Amino Acids (2010) 38:1277–1282
DOI 10.1007/s00726-009-0359-z

REVIEW ARTICLE

Plasma levels and urinary excretion of amino acids by subjects with renal calculi

Stoyanka Slavcheva Atanassova · P. Panchev · M. Ivanova

Received: 30 October 2008 / Accepted: 17 September 2009 / Published online: 29 September 2009

Abstract Plasma levels and urinary amino acid excretions were estimated by high-performance liquid chromatography in 15 control subjects and 36 stone formers (SFs) classified according to the stone type: (1) 22 cases with calcium oxalate stones; (2) four cases with pure uric acid stones; (3) 10 cases with magnesium-ammonium phosphate stones, either pure or mixed with apatite. Some types of stones (namely oxalate and uric acid calculi) are mainly formed as a result of a metabolic deficiency that may affect the amino acid metabolism, and thus may be reflected in the urinary amino acid pattern. Data demonstrated clearly that there is a general tendency towards decreased amino acid excretions in all SFs with all types of stones. As a whole, one can observe a higher percentage of patients with calcium oxalate and phosphate calculosis, who have low urine excretions of amino acids; about 50% are the SFs with lower urine excretion of serine, glycine, taurine and *i*-leucine; the high percentage of patients with CaOX calculi shows lower urine excretions of tyrosine and ornithine.

Keywords Amino acids · Urolithiasis · Renal calculi · Plasma levels · Urine excretion

S. S. Atanassova (✉) · P. Panchev
Department of Urology, Alexandrov's University Hospital, Medical University, "G. Sofiisky" Blvd 1, 1431 Sofia, Bulgaria
e-mail: stoyana_atanassova@abv.bg

M. Ivanova
National Genetic Laboratory, Alexandrov's University Hospital, Sofia, Bulgaria

Introduction

Urolithiasis is a socially significant disease that affects from 1 to 4% of people all over the world and among the European population, in particular. There are two major factors leading to urine supersaturation, and consequently to the biological formation of crystals—renal calculi: the hyperexcretion of ions leading to the formation of crystals (Ca^{2+}, $C_2O_4^{2-}$, PO_4^{3-}, etc.) and a significant decrease in the amount of inhibitors of crystal growth, such as glycosaminoglycans, free amino acids, citric acid, etc. (Bowyer et al. 1979; Ryall et al. 1981). It has been shown, in previous studies, that normal urine contains substances strongly inhibiting crystal growth and aggregation. Urine from recurrent stone formers (SFs) appears to contain less of these substances and the inhibitory activity is lower than in normal urine (Meyer and Smith 1975; Dent and Sutor 1971).

Despite the number of promising hypotheses, the pathogenetic mechanism of intrarenal stone formation remains to be obscure. The solubility of the most widespread of renal calculi—those formed from the various dehydrates of calcium oxalate (CaOX) and magnesium-ammonium phosphate with carbonate-apatite or Ca-phosphate (STR/APA)—has been subject to many investigations. Unfortunately, the alteration of pH within physiologically tolerable limits changes the solubility of CaOX and CaP only slightly (Hammarsten 1921). It also became evident that since Hammarsten's classic studies, many ions, such as Mg^{2+}, citrate, etc., which are normally present in urine, increase the solubility of CaOX in aqueous solutions by forming complexes with either the Ca^{2+} or the $C_2O_4^{2-}$ ions. However, oral administration of these complexing agents does not lead to encouraging clinical results, as they are metabolized in the organism.

In recent years, there has been increasing interest in particular Ca-binding amino acids, with regard to their role in many Ca-dependent physiological processes. For example, dissolution of CaOX calculi with complexon III (Na-EDTA) is possible in the clinical practice only by the difficult technique of the so-called instrumental hemolysis (Timmermann and Kallistratos 1973). The possible inhibiting influence of various amino acids (aspartic, ornithine, tryptophan etc. (Grases et al. 1988) on the CaOX growth process in urine has also often been discussed in literature. These amino acids that are normally present in urine have an inhibiting effect on CaOX and CaP growth even at minimal concentration (Davies and Waind 1950).

Our own in vitro experiments indicated that there are other normally present components of urine, such as hippuric acids, ketoglutaric acid, lysine, etc., that increase the solubility of calcium oxalate in various physiological solutions (Gutzow et al. 1993; Atanassova et al. 1996, 2000). They form a relatively unstable haelate complex with Ca^{2+} in pure water (Budevsky 1979).

