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Evaluation of urine composition and calcium salt crystallization properties in standardized volume-adjusted 12-h night urine from normal subjects and calcium oxalate stone formers

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Abstract The volume of 12-h night urine from ten normal men (NM), ten normal women (NW) and 31 male calcium stone formers (SFM) was adjusted to 750 ml and analysed with respect to supersaturation with calcium oxalate (CaOx) and calcium phosphate (CaP), inhibition of CaOx crystal growth and aggregation, as well as the CaOx and CaP crystallization propensity. Concentrations of oxalate and glycosaminoglycans and AP(CaOx) index, an estimate of the CaOx ion-activity product, were higher and the concentration of citrate lower in NM than in NW. In SFM the directly assessed risk of CaOx crystallization was higher and the inhibition of CaOx crystal growth lower than in NM. There were no differences between the groups regarding inhibition of CaOx crystal growth by 74% dialysed urine or inhibition of CaOx crystal aggregation. SFM with mixed CaOxCaP stones had a higher concentration of phosphate and a higher AP(CaP) index at pH 7.0 than SFM with CaOx stones.

Key words Calcium phosphate · Crystal aggregation · Crystal growth · Kidney stones

Introduction

In routine clinical work conclusions on the course of stone formation and predictions of the risk of further stone formation are usually based on the composition and crystallization properties of 24-h urine samples. Attention has therefore most commonly been paid to supersaturation with calcium oxalate (CaOx) and the inhibitory properties regarding growth and aggregation of such crystals. There are, however, several disadvantages with 24-h urine collections. The samples have to be either acidified or treated in some other way to prevent bacterial growth and counteract precipitation of calcium salts. Such additives as well as storage of urine at room temperature might destroy or alter the structure of important urine constituents. Acidification also makes it impossible to measure pH. For this reason we have routinely assessed the inhibitory and crystallization properties either in 24-h or 4-h urine samples without destructive additives [5, 33]. Recently we have reported our results with analysis of supersaturation in 16-h day-time urine and the inhibitory and crystallization properties in 8-h night urine [40]. Comparison of crystallization and inhibitory properties between samples collected during different periods might, however, be difficult due to the variations in urine flow that occur between different periods.

The inhibitory properties of CaOx crystal growth have been assessed by numerous methods, each with its advantages and disadvantages. Important differences in methodology as well as different degrees of dilution of the samples have made it difficult to establish definitively the role of inhibitors in stone formation [3]. Several studies have shown that the CaOx crystal growth inhibition is lower in urine from stone formers than in normal subjects [2, 7, 11–13, 27, 28, 31] – observations that were not confirmed in other studies [4, 9]. We have previously observed that stone formers had a slightly lower inhibition of CaOx crystal growth in 24-h urine diluted to a standardized concentration of creatinine [34] and a lower inhibition was also recorded in 8-h night urine collections [40].

To avoid the influence of supersaturation in undiluted urine, the inhibition of crystal growth is normally measured in diluted urine samples, usually at concentrations as low as 1%–2%. Although the ensuing information might be valid for crystallization at a high nephron level, where the concentration of urinary mac-

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romolecules is low, for crystallization in whole urine it probably is not [30].

In this investigation we used a 12-h night urine sample, collected without destructive additives, delivered to the laboratory and adjusted to a volume of 750 ml as soon as possible after completion of collection. The main objective with such a procedure was to enable a standardized comparison of crystallization properties between urine from patients who had formed stones containing either only calcium oxalate or mixtures of calcium oxalate and calcium phosphate.

Materials and methods

Urine collection

Urine was collected between 2200 and 1000 hours from ten normal men (NM, mean age 37 years), ten normal women (NW, mean age 35 years) and 31 men with CaOx stone disease (SFM, mean age 42 years). All samples were collected on an out-patient basis in bottles containing 15 ml of 3 mol/l sodium azide as a preservative. No special instructions were given to restrict food or fluid intake during the collection period; all subjects maintained their ordinary diet and drinking habits. None of them took any medication known to affect the risk of calcium salt crystallization. The 31 patients were grouped according to the composition of their stones. Nine patients had formed pure CaOx stones (CaOx-SFM) and 22 had stones composed of a mixture of CaOx and calcium phosphate (CaP). The latter group is referred to hereafter as CaOxCaP-SFM. None of the patients had formed pure CaP stones. The stone composition was in all cases analysed with a wet chemical procedure [21].

