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Chronobiology of urinary citrate excretion amongst stone-formers and healthy males from North Western India*

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Summary. Urinary citrate excretion was estimated colorimetrically from urine samples collected every 3 h for 24 h from 25 healthy adult males (non-stone formers; mean age 39 ± 7 years) and 25 male patients suffering from calcium nephrolithiasis (stone formers; mean age 41 ± 6 years). The 24 h citrate excretion was 2.47 ± 0.65 mmol in non-stone formers and 2.02 ± 0.71 mmol in stone formers. This difference was not significant. However, cosinor rhythmometry revealed a significant circadian rhythmicity in urinary citrate excretion in the healthy males which was absent in the stone formers; the amplitude was 0.06 mmol in non-stone formers and 0.017 mmol in stone formers. The acrophase was located at 14:25 h in non-stone formers and at 23:30 h in stone formers.

Key words: Urinary calculi – Citrate – Circadian rhythm

The concentration of citrate in the urine affects the formation of calcium oxalate and calcium phosphate crystals within the urinary tract. Citrate forms soluble complexes with calcium, thereby decreasing the calcium specific ion activity and the relative supersaturation of both calcium oxalate and calcium phosphate [6]. In addition, citrate has also been shown to exert an inhibitory effect on both calcium phosphate [2] and calcium oxalate crystal growth and aggregation [3]. Urinary citrate levels as reported by various stone laboratories are contradictory: citrate excretion in stone formers has been found both to be normal [10, 11] and to be low [4, 9]. The present investigation was performed to study circadian variations in citrate excretion in patients suffering from calcium urolithiasis and in control subjects. Estimation of total 24 h excretion of citrate in urine will not reveal any circadian fluctuations in the excretion of this inhibitory substance, but investigation of urine samples collected 3-hourly over a period of 24 h will give a better picture of the circadian rhythm and may help to unravel the biological abnormalities present in stone-forming patients.

Subjects, material and methods

The study included 25 healthy adult males (non-stone formers; mean age 39 ± 7 years) and 25 male patients suffering from calcium oxalate nephrolithiasis (stone formers; mean age 41 ± 6 years). None of the study participants was suffering from a urinary tract infection, renal failure, renal tubular acidosis, hyperparathyroidism, or primary or secondary hyperoxaluria. None was taking any drugs such as acetazolamide, thiazides, alkali salts or orthophosphates. The subjects followed their usual diet and levels of activity during the collection period, but were synchronized for diurnal activity from 06:00 h to 22:00 ± 1 h and for nocturnal rest. Urine samples were collected at fixed 3-hourly intervals during a 24-h span beginning at 00:00 h. The volume of 3 h fractions of urine was recorded. Urinary citrate was estimated colorimetrically using the method of White and Davies [14], in which citrate present in urine reacts with acetic anhydride and pyridine to give a coloured complex which is measured at 428 nm. All the urine samples were also analysed for creatinine for assessment of proper urine collection.

Statistical analysis

Conventional methods (mean, *t* test) were used to visualize the circadian and other changes. In addition, time series of experimental data were analysed with the single cosinor method [8], which allows the estimation with 95% confidence limits of the parameters characterizing a circadian rhythm: the period (τ), equal to 24 h, since this corresponds to an average period of the subjects' synchronization; the acrophase (ϕ), the peak time of the cosine function used to approximate the rhythm; the amplitude (A), equal to half of the total rhythmic variability per 24 h; and the mesor (M), the rhythm-adjusted mean, which corresponds to the 24-h mean when sampling is performed at equal intervals, as in this study. M is expressed as mean ± SD.

Computer programs and the least square method were used to find the best-fitting sine function approximating all data. A rhythm identified by this method was regarded as validated if its amplitude differed from zero with a probability of less than 1 in 20 ($p < 0.05$).

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Table 1. Three-hourly urinary citrate excretion (mmol) during a 24-h period in 25 male patients with calcium nephrolithiasis (SF) and 25 healthy subjects (NSF)

	00:00 to 03:00 h	03:00 to 06:00 h	06:00 to 09:00 h	09:00 to 12:00 h	12:00 to 15:00 h	15:00 to 18:00 h	18:00 to 21:00 h	21:00 to 24:00 h
SF	0.31 ± 0.11	0.22 ± 0.07	0.21 ± 0.08	0.29 ± 0.16	0.21 ± 0.06	0.25 ± 0.13	0.24 ± 0.13	0.29 ± 0.10
NSF	0.26 ± 0.12	0.26 ± 0.12	0.23 ± 0.10	0.34 ± 0.23	0.39 ± 0.22	0.35 ± 0.24	0.33 ± 0.24	0.32 ± 0.13

