CLINICO-BIOCHEMICAL INDEXING OF CALCIUM UROLITHIASIS ON THE BASIS OF URINARY CITRATE TO CALCIUM RATIO AND INHIBITION OF MINERALISATION OF URINARY STONE FORMING MINERALS BY HUMAN URINE

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Urinary citric acid and calcium levels have been estimated in the urine of 20 normal healthy persons as well as 12 urinary stone patients. Inhibition efficiency of these urine samples towards the mineralisation of urinary stone forming minerals, viz., calcium phosphate, oxalate or carbonate, has been studied in an experimental model. Statistical correlation of the above data has been made by computing the coefficient of determination and unexplained variance. Clinico-biochemical indexing of calcium urolithiasis risk factor has been attempted in the light of the data.

INTRODUCTION

The level of citric acid in urine has been recognised as an important factor in the control of calcium urolithiasis. It is a natural and potent inhibitor of urolithiasis(1,2). Citrate therapy has been found to be useful in the treatment of calcium oxalate nephrolithiasis(3,4). The mechanism of inhibitory action of citric acid is probably through the chelating of Ca^{++} ions in urine and thus, preventing the latter from combining with stone forming anions like oxalate, phosphate etc. to precipitate out. *In-vitro* studies have shown the sequestration and chelation of urinary stone forming minerals by citric and other related natural acids(5,6). Inhibition of Nucleation and aggregation of calcium oxalate by whole urine has been observed in *in-vitro* studies(7).

The optimum urinary level of citric acid to function as stone inhibitor would also be related to the urinary calcium level. Hence, it would be of significance to estimate calcium as well as citric acid level in urine to find a clinico-biochemical index of urolithiasis risk. A correlation between urinary citric:calcium level and its (urine) inhibition efficiency towards mineralisation of urinary stone forming minerals would throw light on the applicability of this biochemical index as a diagnostic tool in urolithiasis. With these views, we have presently estimated the calcium and citric acid levels in the urine of 20 normal healthy persons and 12 urinary stone patients. We

have also studied these urine samples as *in-vitro* inhibitors of mineralisation of urinary stone forming minerals, viz., calcium phosphate, oxalate or carbonate in an experimental model. The data has been statistically correlated by computing the correlation coefficients, coefficient of determination and unexplained variance. Clinico-biochemical indexing of calcium urolithiasis risk factor has been attempted in the light of the data obtained.

EXPERIMENTAL

COLLECTION OF URINE SAMPLES - Urine samples of 20 healthy persons and 12 urinary stone patients were collected separately in sterilised plastic containers and labelled as U_1 to U_{32} . The healthy persons(controls) were selected in the age group of 18 to 52 years, with no recent report of ill health of any kind and had no past history of urolithiasis, including that in the family. The stone patients were selected at random from among those attending the local clinics and were in the age group of 21 to 65 years. The selection was based on diagnosis by ultrasonography or x-ray. All subjects (healthy persons and stone patients) were in the dietary habit of non-vegetarian food. A 24 hour urine output was collected in each case and 1% thymol was added as a preservative. The samples were used out in minimum possible time after collection. Half of the portion of the sample was treated with 5 ml conc. HCl , and this portion was used for calcium estimation. The sample details including urinary volume, pH and location of stone in case of patients, are mentioned in Table 1.

ESTIMATION OF CITRIC ACID IN URINE - Citric acid in the urine samples was estimated by a

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colorimetric method⁽⁸⁾. This method is based on the principle that citric acid is oxidised to pentabromoacetone (PBA) by bromine. The PBA formed is extracted with petroleum ether. The petroleum ether layer is shaken with thiourea-borax solution (pH 9.2) and the yellow coloured complex formed in the aqueous layer is measured photo-metrically.

ESTIMATION OF CALCIUM IN URINE - Calcium in the urine samples was estimated by Trinder's method⁽⁹⁾. This method is based on the principle that calcium present in the sample is precipitated with naphthylhydroxamic acid (calcium reagent). The precipitate is dissolved in EDTA reagent and calcium from this solution is complexed with ferric nitrate (colour reagent) to give a coloured complex that is measured colorimetrically.