As soon as possible after completion of the urine collection, the pH was measured with a glass electrode (PHM 84, Research pH meter, Radiometer, Copenhagen, Denmark) and the volume recorded. This original urine is designated *Urine O*. The pH was subsequently adjusted to 5.8 with small volumes of sodium hydroxide or hydrochloric acid, and the sample volume increased, by addition of 0.15 mol/l sodium chloride, or reduced to 750 ml by evaporation in a Büchi Rotavapor RE (Büchi, Flawil, Switzerland) at 37°C, after which the pH was readjusted to 5.8. This standardized urine sample is referred to as *Urine S*. The samples were kept frozen at −20°C until analysis, at which time they were thawed, heated to 37°C and carefully mixed.

Estimates of ion-activity products

One aliquot of *Urine S* was acidified to pH 1.0 with hydrochloric acid for analysis of calcium (Ca), oxalate (Ox), citrate (Cit), magnesium (Mg), phosphate (P) and creatinine [6, 14, 15, 20, 22, 44].These variables were subsequently used for calculating approximate estimates of the ion-activity products of CaOx and CaP [35, 39]:

$$\text{AP(CaOx) index} = \frac{2.7 \cdot \text{Calcium}^{0.84} \cdot \text{Oxalate}}{\text{Citrate}^{0.22} \cdot \text{Magnesium}^{0.12} \cdot \text{Volume}^{1.03}}$$

$$\text{AP(CaP) index} = \frac{0.0032 \cdot \text{Calcium}^{1.07} \cdot \text{Phosphate}^{0.70} \cdot (\text{pH} - 4.5)^{6.8}}{\text{Citrate}^{0.20} \cdot \text{Volume}^{1.31}}$$

In these indices the urine variables are expressed in millimoles excreted during 12 h and the urine volume in litres. Calculations were made for both *Urine O* and *Urine S*. In the latter sample AP(CaOx) index is equal to its standardized form AP(CaOx) index(s) [39]. In the standardized form of the AP(CaP) index in *Urine S* the pH was set to 7.0 [42] instead of 5.8, in order to make any differences in CaP-saturation more evident.

The risk of CaOx crystallization

Aliquots of *Urine S* were prepared and used for direct assessment of the risk of crystallization of CaOx (CaOx-CR) [36]. Following dissolution of any precipitate of calcium salts in the sample sediment, the urine was passed through a Millipore filter with a pore size of 0.22 μm (Millipore, Molsheim, France). The appearance of crystals was followed in a Coulter counter with channelyser (Model Z_B, Coulter Electronics, Luton, UK) during standardized increments of the oxalate concentration until 100 crystals in the size range 3.5–5 μm had been recorded. The concentration of oxalate was increased by adding 0.1-ml fractions of a 0.04-mol/l sodium oxalate solution every minute. Crystal counting was carried out 40 s later. The sample was continuously stirred during the experiment and the crystal number determined in a volume of 0.05 ml with a 100-μm tube. The inverted increment in oxalate concentration corresponding to the formation of 100 crystals was used to express CaOx-CR.

The risk of CaP crystallization

The risk of CaP crystallization (CaP-CR) [37] was measured in a similar way with increments in pH achieved by addition of small aliquots of 1 mol/l sodium hydroxide until 1000 crystals in the size range 3.5–5 μm had been recorded. The CaP-CR was expressed as the reciprocal increment in pH necessary for the formation of 500 crystals.

The inhibition of CaOx crystal growth in diluted whole urine

The inhibition of CaOx crystal growth in diluted whole urine (Inh_{GR}U) was assessed by adding 1 ml of Millipore-filtered *Urine S* (pore size 0.22 μm) to 50 ml of a solution metastably supersaturated with respect to CaOx [15]. The rate of crystal growth was followed by measuring the amount of [^{14}C] oxalate remaining in solution during the first 2 h following addition of 2 ml of a suspension of CaOx monohydrate crystals (1 mg/ml). The inhibition was expressed as a percentage of precipitated oxalate in the sample containing urine compared with that in a sample containing 1 ml of 0.15 mol/l saline instead of urine [40]. The samples contained 10 nCi of [^{14}C]oxalate per ml. [^{14}C]oxalic acid had a specific radioactivity of 109 μCi/μmol (Amersham, Buckinghamshire, UK).