Little work has been carried out in the field of amino acid excretions among SFs (Mc Geown 1957; Hum et al. 1962; Zinsser et al. 1971; Shaker et al. 1983; Thomas et al. 1981). There have been two announcements about the maintenance of amino acids in the structure of excreted renal calculi (Heijkenskjold and Molierberg 1956).

The aim of the present work is to categorize the patients studied according to the type of calculi formed as well as to estimate the plasma and urinary amino acid contents to find any possible correlation. Some types of stones (namely oxalate and uric acid calculi) are mainly formed as a result of a metabolic deficiency that may affect the amino acid metabolism, and thus might reflect on the urinary amino acid pattern. The α-amino and α-hydroxyl acids with lower urine contents would be subject to a future research about their complex-forming and/or inhibitory activity on stone formation.

Materials and methods

Stone formers (SFs)

36 patients (pts), 17 male and 19 female subjects, whose age ranged from 19 to 62 years at the beginning, were diagnosed and monitored during a 1-year period. The patients studied had no impairment of renal function or urinary infection. No prior changes in diet or drug prescription for these individuals were made. The patients suffering from urinary lithiasis and clinically free from any hepatic disorders were selected to represent all types of stones and were classified according to the type of stone as follows: (1) 22 cases with calcium oxalate stones (CaOX); these types of stones are usually formed in acid or neutral urine; (2) four cases with uric acid (UA) stones, which are usually formed in more acidic urine, and (3) 10 cases with magnesium-ammonium phosphate stones (struvite—STR) mixed with apatite (APA), which are usually formed in alkaline urine.

Controls

Fifteen healthy subjects, who have never had any urological and hepatic trouble.

Methods

All subjects were hospitalized in the Clinics of the Chair of Urology.

A 24-h collection of urine was obtained from each patient. During the collection period, specimens were refrigerated and aliquots of the 24-h volume were immediately frozen until analyzed.

Serum was obtained from the same people as those whom urine was collected from. Heparinised plasma was separated by centrifugation and was also stored at −25°C.

The amino acid contents of the samples were determined using a Hewlett Packard HPLC 1050 coupled to a fluorescence detector. Ethyl alcohol was added to the plasma and urine specimens to allow the precipitation of proteins and the extraction of free amino acids. An automated precolumn ortho-phthalaldehyde derivation procedure was employed. Separations were done using a reversed-phase column (Waters Corp). Each analysis required 48–52 min of chromatographic run.

Amino acid concentrations of the samples were determined by comparison with values obtained from a standard curve prepared for each amino acid. Amino acid concentrations are expressed in μM.

Statistical analysis

Statistical analyses of data obtained from pts and a control was performed using Student's t test to establish the significance of the difference between means. All results were expressed as mean ± SEM, and the differences were considered significant if $P \leq 0.05$.

Results

The plasma levels of free amino acids, either essential or non-essential in controls and in the different groups of patients with renal calculosis are shown in Table 1. Table 2 shows the urine excretion of amino acids in the same groups. A mathematical estimation of the deviation from the observations on amino acids in patients has been made.

Table 1 Mean ± SEM values (μMol/l) of plasma amino acids in controls and different groups of patients with renal calculosis