INHIBITION EFFICIENCY OF URINE SAMPLES - Crystalloid forming solutions viz., solution of calcium acetate, trisodium phosphate, disodium oxalate and sodium carbonate of 0.01 M concentration were prepared in double distilled water. All chemicals were of analytical reagent grade. An experimental model was designed in which the two salt forming solutions, e.g. sodium phosphate and calcium acetate (for calcium phosphate) were taken in two separate burettes (50 ml) and were allowed to fall simultaneously and slowly (drop wise) with equal speed into a 250 ml beaker containing 50 ml. of urine sample (inhibitor). The whole operation took about 40 minutes. At the end the contents of beaker were digested in a hot water bath for 10 minutes, cooled to room temperature and the precipitate was collected into a pre-weighed centrifuge tube by centrifuging small volumes at a time and rejecting the supernatant liquid. Next, the tube with the precipitate was dried in air oven at 120°C, cooled to room temperature and weighed till constant weight. Weight of the precipitate was determined.

Simultaneous blank experiments with water in place of urine sample were also carried out for evaluating the inhibition efficiency of urine compared to that of water. All experiments were carried out at room temperature (20-25° C).

Percentage efficiency of inhibition by the urine sample was calculated using the formula,

$$\text{Inhibition} = \frac{\text{Wt. of ppt. in blank set} - \text{Wt. of ppt. in exptal. set}}{\text{Wt. of ppt. in blank set}} \times 100$$

Blank set = Water as inhibitor

Exptal. set = Urine sample as inhibitor

STATISTICAL CORRELATION OF DATA - The associative correlation coefficient between urinary citrate:calcium values and corresponding inhibition efficiency values of urine samples towards mineralisation of stone forming minerals, was calculated using standard method⁽¹⁰⁾. Coefficient of determination and unexplained variance were also calculated.

RESULTS AND DISCUSSION

The data concerning urinary citrate and calcium contents of healthy persons and urinary stone patients are recorded in Table 1. The inhibition efficiency of urine samples towards mineralisation of stone forming minerals is recorded in Table 2. The statistical analysis of the data is presented in Table 3. Values of calcium urolithiasis risk index are presented in Table 4.

A study of Table 1 suggests that the urinary citrate of normal healthy persons range from 476 to 278 mg where as the range of calcium is 245 to 96 mg. The weight ratio (citrate:calcium) lies in the range 3.29 to 1.65. The corresponding mole ratio ranges from 0.70 to 0.35. Generally the urinary excretion of calcium lies between 100 to 300 mg per day⁽¹¹⁾. In fact, calcium absorption depends on reaction of the intestinal contents. Increased alkalinity of intestinal contents causes its precipitation as phosphate, which is lost in the faeces. As such urinary excretion of calcium is also decreased. Calcium absorption increases with increased acidity of intestinal contents. Out of the 20 normal persons presently studied, only 4 subjects seem to suffer from mild hypercalciuria. However, these subjects have also been found to have relatively higher citraturia levels. This perhaps is a compensatory factor for preventing calcium precipitation and stone formation. The urinary citrate levels are, in fact, subject to wide variations and depend to some extent on dietary habits. Larger intake of animal protein and sodium generally reduces the urinary citrate level. Presently we observed severe hypocitraturia in almost all stone patients. This might be because all these patients are non-vegetarians and are in the habit of eating non-vegetarian food frequently.

The mean weight ratio of citrate to calcium for healthy persons has been found to be 2. The corresponding mole ratio comes to 0.4. For urinary stone formers, the mean of citrate to calcium weight ratio has been found to be 0.9 and the corresponding mole ratio is 0.2. Thus, it seems, as the calcium level of the urine increases and corresponding citrate level decreases, the urinary stone formation sets-in. It is apparent from the data of stone formers that most of the patients present with low urinary citrate levels and relatively increased calcium levels on the other hand. This results in a loss of balance between citrate and calcium levels. Citrate's capacity to sequester calcium ions is lost. The stone forming anions of urine viz., oxalate, phosphate, etc. now get an upper hand to react with Ca^{++} and thus precipitate it out. Thus, it seems, out of the two opposing competitors for Ca^{++} in urine namely, oxalate/ phosphate (precipitating anions) and citrate (inhibiting anion), the latter dominates the scene. As long as the citrate level in urine is sufficient (relative to calcium) the calcium ions remain in solution in equilibrium with it (citrate). It seems, that despite a person suffering from hyperoxaluria, the stone formation may not set-in as long as he is normocitratuariac. In case of hypercalciuria, however, the urinary levels of precipitating anions like oxalate, phosphate become more significant.