Preparation of dialysed urine

Dialysis of urine was carried out by pouring 100 ml of *Urine S* into Spectrapore No. 3 dialysis tubing (Spectrum Medical Industries, Houston, Texas). Each tube was subsequently kept overnight in 1 l of deionized water with continuous stirring. During the following day Milli-Q-filtered water was exchanged eight times and during day 2 three times, after which the samples were dialysed against a solution of 0.15 mol/l sodium chloride. The original volume was reestablished by adding saline up to 100 ml.

Inhibition of CaOx crystal growth by a high concentration of urinary macromolecules

The inhibition of CaOx crystal growth in the presence of a 74% concentration of dialysed urine (Inh_{GR}dU) was assessed by measuring the concentration of ^{45}Ca (4 nCi in each sample) remaining in solution 30 min after the addition of 1 ml of a suspension of CaOx monohydrate seed crystals (1 mg/ml) and 0.1 ml of sodium oxalate solutions with concentrations between 10 and 80 mmol/l to a series of samples of dU. The samples were continuously mixed during the crystallization experiment [38].

Inhibition of CaOx crystal aggregation

The inhibition of CaOx crystal aggregation (Inh_{AGG}) was measured in diluted urine as the rate of crystal sedimentation in a 0.5 mg/ml CaOx monohydrate crystal suspension, mainly according to the principles described by Hess and coworkers [17, 40]. The suspension was carefully prepared to ensure that approximately 15–20% of the crystals had a diameter of less than 5.5 μm and that all crystals were smaller than 14 μm. The crystals were suspended in a 10-mmol/l TRIS-hydrochloric acid buffer with a pH of 7.2, containing 90 mmol/l of sodium chloride. We added 0.5 mL of whole *Urine S* to 14.5 ml of this suspension. Following vigorous stirring, 1.5 ml of the suspension was immediately transferred to a cuvette and placed in a spectrophotometer (Spectrometer Lambda 2, Perkin Elmer, Überlingen, Germany) The sample in the cuvette was magnetically stirred at a rate of 1100 rpm. The absorbance at 690 nm was recorded during 10 min of continuous stirring. Inh_{AGG} was expressed as a percentage of absorbance after 600 s in the sample containing urine compared with that in a sample containing 0.5 ml of 0.15 mol/l sodium chloride with 10^{-7} mol/l heparin. This low concentration of heparin was used to increase the reproducibility of the measurements in the urine-free solution and the effect of this concentration of heparin on crystal aggregation was negligible.

Measurement of glycosaminoglycans

The concentration of glycosaminoglycans (GAGs) was determined as Alcian blue precipitable polyanions [46]. The samples were centrifuged at 1700 *g* for 20 min. To 100 ml of the sample was added 1.2 ml of Alcian blue buffered to pH 5.8 after which the sample was carefully mixed with air. The sample was left overnight and centrifuged at 1100 *g* the next day. The sediment was dissolved in 1 ml of 7.5 g/l sodium lauryl sulphate after which the absorbance was measured in a spectrophotometer at 678 nm.

Calculation of supersaturation/inhibition quotients

The quotients Q_1–Q_4 were calculated to express the net effect of the supersaturation and the crystallization inhibitory properties [40]:

$$Q_1 = \frac{10^2 \cdot \text{AP(CaOx) index(s)}}{Inh_{GR}U}$$

$$Q_2 = \frac{10^2 \cdot \text{AP(CaOx) index(s)}}{Inh_{AGG}}$$

$$Q_3 = \frac{10^4 \cdot \text{AP(CaOx) index(s)}}{Inh_{GR}U \cdot Inh_{AGG}}$$

$$Q_4 = \frac{10^2 \cdot \text{CaOx} - \text{CR}}{Inh_{AGG}}$$

Estimate of the ion-activity product of CaP in distal tubular urine

According to calculations presented elsewhere [42] the ion-activity product of CaP (AP_{CaP}) at pH 6.45 in distal tubular urine (DTd) might roughly be derived from the AP(CaP) index(s) measured in final urine according to the following relationship:

$$10^{14} \cdot \text{DTd-}AP_{CaP} = -0.96 + 0.20 \cdot \text{AP(CaP) index(s)}$$

This formula was used to obtain approximate estimates of DTd-AP_{CaP} in NM, CaOx-SFM and CaOxCaP-SFM at pH 6.5, 6.8, 7.0 and 7.3 (pH_x) by inserting the DTd-AP_{CaP} (pH 6.45) value in the following expression:

$$\text{DTd} - AP_{CaP}^{pHx} = \text{DTd} - AP_{CaP}^{pH\ 6.45} \cdot (pH_x - 5.45)^{4.00}$$

This relationship was obtained by comparing the DTd-AP_{CaP} at pH 6.45 with DTd-AP_{CaP} at other pH levels. The EQUIL 2 programme was used for these computations, from which the factors 5.45 and 4.00 gave the best fit.