Amino acid × [μmol/l]	Controls ($n = 15$)	Calcium oxalate stones ($n = 22$)	Uric acid stones ($n = 4$)	Strive/apatite stones ($n = 10$)
Aspartic acid	12–37	23.1 ± 17.4	21.0 ± 12.6	40.0 ± 11.3*
Glutamic acid	22–177	148.5 ± 140.3	180.0 ± 71.3*	193.0 ± 78.4*
Asparagine	24–91	58.9 ± 36.1	45.0 ± 20.1	43.3 ± 23.9
Serine	58–181	95.3 ± 59.7	93.0 ± 41.3	103.7 ± 34.7
Histidine	23–127	66.9 ± 45.8	79.0 ± 41.2	75.3 ± 53.4
Glutamine	340–1,238	220.0 ± 131.9*	88.0 ± 73.2*	223.0 ± 138.7*
Glycine	107–885	185.9 ± 94.2*	208.0 ± 68.4*	207.7 ± 70.0*
Threonine	52–276	105.8 ± 84.2	97.0 ± 42.9*	52.0 ± 21.3*
Arginine	72–91	109.9 ± 39.4*	106.0 ± 32.1*	68.7 ± 44.1
Taurine	0–51	72.3 ± 66.8*	104.0 ± 63.2*	61.7 ± 25.1*
Alanine	139–825	257.9 ± 136.6*	277.0 ± 83.6*	234.7 ± 89.7*
Tyrosine	16–167	43.9 ± 27.7*	44.0 ± 27.4*	32.3 ± 19.6*
Methionine	0–70	43.69 ± 19.96	91.0 ± 32.3*	26.00 ± 19.51
Valine	86–293	169.9 ± 109.6	179.0 ± 103.0	109.0 ± 39.0
Phenylalanine	22–103	61.6 ± 36.5	72.0 ± 30.1	107.7 ± 54.5*
i-Leucine	21–91	66.9 ± 32.4	79.0 ± 10.7	46.7 ± 22.2
Leucine	46–183	109.6 ± 70.2	101.0 ± 53.2	105.3 ± 61.4
Ornithine	41–131	72.0 ± 41.4	152.0 ± 33.2*	64.0 ± 31.3
Lysine	85–284	115.8 ± 52.4	114.0 ± 58.3	126.7 ± 65.1

* Statistically different from controls ($P < 0.05$)

Table 2 Mean ± SEM values (μMol/24 h) of urinary amino acids in controls and different groups of patients with urolithiasis

Amino acid × [μmol/24 h]	Controls ($n = 15$)	Calcium oxalate stones ($n = 22$)	Uric acid stones ($n = 4$)	Strive/apatite stones ($n = 10$)
Aspartic acid	10–220	59.2 ± 45.4*	241.5 ± 42.6*	68.2 ± 30.7*
Glutamic acid	–	214.9 ± 123.8	142.5 ± 58.5	105.6 ± 52.3
Asparagine	–	148.6 ± 96.3	87.0 ± 47.2	93.4 ± 51.9
Serine	110–620	240.6 ± 202.6	168.0 ± 56.2*	112.8 ± 94.3*
Histidine	130–1,370	632.9 ± 325.5	415.0 ± 132.6	406.1 ± 175.1
Glutamine	–	188.4 ± 200.7	61.5 ± 43.2	72.9 ± 48.8
Glycine	710–4,160	841.5 ± 439.9*	499.0 ± 262.6*	485.7 ± 286.5*
Threonine	20–300	70.9 ± 52.2	73.5 ± 50.5	64.2 ± 41.3
Taurine	220–1,850	400.0 ± 276.1*	1,798.0 ± 389.5	220.0 ± 68.0*
Alanine	60–500	206.0 ± 151.5	163.5 ± 63.8	148.4 ± 73.5
Tyrosine	40–150	86.8 ± 57.3	96.0 ± 50.2	81.6 ± 40.3
Tryptophane	–	54.9 ± 45.4	51.0 ± 38.8	13.5 ± 10.7
Valine	0–260	57.6 ± 50.3*	40.5 ± 34.2*	65.0 ± 41.6*
Phenylalanine	40–250	63.6 ± 54.9*	31.5 ± 28.4*	96.1 ± 50.2
i-Leucine	40–180	44.3 ± 32.4*	33.0 ± 28.6*	59.7 ± 37.1*
Leucine	10–150	51.4 ± 43.2	57.0 ± 50.4	94.1 ± 67.0
Ornithine	10–80	35.7 ± 28.5*	108.0 ± 54.6*	72.1 ± 70.3
Lysine	0–120	174.7 ± 140.6*	169.5 ± 130.6*	226.5 ± 144.7*

– There is no data for the control people

* Statistically different from controls ($P < 0.05$)