A study of the inhibition efficiency of urine samples towards mineralisation of stone forming minerals (Table 2) indicates that the human urine has some efficiency in itself to inhibit the mineralisation. The efficiency, however, is markedly higher for phosphate and carbonate. The inhibition efficiency varies in the range 23 to 64% for phosphate and 37 to 80% for carbonate by the healthy urine samples. For the stubborn mineral, oxalate, the urine generally has low inhibition efficiency (17-46% by healthy urine samples). Study of urinary citrate:calcium values vis-à-vis inhibition efficiencies suggest that the two factors are directly proportional to each other. A higher Citrate:Calcium value of urine invariably shows higher inhibition efficiency. The urine of stone formers show very little inhibition. The observed inhibition efficiencies are 7-23% for phosphate, 8-28% for carbonate and 4-12% for oxalate. This may, presumably, be due to very low Citrate:Calcium values of these urine samples.

From statistical point of view (Table-3), there is a clear associative correlation between urinary citrate:calcium levels and inhibition efficiencies.

Correlation coefficient is clear indicator of this. For phosphate and oxalate the associative correlation is significant at as low as 0.1% level, for both, healthy as well as stone patients. For carbonate, however, the data of correlation is significant at 5% level for healthy urine. However, even in case of carbonate inhibition by the urine of stone patients, the correlation is significant at 0.1% level. The corresponding unexplained variances of the data are also quite low (3.6 to 17.4%) except in case of carbonate and healthy urine, where in it is 73.6%. A mostly very low values of unexplained variance indicate a definite relation between the urinary Citrate:Calcium levels and mineralisation-inhibition efficiencies. Thus, urinary Citrate:Calcium level can be confidently used as an index for calcium urolithiasis risk. Higher the former (Citrate:Calcium) lower will be the risk factor.

As we have observed a linear relation between citrate:calcium and inhibition efficiency of urine towards stone forming minerals, a general hypothesis can be made as follows.

Hypothesis : The urinary citrate to calcium ratio is directly proportional to the inhibition efficiency of urine towards the mineralisation of stone forming minerals.

Let, R = Urinary citrate:calcium ratio

I = Inhibition efficiency of urine towards stone forming mineral

According to hypothesis,

$$R \propto I$$

or, $R = K.I$, where K = Inhibition coefficient (a constant for a given mineral)

Inhibition coefficient may be defined as that urinary citrate to calcium ratio which can cause 1% inhibition of the mineral by the urine.

On the basis of our present data, we have calculated the inhibition coefficients for phosphate, oxalate and carbonate minerals of calcium and termed as K_{phos} , K_{ox} and K_{carb} respectively. We have used the average values (of all 32 samples) for R and I in our calculations of inhibition coefficients, for different minerals. Considering these values of inhibition coefficients on one hand and average inhibition of the minerals by the urine of healthy and stone patients (separately) on the other hand, we have calculated the urinary Citrate:Calcium values (Index

values) for calcium urolithiasis risk. Various inhibition coefficients as well as the index values for calcium urolithiasis risk/immunity, as calculated by us, are recorded in Table 4.

CONCLUSION

Calcium urolithiasis risk is related to hypocitraturia because citric acid is a dominant inhibitor of stone formation in the urinary tract. It is very difficult to diagnose the actual stage when the stone formation would start. However, as observed presently, this risk stage can be diagnosed, with a fair amount of accuracy, by estimating urinary citrate and calcium levels or, alternatively, inhibition efficiency of urine towards stone forming minerals. This would tell us

the stage of calcium urolithiasis risk to which the patient is exposed to.

The mechanism of inhibition of calcium urolithiasis by citric acid is basically through the chelation of Ca^{++} ions. Our present experimental model too reflects the net calcium-chelating ability (inhibition) of urine as a function of its Citrate:Calcium level. As such, this model would be of diagnostic relevance in calcium urolithiasis. As per present study, it can be concluded, tentatively, that a urinary citrate:calcium weight ratio of 2.4 (mole ratio of 0.5) or more is an indicator of immunity from calcium stone formation. Any value less than this would definitely be a calcium urolithiasis risk factor.