Statistical methods

The excretion of urine variables was expressed as mean, median, range and standard deviation. The Mann-Whitney test for unpaired groups was used for group comparison.

Results

Urinary findings in normal men and women

As shown in Table 1 *Urine S* from NW had a significantly lower concentration of oxalate ($P = 0.002$) and a higher concentration of citrate ($P = 0.028$) than that from NM. There was also a significantly lower concentration of GAGs in urine from NW. The mean (SD) pH in *Urine O* was significantly higher in NW [5.99 (0.37) vs 5.62 (0.46); $P = 0.045$].

AP(CaOx) index in *Urine S* was significantly higher in NM than in NW (Table 2), but the numerically higher AP(CaOx) index in *Urine O* from NM was not significantly different from that in NW. There were no differences in terms of AP(CaP) index or AP(CaP) index(s). The similarity in 12-h *Urine O* volumes in NM and NW is noteworthy. The $Inh_{GR}U$ did not differ between NM and NW, neither did $Inh_{GR}dU$ or Inh_{AGG}. No differ-

Table 1 Median (range) and mean (SD) concentrations of different urine variables in *Urine S* from normal men and normal women. *GAGs* glycosaminoglycans

	NM Median (range) Mean (SD)	NW Median (range) Mean (SD)	Significance of difference
Phosphate (mmol/l)	16.15 (1.71–37.2) 16.75 (14.03)	2.88 (1.25–19.1) 8.01 (7.78)	$P = 0.14$
Calcium (mmol/l)	3.30 (1.48–5.49) 3.43 (1.51)	3.47 (0.82–6.6) 3.40 (1.74)	$P = 0.94$
Oxalate (mmol/l)	0.26 (0.19–0.35) 0.26 (0.05)	0.17 (0.09–0.24) 0.17 (0.05)	$P = 0.002$
Citrate (mmol/l)	1.98 (0.71–2.92) 1.70 (0.65)	2.29 (1.60–3.74) 2.39 (0.61)	$P = 0.028$
Magnesium (mmol/l)	3.25 (2.40–4.12) 3.35 (0.53)	3.03 (1.16–4.64) 2.99 (1.13)	$P = 0.597$
GAGs (mg/l)	16.2 (9.4–20.0) 14.78 (3.06)	10.6 (5.0–16.0) 10.8 (2.9)	$P = 0.014$

Table 2 Median (range) and mean (SD) ion-activity product [AP (CaOx), AP(CaP)] indices, crystallization (CR) and inhibitory (Inh_{GR}, Inh_{AGG}) properties in *Urine O* and *Urine S*, *CaOx* Calcium oxalate, *CaP* calcium phosphate

Variable	NM Median (range) Mean (SD)	NW Median (range) Mean (SD)	Significance of difference
Urine O			
AP(CaOx) index	1.24 (0.38–3.17) 1.48 (0.86)	0.79 (0.37–1.49) 0.83 (0.42)	$P = 0.064$
AP(CaP) index	0.03 (0.01–1.56) 0.44 (0.69)	0.37 (0.46–2.05) 0.70 (0.78)	$P = 0.059$
12-h urine volume (ml)	707 (326–1249) 769 (303)	723 (399–1182) 763 (272)	$P = 0.94$
Urine S			
AP(CaOx) index	1.31 (0.62–2.07) 1.31 (0.55)	0.73 (0.2–1.26) 0.78 (0.36)	$P = 0.023$
AP(CaP) index(s)	25.61 (4.97–109.4) 38.55 (36.73)	9.0 (3.47–78.8) 19.67 (24.3)	$P = 0.241$
CaOx-CR	0.80 (0.54–1.18) 0.85 (0.26)	1.0 (0.49–1.66) 1.04 (0.38)	$P = 0.385$
CaP-CR	0.82 (0.46–1.67) 0.91 (0.34)	0.79 (0.42–1.16) 0.76 (0.24)	$P = 0.521$
InhGR-U (%)	51.5 (43.0–59.0) 51.5 (5.85)	53.0 (43.0–73.0) 54.3 (9.52)	$P = 0.762$
InhGR-dU (%)	44.0 (42.0–50.0) 44.4 (2.27)	46.5 (40.0–61.0) 47.6 (6.4)	$P = 0.227$
InhAGG (%)	74.0 (68–85) 75.5 (6.7)	73.4 (62–86) 74.47 (9.1)	$P = 0.909$

ences between the two sexes were recorded in terms of CaOx-CR or CaP-CR.