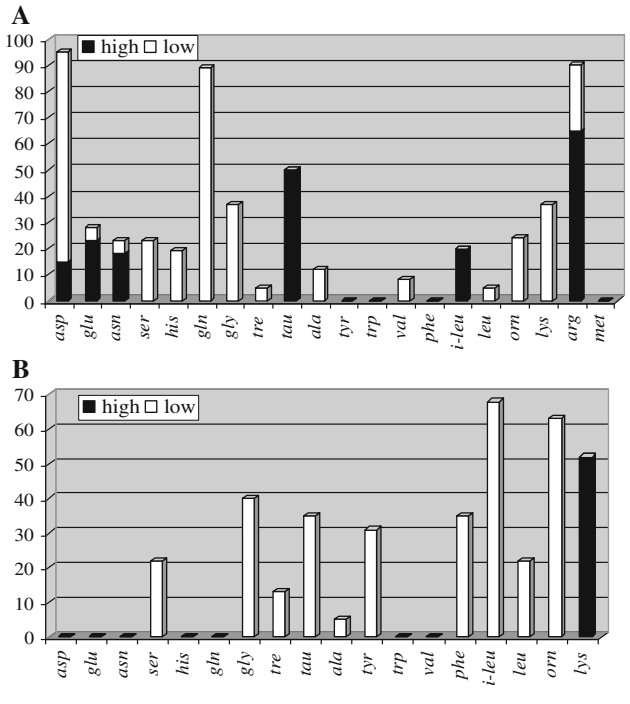

Fig. 1 **a** Percentage of pts with CaOX calculi showing lower and/or higher plasma levels of some amino acids. **b** Percentage of pts with CaOX calculi with lower or higher urine excretions of some amino acids

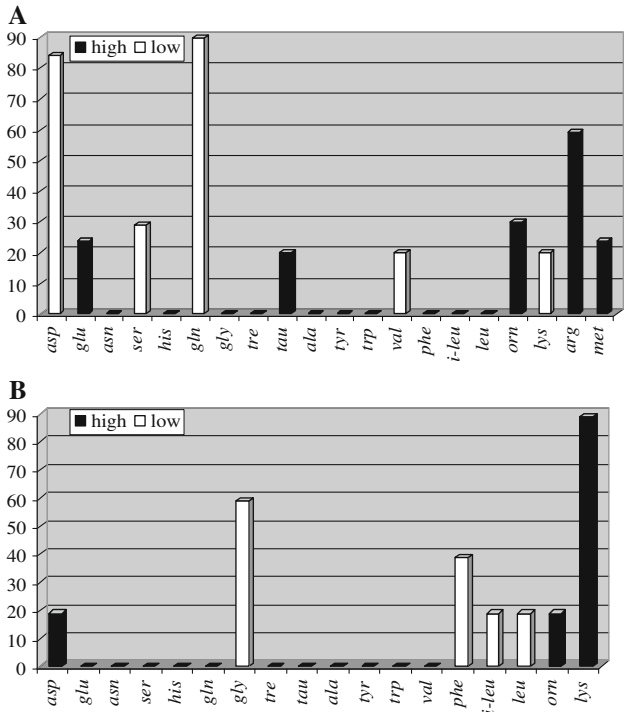

Fig. 2 **a** Percentage of UA-SFs showing lower or higher plasma levels of some amino acids. **b** Percentage of UA-SFs with higher or lower degrees of urine excretions of some amino acids

Figures 1, 2, 3 represent the percentage of patients with amino acid levels below and/or above the value of the control group.

Arginine (in about 60% of all SFs), glutamic acid (in 20% of CaOX-SFs and UA-SFs and about 60% of STR/APA-SFs), aspartic acid (in 60% of STR/APA-SFs) and phenylalanine (in 40% of STR/APA-SFs) showed to be the predominant amino acid levels in the plasma of SFs with citated renal calculosis. Increased plasma taurine was observed in all groups of the SFs. Higher blood methionine and ornithine were observed only in the group of UA-SFs. In contrast, the lower plasma levels (in all groups) of glutamine and lysine as well as of serine and glycine in CaOX-SFs, and serine and histidine in group of the UA-SFs were observed.

One can observe greater number of pts with lower serum concentration mainly of non-essential amino acids eliminating UA and STR/APA calculi (Fig. 3a).