TABLE 1
24 HOUR URINARY CITRATE AND CALCIUM CONTENT OF HEALTHY PERSONS AND URINARY STONE PATIENTS

Sample No.	Sex(Age)/ Health State	24 h. Urinary volume	Citrate (mg)	Calcium (mg)	Citrate: Calcium (Wt.Ratio)	Citrate: Calcium (Mole Ratio)
U ₁	Male (40) Healthy	1612 (6.4)	415	204	2.03	0.43
U ₂	Male (29) Healthy	1580 (6.2)	465	226	2.06	0.44
U ₃	Male (18) Healthy	1592 (6.0)	396	192	2.06	0.44
U ₄	Female(25) Healthy	1476 (5.7)	384	187	2.05	0.43
U ₅	Male (44) Healthy	1712 (5.8)	392	238	1.65	0.35
U ₆	Female(38) Healthy	1671 (6.1)	476	245	1.94	0.41
U ₇	Male (24) Healthy	1705 (6.1)	306	124	2.47	0.52
U ₈	Female(30) Healthy	1670 (6.3)	292	132	2.21	0.47
U ₉	Female(29) Healthy	1530 (6.4)	343	168	2.04	0.43
U ₁₀	Female(25) Healthy	1382 (6.0)	405	156	2.60	0.55
U ₁₁	Male (32) Healthy	1526 (5.9)	288	114	2.53	0.54
U ₁₂	Male (45) Healthy	1636 (5.9)	314	158	1.99	0.42
U ₁₃	Male (52) Healthy	1530 (5.8)	392	186	2.11	0.45
U ₁₄	Female(38) Healthy	1473 (5.9)	328	110	2.98	0.63
U ₁₅	Male (27) Healthy	1350 (6.2)	278	96	2.90	0.61
U ₁₆	Male (36) Healthy	1560 (6.2)	289	104	2.78	0.59
U ₁₇	Male (34) Healthy	1728 (5.6)	342	132	2.59	0.55
U ₁₈	Female(33) Healthy	1476 (6.3)	451	181	2.49	0.53
U ₁₉	Male (30) Healthy	1680 (6.8)	408	124	3.29	0.70

TABLE 1 [Contd..]

U ₂₀	Male (42) Healthy	1549 (6.2)	358	172	2.08	0.44
U ₂₁	Male (65) Stone patient (Stone in renal pelvis)	1633 (6.4)	231	172	1.34	0.28
U ₂₂	Female (38) Stone patient (Stone in renal pelvis)	1442 (6.5)	162	189	0.86	0.18
U ₂₃	Male (30) Stone patient (Stone in ureter)	1358 (6.0)	204	236	0.86	0.18
U ₂₄	Male (20) Stone patient (Stone in renal pelvis)	1310 (5.8)	148	212	0.70	0.15
U ₂₅	Male (32) Stone patient (Stone in ureter)	1730 (6.8)	291	242	1.20	0.25
U ₂₆	Male (36) Stone patient -(Stone in renal pelvis)	1338 (5.7)	94	248	0.38	0.08
U ₂₇	Female (42) Stone patient (Stone in renal pelvis)	1550 (6.8)	206	215	0.96	0.20
U ₂₈	Male (32) Stone patient (Stone in bladder)	1298 (6.8)	382	278	1.37	0.29
U ₂₉	Female (21) Stone patient (Stone in bladder)	1482 (7.1)	163	291	0.56	0.12
U ₃₀	Female (35) Stone patient (Stone in renal pelvis)	1403 (6.4)	149	307	0.49	0.10
U ₃₁	Female (45) Stone patient (Stone in Bladder)	1520 (7.2)	275	196	1.40	0.30
U ₃₂	Male (28) Stone patient (Stone in ureter)	1423 (6.4)	114	186	0.61	0.13

TABLE 2

INHIBITION OF MINERALISATION OF URINARY STONE FORMING MINERALS BY URINE SAMPLES OF HEALTHY PERSONS AND URINARY STONE PATIENTS

Urine Sample	<u>Inhibition efficiency (%) towards the mineral</u>		
	Calcium Phosphate	Calcium Oxalate	Calcium Carbonate
U ₁	26	19	41
U ₂	26	20	44
U ₃	25	19	42
U ₄	24	18	40
U ₅	24	17	37
U ₆	23	18	39
U ₇	49	24	51
U ₈	37	20	46
U ₉	27	19	43
U ₁₀	50	23	54
U ₁₁	50	22	52
U ₁₂	25	17	39
U ₁₃	27	20	44
U ₁₄	58	42	62
U ₁₅	54	35	60
U ₁₆	51	33	55
U ₁₇	48	21	54
U ₁₈	47	19	51
U ₁₉	64	46	80
U ₂₀	28	19	46
U ₂₁	19	10	23
U ₂₂	14	7	15
U ₂₃	13	6	16
U ₂₄	10	5	14
U ₂₅	17	8	20
U ₂₆	7	4	8
U ₂₇	16	7	17
U ₂₈	21	10	28
U ₂₉	9	5	12
U ₃₀	8	4	10
U ₃₁	23	12	26
U ₃₂	10	5	12