Comparison of urinary findings between normal men and stone-forming men

The $Inh_{GR}U$ was significantly lower in SFM than in NM, but there was no difference in the concentration of GAGs (Table 3). There was a significantly higher CaOx-CR in urine from SFM than in urine from NM. In comparison with NM there was an unexpected lower concentration of oxalate in *Urine S* from SFM. The AP(CaOx) index in *Urine S* (Table 3) as well as in *Urine O* (Table 4) was not different between the groups. The quotients Q_1, Q_2, Q_3 and Q_4 in *Urine S* were not different between NM and SFM.

Comparison of urinary findings in normal men and stone formers with pure CaOx stones and CaOxCaP mixed stones

There was no significant difference in *Urine S* oxalate concentration between NM and CaOx-SFM (Table 3). A higher phosphate concentration was recorded in CaOxCaP-SFM compared with CaOx-SFM. Although $Inh_{GR}U$ was lower in CaOx-SFM than in NM, there was no difference in $Inh_{GR}U$ between CaOx-SFM and CaOxCaP-SFM. It is noteworthy that neither $Inh_{GR}dU$ nor Inh_{AGG} disclosed any differences between the two groups of SFM, although CaOx-SFM had the numerically lowest Inh_{AGG} levels.

There was a pronounced difference in AP(CaP) index(s) in *Urine S* between CaOxCaP-SFM and CaOx-SFM as well as between CaOxCaP-SFM and NM. (Table 3). Such a difference was also recorded for AP(CaP) index in *Urine O* (Table 4). In contrast CaP-CR did not differ between the groups. No differences were recorded in terms of AP(CaOx) index either in *Urine S* or in *Urine O*, but CaOx-CR was significantlly higher in *Urine S* from CaOxCaP-SFM than in NM. However, CaOx-CR was not different between CaOx-SFM and CaOxCaP-SFM.

As could be expected from the lack of differences in CaOx supersaturation the quotients Q_1, Q_2, Q_3, and Q_4 in *Urine S* were at the same level in CaOx-SFM as in CaOxCaP-SFM.

Estimated levels of APCaP in DTd urine

As shown in Table 5 the DTd-AP_{CaP} at different pH levels were highest in CaOxCaP-SFM and a risk of CaP crystallization might be expected only in these patients.

Discussion

Stone formation is thought to be the end result of nucleation induced by the driving force in the supersaturated solution with or without a contribution from urinary macromolecules, crystal growth, crystal aggregation and some mechanism leading to crystal retention [24, 41]. With various analytical techniques, the level of supersaturation [35, 39, 42], the inhibition of crystal growth [28, 34] and the inhibition of crystal aggregation

Table 3 Median (range) and mean (SD) concentrations of different urine variables and risk expressions in *Urine S* from normal mean (NM) and male calcium stone formers (SFM) with stones composed of CaOx or mixtures of CaOx and CaP