As shown in Table 2, most of the total essential and non-essential amino acids in urine are significantly decreased in observed groups of SFs in comparison with our controls. Thus, in the CaOX group, the following amino acids were significantly decreased such as glycine, taurine and phenylalanine (in about 40% of SFs), tyrosine (in 30% of SFs),

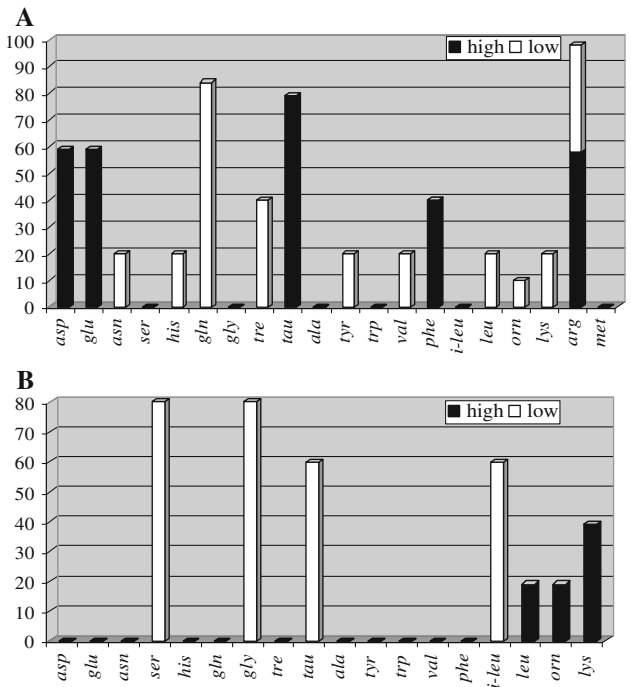

Fig. 3 **a** Percentage of STR/APA-SFs with lower and/or higher plasma levels of some amino acids. **b** Percentage of STR/APA-SFs showing lower or higher urine excretions of some amino acids

i-leucine and ornithine (in above 50% of SFs) (see also Fig. 1b). In the UA group (Fig. 2b), only glycine (in 60% of SFs), phenylalanine (in 40% of SFs) and *i*-leucine (in 20% of SFs) were significantly decreased. As to the group of STR/APA-SFs, significant lower urine excretions of serine and glycine (in above 70% of STR/APA-SFs), taurine and *i*-leucine (about 50% of STR/APA-SFs) were observed (see Fig. 3b). In contrast, the higher urinary excretions of lysine and aspartic acid of UA-SFs (see Table 2; Fig. 2b) were observed. This stand out against the background of lower and/or normal plasma levels of the others mentioned above. High urine excretions of lysine can be estimated in the other two groups of SFs with CaOX and STR/APA lithiasis (see Table 2; Figs. 1b, 3b) as well.

As a whole, one can observe a higher percentage of patients with calcium oxalate and phosphate calculosis, who have low urine excretions of amino acids; about 50% are the SFs with lower urine excretions of serine, glycine, taurine and *i*-leucine; according to the mentioned, the high percentage of patients with CaOX calculi show lower urine excretions of tyrosine and ornithine, which is of particular interest.

Two different amino acids observed in the plasma of patients (arginine and methionine) as well as citric acids have not been registered in the urine samples.

Discussion

There are two main factors that characterize the renal calculus as the major problem of our age; on the one hand, its frequency and high rate of recurrence, and on the other hand, the lack of efficient therapeutical effect from dietary or pharmacological approaches.

Although, presently, there are diverse instrumental methods for the extraterrestrial breaking and removal of renal calculi, an efficient metaphylaxis based on biologically significant urine constituents would lead to a significant decrease in the recedive of urolithiasis. In that sense, amino acids may have a special role in the desegregation or act as important inhibitory agents of kidney stone formation.

Data demonstrated clearly that there was a general tendency towards decreased amino acid excretions in all SFs with all types of stones, however, individual amino acid patterns varied according to the type of stone formed. It is evident from Tables 1 and 2 that there was a similar pattern, both quantitatively and qualitatively, in the plasma levels and urine excretions of amino acids by unaffected subjects (controls) and those suffering renal calculosis.

Of great interest for scientists is the significant decrease in glycine and serine excretions in the CaOX group, which stands out against the background of their plasma levels; about 40% of pts with CaOX lithiasis have a decreased exception and show lower plasma values of glycine and serine. Our data corresponded with the observation reported by Crawhall et al. (1959), Mayer et al. (1968) and Zinsser et al. (1971), who demonstrated certain defects in the metabolic pathway of glycine resulting in increased production of oxalate, which is in turn possibly leading to the formation of oxalate renal stones. In addition, serine conversion to glycine may favor the shift towards increased oxalate formation and would, thus, indirectly participate in oxalate stone formation. About 40% of pts from the CaOX group are in good agreement with these results.