All values are mean ± SD. Mean excretion for SF = 0.26 mmol/3 h & NSF = 0.31 mmol/3 h

Table 2. Circadian rhythm of citrate excretion (1 cycle = 24 h) in stone formers (SF) and non-stone formers (NSF)

NSF	SF
M = 0.31	M = 0.26
A = 0.06	A = 0.017
ϕ = +216	ϕ = +352

Equation: Y (mmoles/3 h) = M + A cos (angle + ϕ)

Angle	NSF	SF
0°	0.261	0.276
45°	0.249	0.270
90°	0.277	0.258
135°	0.325	0.246
180°	0.369	0.244
225°	0.381	0.249
270°	0.354	0.263
315°	0.306	0.273
360°	0.261	0.276

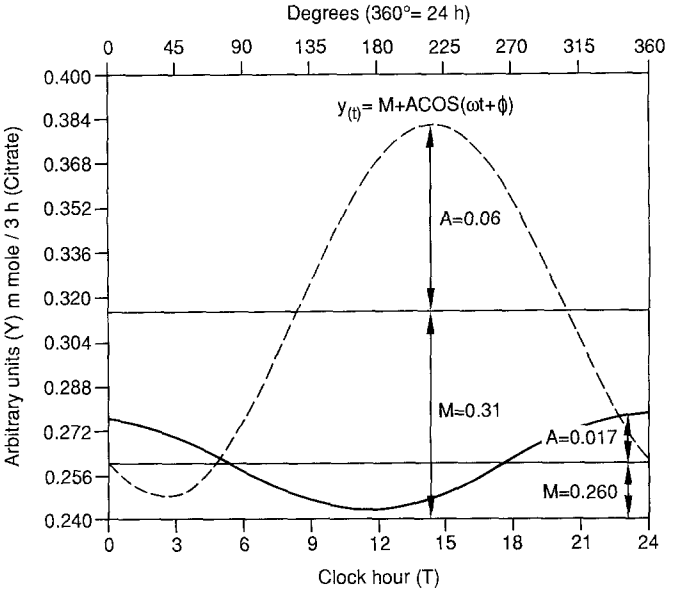

Fig. 1. Estimation of rhythm parameters by least squares fitting of cosine function amongst non-stone formers (– – –) and stone formers (———). M = mesor; A = amplitude; ω = angular velocity (15°/h); ϕ = computative acrophase (degrees from 00:00 h)