Variable (mmol/l)	NM Median (range) Mean (SD)	CaOx-SFM Median (range) Mean (SD)	CaOxCaP-SFM Median (range) Mean (SD)	SFM Median (range) Mean (SD)	NM vs SFM	NM vs CaOx-SFM	CaOx-SFM vs CaOxCaP-SFM	NM vs CaOxCaP-SFM
Calcium (mmol/l)	3.3 (1.48–5.49) 3.43 (1.51)	4.36 (1.42–4.78) 3.84 (1.19)	4.43 (2.2–8.32) 4.68 (1.73)	4.36 (1.42–8.32) 4.44 (1.62)	$P = 0.101$	$P = 0.568$	$P = 0.384$	$P = 0.056$
Oxalate (mmol/l)	0.26 (0.19–0.35) 0.26 (0.05)	0.21 (0.12–0.32) 0.21 (0.06)	0.17 (0.12–0.25) 0.17 (0.04)	0.19 (0.12–0.32) 0.18 (0.05)	$P = 0.0005$	$P = 0.060$	$P = 0.192$	$P = 0.0002$
Citrate (mmol/l)	1.98 (0.71–2.92) 1.70 (0.65)	1.77 (0.58–4.06) 1.91 (0.90)	1.34 (0.58–4.08) 1.53 (0.87)	1.68 (0.58–4.08) 1.63 (0.88)	$P = 0.421$	$P = 0.935$	$P = 0.286$	$P = 0.264$
Magnesium (mmol/l)	3.25 (2.40–4.12) 3.35 (0.53)	3.1 (1.12–6.3) 3.41 (1.40)	3.2 (1.2–4.8) 3.21 (1.09)	3.14 (1.12–6.3) 3.27 (1.17)	$P = 0.649$	$P = 0.744$	$P = 0.948$	$P = 0.655$
Phosphate (mmol/l)	16.15 (1.7–37.2) 16.75 (14.03)	2.8 (1.85–28.0) 9.36 (10.67)	21.8 (9.2–34.5) 21.55 (6.38)	18.9 (1.85–34.5) 18.01 (9.51)	$P = 0.785$	$P = 0.289$	$P = 0.0009$	$P = 0.371$
GAGs mg/l	16.2 (9.4–20.0) 14.78 (3.06)	16.2 (6.0–25.8) 15.64 (5.74)	16.1 (5.4–20.0) 14.19 (4.04)	16.2 (5.4–25.8) 14.61 (4.54)	$P = 0.976$	$P = 0.807$	$P = 0.486$	$P = 0.871$
CaP-CR	0.82 (0.46–1.67) 0.91 (0.34)	1.0 (0.56–1.22) 1.0 (0.21)	0.97 (0.65–1.43) 0.99 (0.23)	1.0 (0.56–1.43) 0.99 (0.22)	$P = 0.141$	$P = 0.131$	$P = 0.557$	$P = 0.223$
CaOx-CR	0.80 (0.54–1.18) 0.85 (0.26)	1.2 (0.54–1.21) 0.998 (0.25)	1.1 (0.72–1.48) 1.12 (0.21)	1.1 (0.54–1.48) 1.09 (0.23)	$P = 0.010$	$P = 0.094$	$P = 0.223$	$P = 0.009$
AP(CaOx) index	1.31 (0.62–2.07) 1.31 (0.55)	1.1 (0.47–2.15) 1.17 (0.55)	1.1 (0.55–2.28) 1.18 (0.44)	1.1 (0.47–2.28) 1.18 (0.47)	$P = 0.585$	$P = 0.683$	$P = 0.794$	$P = 0.597$
AP(CaP) index(s)	25.6 (4.97–109.4) 38.55 (36.73)	13.3 (4.3–59.13) 23.85 (20.98)	61.6 (19.71–140.74) 65.74 (29.81)	55.7 (4.29–140.74) 53.57 (33.36)	$P = 0.213$	$P = 0.540$	$P = 0.0007$	$P = 0.046$
Inh_{GR}-U (%)	51.5 (43.0–59.0) 51.5 (5.85)	46 (39–51) 45.33 (3.91)	44 (34–73) 46.27 (10.36)	44 (34–73) 46.0 (8.91)	$P = 0.016$	$P = 0.031$	$P = 0.514$	$P = 0.903$
Inh_{GR}-dU (%)	44 (42–50) 44.4 (2.27)	42 (33–49) 41.11 (5.37)	45 (36–57) 44.47 (5.26)	43 (33–57) 43.5 (5.43)	$P = 0.649$	$P = 0.142$	$P = 0.122$	$P = 0.059$
Inh_{AGG} (%)	74 (68–85) 75.5 (6.7)	69.5 (53–87) 70.75 (10.43)	73 (59–88) 73.24 (8.47)	73 (53–88) 72.55 (8.93)	$P = 0.459$	$P = 0.328$	$P = 0.981$	$P = 0.612$

Table 4 Median (range) and mean (SD) ion-activity products, volume and pH of *Urine O*