The significant decrease in tyrosine (above 30% of pts with CaOX renal calculi) is in good agreement with the results of Zinsser et al. (1971), who attributed this decrement to increased tyrosine degradation to hydroxyphenyl pyruvic acid. On the other hand, tyrosine takes part in synthesis of hippuric acid, which is made of helate, combined with Ca^{2+} (Gutzow et al. 1993). We consider that the lower excretion of tyrosine explains well the lower inhibitory capacity of urine in the process of CaOX stone formation. In the other two groups of pts (the UA group and STR/APA group), there was no data of lower tyrosine excretions.

Taurine was the amino acid with the highest plasma levels amounting to all subjects of our groups. We attribute this increased taurine as a possible consequence of a high dietary intake of meat before clinical analyses. However, significantly lower urine excretions of taurine in CaOX and STR/APA-SFs were observed. Glycine and taurine are the products of holic acid disintegration; glycoholic acid breaks up to glycine, but instead of glycine, tauroholic acid further disintegrates to taurine. According to the mentioned above, the increased production of oxalate in CaOX-SFs results from the metabolic pathway of glycine, the decreased excretion of taurine at the expense of glycine, and indirectly explains higher oxalate production.

Glutamine was the amino acid with lower level in the plasma of our groups, a result of probably a low vegetable dietary intake, mainly corn. Glutamine is the basis for the production of nucleic acids and glycosamineglycanes, which are the most important inhibitors of calcium oxalate renal formation. The latter explained the decrease in inhibitory activity mainly of CaOX supersaturated urine.

Significantly higher were the plasma levels of arginine in most of SFs. The detection of the amino acid known as arginine was quite interesting, because ornithine which is the amino acid with a determined capacity to form compounds, was not found in proteins, but at the same time, it is a major component of arginine; which explained the lack of any concentration of ornithine in all urine probes. Taking into account the possibility of arginine to disintegrate to ornithine by the enzyme arginase and in this aspect, it is of special interest to find a decrease in urinary

ornithine excretion (in above 50%) in the group of CaOX-SFs. As it is well known, ornithine is a good complex-forming agent, and in this connection, his lower urinary excretion in pts with CaOX calculi may explain the shift towards increased calcium oxalate formation in these patients (Gutzow et al. 1993).

The possibility of the precipitation and/or adsorption of some free amino acids in the calculus material, itself, may explain their lower urine contents in most SFs. This suggestion is based on the observation reported by Heijkenskjold and Molierberg (1956), who demonstrated that the available free amino acid contents in renal calculi ranged from 0.39 to 3.98% of their total weight. Alanine, lysine, histidine, valine, methionine, tryptophan, leucine and isoleucine have been the amino acids occurring in renal stones, which may explain our data on their lower excretion.

It is essential to study the bacterial urine infections that influenced the amino acid urine contents. According to data of Mc Geown (1957) and our results, the total excretions of amino acids in the STR/APA-SFs, where urinary tract infections were more prevalent, were not statistically different from that of the other two groups.

In conclusion, we may say that a really successful metaphylaxis of renal stone formation (for example that of oxalate and uric acid calculi), which forms as a result of metabolic deficiency, can be achieved by the improvement of dissolving and inhibitory effects of some normally present substances in the urine. We would need data about the low excretions of these urine substances, which may play a role as complex-forming agents and/or have a good inhibitory activity (Azoury et al. 1985; Davies and Waind 1950). On the whole, many of the essential and non-essential amino acids (mainly α- and β- ones) are normal constituents of human urine and in this way they might be potential complex-forming agents, which form a helate relatively unstable complex with Ca^{2+} or $C_2O_4^{2-}$ ions (Budevsky 1979). Our own in vitro experimental results are a good evidence of the matter concerned in the previous sentence (Gutzow et al. 1993; Atanassova et al. 1996, 2000). The present paper gives us new data about concentrations of tyrosine, arginine and ornitine, glutamic acid, lysine, aspargine. Of course, it is of great interest to study the metabolic disorders in the cases of higher or normal plasma levels of some amino acids (such as taurine, lysine, aspartic acid) with regard to their lower urine excretions and the opposite.