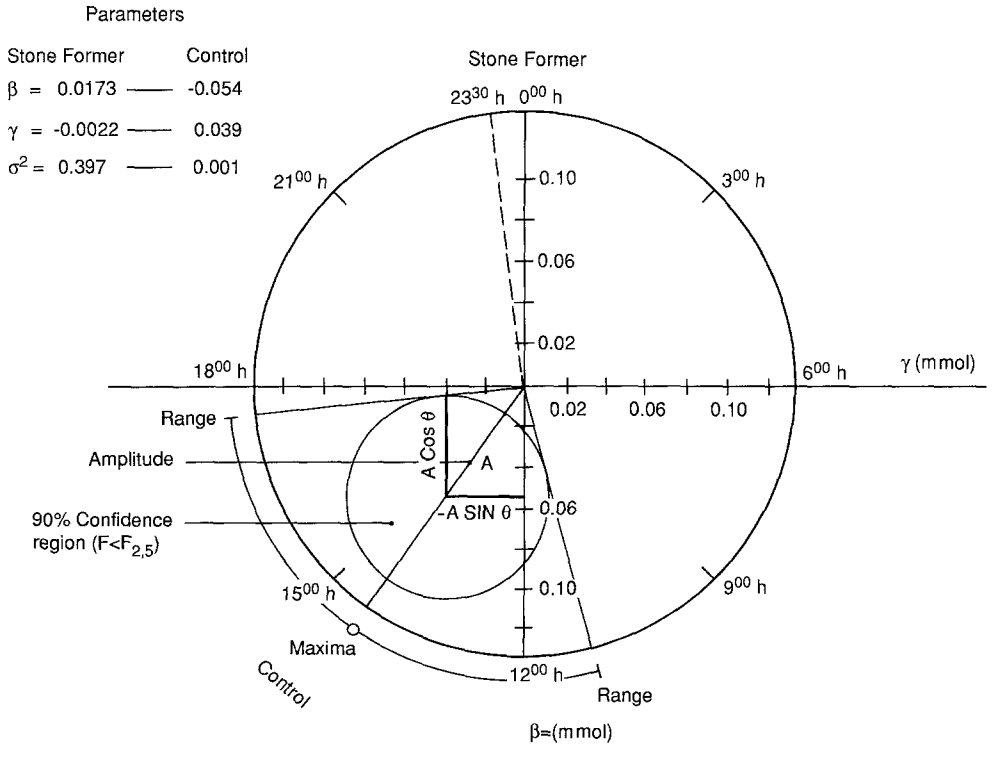

Fig. 2. Rectangular (β, γ) and polar (A, ϕ) representation of circadian rhythm of citrate excretion amongst stone formers and non-stone formers with joint confidence regions. Based on cosine function $Y_{(t)} = M + A \cos(\omega t + \phi)$ where A = amplitude, ϕ = acrophase, M = mesor, ω = 15°/h

Results

The urinary citrate excretion during 3-h periods over a 24-h period in the healthy subjects and in the stone formers is shown in Table 1. No significant difference was found between the two groups in total 24-h citrate excretion (2.47 ± 0.65 mmol in non-stone formers and 2.02 ± 0.71 mmol in stone formers).

Cosinor rhythmometric analysis of citrate excretion revealed a significant circadian rhythmicity in the non-stone formers ($F_{2,5} = 17.73$; $p < 0.025$) which was not detected in stone formers. The cosinor rhythmometric data for both groups are given in Table 2.

Maximum citrate excretion among control subjects was found to be at 14:25 h, with a broad range of 10:55 h to 17:35 h (Fig. 1). Although the 3-h urinary excretion of citrate was found to be low in stone formers as compared to control subjects, this difference was not significant because of high standard deviations. Rectangular (β, γ) and polar (A, ϕ) representation of the data was also carried out to pinpoint the significant confidence regions of the cycle where the excretion was at its maximum (Fig. 2).

Discussion and conclusions

Hypocitraturia in patients with calcium nephrolithiasis has been reported by several workers [2, 3]. However, hypocitraturia as defined by the presence of citrate levels lower than those in 95% of the normal populations was detected only in 7 out of 46 patients with recurrent calcium oxalate stones [5]. In the present study, no significant difference in 24-h citrate excretion was observed between the healthy men and male patients suffering from calcium nephrolithiasis. On the other hand, the latter did not show the circadian rhythmicity in citrate excretion which was present in the healthy subjects.

Alterations in circadian rhythmicity in urinary excretion of lithogenic substances and inhibitors in patients suffering from urinary calculus disease will be detected only if urine samples are at collected at repeated intervals over 24 h and analysed separately [12]. Such subtle changes in the urinary excretion of citrate, which is a potent inhibitor of calcium oxalate and calcium phosphate crystal formation and growth, will be missed unless chronobiological studies are performed. An earlier study [7] of patients with calcium oxalate nephrolithiasis revealed that the acrophase for urinary calcium excretion was at 11:51 h, which coincides with the nadir [8] for urinary citrate excretion at 11:30 h. This indicates a high risk period for calcium oxalate or calcium phosphate crystallization.

In the present study, in healthy control subjects, the acrophase for urinary citrate excretion was at 14:25 h and the nadir at 02:25 h, an observation similar to that of Bach and associates [1], who also found a maximum citrate excretion during the day time and a minimum between 02:00 h and 08:00 h in healthy controls.

Rectangular (β, γ) and polar (A, ϕ) representation of the circadian rhythm of citrate excretion amongst both these groups revealed a statistically significant 95% confidence region where F values were found to be less than the calculated $F_{2,5}$ value. The region (represented by a circle in Fig. 2) corresponds to the region of maximum excretion during the 24-h span, whereas β and γ correspond to the amplitude of this excretion on the x-axis and y-axis respectively. The above observation reveals that during the period from 11:00 h to 18:00 h the relative excretion of citrate in control subjects is at its maximum. However, our previous observation [7] shows that the risk of increase in the concentration of lithogenic substances is also at its maximum during this period.

In the stone forming patients in the present study, the acrophase for urinary citrate excretion was at 23:30 h and the nadir was at 11:30 h, but the rhythm was not found to be significant, with no confidence regions. However, Vahlensieck et al. [13] found a distinct elevation in urinary citrate excretion during the day time and a minimum between 05:00 h and 08:00 h in stone formers as well. This indicates possible geographical differences in the circadian rhythmicity of the urinary excretion of inhibitory substances in patients with urolithiasis. This difference in the acrophase of urinary citrate excretion between stone-forming patients in Westen Germany and North-Western India emphasizes the need for study of the circadian biology of patients with urinary calculus disease separately for each geographical region. Extrapolation of results from one region to another, where dietary habits may be different, may not be valid.

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