Variable	NM Median (range) Mean (SD)	CaOx-SFM Median (range) Mean (SD)	CaOxCaP-SFM Median (Range) Mean (SD)	SFM Median (Range) Mean (SD)	NM vs SFM	NM vs CaOx-SFM	CaOx-SFM vs CaOxCaP-SFM	NM vs CaOxCaP-SFM
AP(CaOx) index	1.21 (0.38–3.17) 1.48 (0.86)	1.2 (0.5–3.2) 1.58 (0.99)	1.3 (0.46–3.02) 1.50 (0.64)	1.3 (0.46–3.16) 1.53 (0.74)	$P = 0.716$	$P = 0.935$	$P = 0.728$	$P = 0.611$
AP(CaP) index	0.03 (0.01–1.56) 0.44 (0.67)	0.11 (0.00–0.92) 0.243 (0.34)	1.0 (0.03–33.44) 3.34 (7.18)	0.6 (0.00–33.4) 2.43 (6.18)	$P = 0.052$	$P = 0.935$	$P = 0.005$	$P = 0.011$
12-h volume (ml)	707 (326–1249) 769 (303)	517 (309–1308) 687 (372)	633 (300–1363) 674 (307)	611 (300–1363) 678 (321)	$P = 0.347$	$P = 0.414$	$P = 0.931$	$P = 0.393$
pH	5.4 (5.2–6.5) 5.62 (0.46)	5.5 (5.25–5.95) 5.59 (0.27)	5.75 (5.2–6.7) 5.83 (0.41)	5.75 (5.2–6.7) 5.76 (0.39)	$P = 0.187$	$P = 0.596$	$P = 0.170$	$P = 0.133$

Table 5 Ion-activity products for CaOx-SFM and CaOxCaP-SFM in the distal part of distal tubule calculated for different values of pH

	pH = 6.50	pH = 6.80	pH = 7.00	pH = 7.30
NM	8.2	22.4	39.0	79.1
CaOx-SFM	4.7	12.8	22.2	45.0
CaOxCaP-SFM	15.0	41.2	71.6	145.2

[17, 29, 40] are possible to assess in urine. Information on the balance between supersaturation and the inhibition of crystallization and aggregation is of great value in the clinical management of these patients [30, 34, 40]. Unfortunately, the collection of urine puts the patient in an unusual situation and, merely as a result of the sampling procedure, the drinking habits and thus the urine flow might differ from what is normal for the individual patient. The use of a sample not reflecting the normal urine production makes interpretation of analytical data difficult and can certainly lead to incorrect conclusions. These circumstances are of fundamental importance in the risk evaluation because although the supersaturation level can be corrected mathematically for such deviations [32, 35, 39] this is not equally simple for the measurements of inhibition and crystallization.

It has been shown in several studies that stone formers have urine volumes that are greater than those recorded in normal subjects [43, 45]. If this is attributable to an excessive fluid intake during the collection period, the result will undoubtedly be an underestimation of the crystallization propensity and the inhibitory potential. The volume in stone formers urine in this study was, however, not higher than in normal subjects.

Attempts have been made to circumvent this problem by carrying out assessment of crystal growth inhibition in samples diluted to a standardized concentration of creatinine [34]. Such an artifice is, however, not without objections because the excretion of creatinine differs between men and women, is affected by changes in renal function and increases with the dietary load of protein.

Although there is no linear relationship between the inhibitory properties and degree of urine dilution we believe that a standardized comparison between different samples can be useful. In our series of experiments the inhibition of growth was measured in 2% whole urine and the inhibition of aggregation in 3% whole urine. Direct estimates of CaOx and CaP crystallization was obtained from 98% whole urine. Whereas the latter measurements are of relevance only for the crystallization in distal collect duct and pelvic urine, the inhibition recorded in diluted urine might reflect the situation at higher nephron levels.

It should be pointed out that the preparations of urine might have affected its composition regarding urinary macromolecules. Samples from stone formers and normal subjects, however, were treated in the same way. Differences in behaviour of macromolecules between stone formers and normal subjects might invalidate a comparison between the two groups, but filtration was carried out only after pH adjustment to pH 5.8. The filtration was considered necessary to avoid the risk of heterogeneous crystallization.

The main objective of this study was to get information on the crystallization of CaOx and CaP as well as the inhibition of CaOx crystallization in samples standardized with respect to volume and pH. We therefore used 12-h urine, most of which had been produced during the night, to avoid long periods of inappropriate sample storage, and to enable delivery of the samples for analysis close to the end of the collection period. It needs to be emphasized that in applying these principles for volume adjustment an increased supersaturation caused by a low urine flow will escape detection. This was obviously also the case for some patients in whom the highest AP(CaOx) index levels were observed in *Urine O*. One great advantage in addition to the standardization is that the urine composition during the night period is subject to less dietary influences than the daytime urine and *Urine S* therefore should be particularly well suited for measurements of inhibitory properties.

The accuracy of our measurements depends entirely on the correct collection of urine. We cannot prove that no urine was lost or that urine was not collected during a period exceeding the 12-h period, but the patients were carefully instructed and the volumes of *Urine O* appeared to be consistent with a 12-h urine production.