References

Atanassova SS, Neykov KG, Gutzow IS (1996) Solubility, inhibited growth and dissolution kinetics of CaOX crystals in solutions, containing α-ketoglutaric acid. J Cryst Growth 160:148–153. doi:10.1016/0022-0248(95)00545-5

Atanassova SS, Neykov KG, Gutzow IS (2000) Solubility and dissolution kinetics of CaOX renal calculi in solutions, containing DL-lysine: in vitro experiments. J Cryst Growth 212:233–238. doi:10.1016/S0022-0248(99)00883-0

Azoury K, Goldwasser B, Wax Y, Perlberg S, Garti N, Sarig S (1985) Evaluation of the relative inhibitory potential of low fractioned urinary macromolecules. Urol Res 13:199–205. doi:10.1007/BF00261824

Bowyer RC, Brockis JG, McCulloch RK (1979) Glycosaminoglycans as inhibitors of calcium oxalate crystal growth and aggregation. Clin Chim Acta 95:23–27. doi:10.1016/0009-8981(79)90332-2

Budevsky O (1979) Foundation of chemical analysis. Willy, NY

Crawhall JG, Scowen EF, Watts RW (1959) Conversion of glycine to oxalate in primary hyperoxaluria. Lancet ii:806–809. doi:10.1016/S0140-6736(59)90751-2

Davies CW, Waind GM (1950) Calcium salts of some amino acids and dipeptides. J Chem Soc 50:301–303. doi:10.1039/jr9500000301

Dent CE, Sutor DJ (1971) Presence or absence of inhibitor of calcium oxalate crystal growth in urine of normal and stones-formers. Lancet 2:775–778. doi:10.1016/S0140-6736(71)92737-1

Grases F, March JG, Bibiloni F, Amat E (1988) The crystallization of CaOX in the presence of amino acids. J Cryst Growth 87:299–302. doi:10.1016/0022-0248(88)90179-0

Gutzow IS, Atanassova SS, Budevsky GP, Neykov KG (1993) Solubility, inhibited growth and dissolution kinetics of calcium oxalate crystals in solutions, containing hippuric acids. Urol Res 21:181–185. doi:10.1007/BF00590034

Hammarsten G (1921) On calcium oxalate and its solubility in the presence of inorganic salts with special reference to the occurrence of oxaluria. Comp Rend Trav Lab Carlsberg Copenh 17:1

Heijkenskjold F, Molierberg H (1956) The presence of amino acids in urinary calculi. Scand J Clin Lab Invest 8:230–233. doi:10.3109/00365515609049276

Hum BAL, Huntsman RG, Lehmann H, Liddell J, Rose DB (1962) Urinary amino acids and calcium in relation to renal stone and renal damage. Clin Sci 22:195–208

Mayer GG, Chase T, Farvar B, Al-Waidh M, Longo F, Karp F, Zinsser HH (1968) Metabolic studies on the formation of calcium oxalate stones with special emphasis on vitamin B_6 and uric acid metabolism. Bull N Y Acad Med 44:28–44

Mc Geown MG (1957) The urinary amino acids in relation to calculus disease. J Urol 78:318–322

Meyer JL, Smith LH (1975) Growth of calcium oxalate crystals. II. Inhibition by natural urinary crystal growth inhibitors. Invest Urol 13:36–39

Ryall RL, Harnett RM, Marshall VR (1981) The effect of urine, pyrophosphate, citrate, magnesium, and glycosaminoglycans on the growth and aggregation of calcium oxalate crystals in vitro. Clin Chim Acta 112:349–352. doi:10.1016/0009-8981(81)90458-7

Shaker YM, El-Hawary MFS, Zahran MM, Mostafa SO (1983) Urine and calculus amino acids in the different types of urolithiasis. Urol Int 38:51–54

Thomas WC, Malagodi MH, Rennert OM (1981) Urinary excretion of aminoacids by subjects with renal calculi. In: Smith LH, Robertson WG, Finlayson B (eds) Urolithiasis: clinical and basic research. Plenum press, London, pp 77–80

Timmermann A, Kallistratos G (1973) In: Hienzsch F, Schneider H-J (eds) Der Harnstein. Instrumentalle Nierensteinchemolyse. Fischer, Jena. p 235

Zinsser HH, Stem F, Marshall S, Karp F, Seneca E, Gursel E (1971) Urinary organic acids found in B_6 deficient rats and calcium oxalate calculus patients. Br J Urol 43:523–535. doi:10.1111/j.1464-410X.1971.tb10038.x