions resulting in insufficient urine acidification.

In these urines, from yet unknown reasons, co-precipitation of amorphous calcium phosphate may occur. So, we recommend that this subgroup will benefit from a thorough diagnostic work-up, and that some test aimed at crystallization of calcium phosphate is more important.

A.L. Rodgers: Does the incorporation of citrate into the crystal structure affect the morphology of calcium oxalate crystals?

Authors: We did not state that “incorporation of citrate” has taken place, because this would signal that citrate did alter the crystal lattice, ultimately leading to a crystal different from calcium oxalate. However, there was a change of morphology of calcium oxalate crystals: at pH 5 of urine, there was a predominance of the monohydrate, at pH 7 of the dihydrate form (Fig. 2). Although not directly studied, the increase of water content of calcium oxalate crystals and the concomitant pH-dependent increase of citrate deprotonation reflect that there was increasing adhesion of some citrate species, the nature of which depends on the degree of ionisation. Therefore, citrate may be an effector of calcium oxalate phase transformation. In line with this assumption are the EQUIL data (Table 2), showing no substantial fall of ionised calcium and complexed citrate, respectively.

A.L. Rodgers: Are there specific cut-off values for the calcium/citrate ratio in urine which indicate whether chelation or complex formation is occurring?

Authors: We cannot give a sufficiently helpful answer, because neither did we calculate (by use of EQUIL) the free calcium, free citrate, and the various calcium-containing citrate complexes formed upon varying the urinary molar calcium/citrate ratio by loading with the citrate-containing preparations, nor did we analyze the formed crystals. According to the physico-chemistry literature calcium ions

have a rate constant for chelation about 1000 times faster than the one of magnesium ions, suggesting that the amount and nature of chelates and calcium citrate complexes are dictated by the citrate ligand present at a given pH. Figure 5 illustrates that a molar ratio of 1.0 (urinary total calcium/urinary total citrate), and less, may guarantee a low growth rate of calcium oxalate crystals. More insight into the situation is desirable; this would also allow to give more practically oriented recommendations with respect to composition and dosage of citrate preparations, especially the alkali-containing calcium citrate.