A comparison between NM and NW is of interest in view of the much higher stone incidence in men. The higher concentration of oxalate and the lower concentration of citrate in *Urine S* from NM are in agreement with other observations [8, 43] and explain the higher AP(CaOx) index in NM.

The lower oxalate excretion in SFM in comparison with NM was surprising, but might partly be explained by the fact that only night urine samples were included in the study. Although the small number of subjects in each group makes conclusions difficult it is nevertheless highly interesting that the lowest oxalate levels were recorded in CaOxCaP-SFM. There was, however, no significant difference in oxalate excretion between NM and CaOx-SFM, albeit the oxalate concentration was numerically lower in the latter group. A precipitation or retention of CaOx crystals within the nephron and subsequently a reduction of crystals might have contributed to such an outcome [16], a risk that might be particularly high during the night.

There is a paucity of information on the composition of night urine samples. Repeated analysis of urine during a 24-h period suggested a peak supersaturation during early morning and late night [1], but this is to a great extent the result of a reduced urine flow during the latter period. It is reasonable to assume that the risk during the day is a result of the dietary load with calcium, oxalate, phosphate and alkali. Support for this assumption has recently been obtained by comparing 16-h daytime urine with 8-h night urine [40].

The lower $Inh_{GR}U$ in SFM is a finding supported by numerous reports in the literature of an insufficient inhibition of CaOx crystal growth in stone formers [2, 7, 11–13, 27, 28, 31, 40]. The difference in $Inh_{GR}U$ was, however, numerically small and at a level commensurate with our previous results of CaOx crystal growth inhibition at a fixed concentration of creatinine [34] and in 8-h urine (40). Albeit the lowest $Inh_{GR}dU$ values were observed in SFM, the lack of differences in $Inh_{GR}dU$ between SFM and NM was in accordance with our previous measurements of the inhibition of CaOx crystal growth in the presence of high concentrations of dialysed urine [4]. This is in agreement with previously reported observations that high concentrations of urine have a great inhibitory capacity resistant to addition or subtraction of inhibitors [19]. For this reason it is unlikely that minor inhibitory differences would be detected, at least not with the methodology used in this study. The assessment of CaOx crystal growth inhibition in undiluted urine is therefore probably of doubtful value in the routine evaluation of stone formers. Growth inhibition might play a greater part during the early phase of the stone-forming process which is assumed to occur at a nephron level where the concentrations of inhibitors probably approach those used in the $Inh_{GR}U$ method. Despite the similarity in CaOx supersaturation in *Urine S* between SFM and NM expressed in terms of AP(CaOx) index, the CaOx-CR was higher in SFM than in NM. Whether this is an effect that can be ascribed to the lower $Inh_{GR}U$ is not proved, but reasonable [39]. Other factors might, however, also be operative such as macromolecules promoting the nucleation [41].

In contrast to our previous results and expectation Inh_{AGG} was not significantly different between SFM and NM or between SFM with different types of calcium stones. The small number of normal subjects included in the study might have contributed to this result as well as the fact that both pH and urine volume were standardized. Furthermore citrate, which is known to be of importance for CaOx crystal aggregation, was not different between the groups. The excretion of citrate cannot, however, be the only explanation inasmuch as Inh_{AGG} was similar in NM and NW despite the higher citrate concentration encountered in NW.

It is noteworthy that the concentration of GAGs did not differ between SFM and NM despite the lower $Inh_{GR}U$ in SFM. There is no consensus in the literature regarding any difference in GAG excretion between normal subjects and stone formers [18, 25], but information on the different types of GAGs might be more relevant [47]. Routine analysis of the total excretion of GAGs is apparently not justified.

The higher phosphate concentration in CaOxCaP-SFM and the higher AP(CaP) index(s) in these patients compared with CaOx-SFM is highly interesting. Recent evidence has suggested that calcium stones might be initiated by a nucleation of CaP at a nephron level [10, 23]. The findings in CaOxCaP-SFM give further support to such a mechanism, particularly in view of the AP_{CaP} levels obtained by extrapolation to DTd urine. The higher phosphate excretion in CaOxCaP-SFM is in line with a previous idea that pure CaOx stones have an aetiology other than CaOxCaP stones [26].

Although limited in number of patients and normal subjects the results in this study emphasize the usefulness of $Inh_{GR}U$ and CaOx-CR in the risk evaluation of patients with calcium stone disease, and also show that analysis of phosphate and calculation of AP(CaP) index(s) might be useful for this purpose, but the latter statement needs to be validated by additional experimental and clinical studies.

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