U₂₁ TO U₃₂ -> URINARY STONE PATIENTS

TABLE 3

ASSOCIATION BETWEEN URINARY CITRATE TO CALCIUM MOLE RATIO AND MINERALISATION-INHIBITION EFFICIENCY OF URINE OF HEALTHY PERSONS AND URINARY STONE PATIENTS

Inhibited Mineral	Correlation Coefficient (r)		Test of Significance		Coefficient of Determination (r ²)		Unexplained Variance (100 K ²)	
	Healthy	Stone Patients	Healthy	Stone Patients	Healthy	Stone Patients	Healthy	Stone Patients
Calcium Phosphate	0.9817	0.9809	$r > r_{0.001}$ significant at 0.1% level	$r > r_{0.001}$ significant at 0.1% level	0.9637	0.9622	3.6%	3.8%
Calcium Oxalate	0.909	0.960	$r > r_{0.001}$ significant at 0.1% level	$r > r_{0.001}$ significant at 0.1% level	0.826	0.9216	17.4%	7.8%
Calcium Carbonate	0.5141	0.9764	$r > r_{0.05}$ significant at 5% level	$r > r_{0.001}$ significant at 0.1% level	0.2643	0.9534	73.6%	4.7%

TABLE 4

CALCIUM UROLITHIASIS RISK INDEX ON THE BASIS OF URINARY CITRATE TO CALCIUM WEIGHT RATIO (MOLE RATIO)*

Immunity / risk Factor	Index Value		
	Phosphate Inhibition basis (Inhibition Coefficient, $K_{phos}=0.06$ wt. Ratio % ⁻¹)	Oxalate Inhibition basis (Inhibition Coefficient, $K_{ox}=0.10$ wt. Ratio % ⁻¹)	Carbonate Inhibition basis (Inhibition Coefficient, $K_{carb}=0.04$ wt. Ratio % ⁻¹)
Ideal Immunity (100% Inhibition)	6 (1.26)	10 (2.10)	4 (0.84)
General Immunity (No Risk)	2.28 (0.47)	2.35 (0.49)	1.96 (0.41)
No Immunity (Risk State)	< 2.28 (0.47)	< 2.35 (0.49)	< 1.96 (0.41)
Stone Formation (High Risk State)	0.83 (0.17)	0.69 (0.14)	0.67 (0.14)

* Figures in parentheses denote corresponding mole ratio values.

REFERENCES

1. Y.M.Fazil Marickar, Essentials of Nephrology and Urology (Ed. R.Kasivishwesar), Churchill Livingstone Pvt. Ltd., New Delhi, pp. 329, 1994.
2. B.Francois, Cahen and B.Pascal, Inhibitors of Urinary stone formation in 40 recurrent stone formers. *Brit. J. Urol.*, 58, 479-483, 1986.
3. C.Y.C. Pak and C. Fuller, Idiopathic hypocitraturic calcium-oxalate nephrolithiasis successfully treated with potassium citrate. *Ann. Intern. Med.*, 104, 32-37, 1986.
4. C.Y.C. Pak and R. Peterson, successful treatment of hyperuricosuric calcium oxalate nephrolithiasis with potassium citrate. *Arch. Intern. Med.*, 146 863-867, 1986.
5. T.V.R.K.Rao, Reactions of calcium phosphate with some natural acids. *J. Inst. Chemists (India)*, 72, 166-168, 2000.
6. T.V.R.K Rao and Sofia Bano, Sequestration and Complexation of Brushite by Some Naturally Occurring Acids. *J. Indian Council Chemists*, 17, 35-37, 2000.
7. J.M. Baumann, B. Affolter. U. Caperez, D. Lauper, F. Maier, H.P. Siegrist and T. Zysset. Nucleation and Aggregation of calcium Oxalate in whole Urine; Spectrophotometric sedimentation analysis: A new approach to study the aggregation of calcium oxalate dihydrate. *Urol. Res.*, 28, 147-154. 2000.
8. S. Natelson. J.B. Pincus and J. Lugovoy. *J.Biol. Chem.*, 175, 745, 1948.
9. I.D.P. Wootton, Microanalysis in Medical Biochemistry, 5th Edition, Churchill Livingstone, U.K., pp. 68, 1974.
10. N. Raghuramulu, K. Madhavannair and S. Kalyanasundaram, A Manual of Laboratory Techniques, National Institute of Nutrition (ICMR), Hyderabad, pp. 295, 1960.
11. M.N.Chatterjee and Rana Shinde, Text Book of Medical Biochemistry (4th Edition), Jaypee Brothers, New Delhi, pp. 671, 2000.