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Detection of Crystallization Inhibitory Activity of Whole Urine with a Gel Model

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Summary. The inhibitory activity of urine on calcium oxalate crystallization was measured as an inhibitory index using a gel model modified from Schneider et al. [8]. Urine samples from 36 recurrent stone formers and 21 controls in 3 separate periods (morning, afternoon and evening) were tested. Mean inhibitory indices of > 0 were observed among normal controls in the 3 sampled periods and among stone formers in the morning and afternoon samples. A significantly lower (negative) value was observed in the evening samples of stone formers, indicating a higher tendency towards crystallization than normal. The gel method may be applied to identify individuals at risk of stone formation if sufficient numbers of period-defined urine samples from the individual are tested for a statistically significant mean inhibitory index but whether this is practical in a clinical laboratory will need further evaluations.

Key words: Gel model, Calcium oxalate crystallization, Inhibitory index.

Introduction

Many methods have been used to detect the inhibitory activity of urine on calcium oxalate crystallization. They varied from the simple experiments of calcification of cartilage [3] to the sophisticated continuous crystallizer [2] using artificial urine. These methods require either special material or apparatus that may not be easily available. We therefore attempted to detect the inhibitory activity of whole urine using a relatively simple gel model [8]. In addition, we also attempted to differentiate stone formers from normal subjects by the measured inhibitory activities and to detect possible diurnal variation of the activity by collecting urine at three physiological periods which are related to meals and to acticity.

Materials and Methods

Urine Specimens

Urine was collected from 2 groups of male subjects: 36 recurrent calcium oxalate stone formers (age 20–38) and 21 normal controls (age 20–33). Subjects were on free diets without previous advice on low calcium and low oxalate foods, before and during the period of urine collection. Collections were made in three periods namely morning, afternoon and evening. The morning sample was collected after the early morning specimen until lunch. The afternoon period started from after lunch and lasted till dinner while the evening period was the time from dinner to the next early morning specimen. The samples were collected into bottles containing 5 ml of 0.1% hibitane and stored in the freezer (−20 °C). Undiluted whole urine was used for analysis.

Gel Procedure

We used a method modified from Schneider et al. [8]. Each slide was first coated with 1 ml of 0.2% Bactoagar and oven-dried (40 °C for 1 h) before it was coated with an additional layer of 3 ml of 1% Bactoagar. Four wells, each of 4 mm diameter, were punched out of the gel, two at a distance of 2 cm apart along the longitudinal midline and two at a distance of 1.4 cm apart along the horizontal midline. Twenty microlitres of 0.2 M calcium chloride solution (pH 5.5) were pipetted into the well on the right on the longitudinal axis and 20 µl of 0.2 M ammonium oxalate solution (pH 7.0) into the well on the left. Twenty microlitres of each urine sample were pipetted into each of the remaining two wells (sample wells). The preparation was duplicated for each urine sample. The slides were allowed to stand in moist chambers at room temperature (23 °C) for 24 h and scanned densitometrically longitudinally between the calcium and oxalate wells with a LKB 2202-002 Ultroscan Laser Densitometer at scan speed of 10 mm/s and Absorbance Units Full Scale of 0.5. Analytical grade chemicals were used.

The densitometric profile was examined for a relationship with the concentration of calcium and oxalate ions in the gel for crystallization. For this purpose, calcium and oxalate solutions, paired for concentrations of (0–0.2 M) were applied in the respective wells of individual slides; distilled water was applied instead of urine in the sample wells. The profiles of indentical preparations showed variable heights and breadths – the lower the height, the wider the base and vice versa. Consequently, the ratio of height to half base yielded errors of more than 5% but the area under the profile showed errors

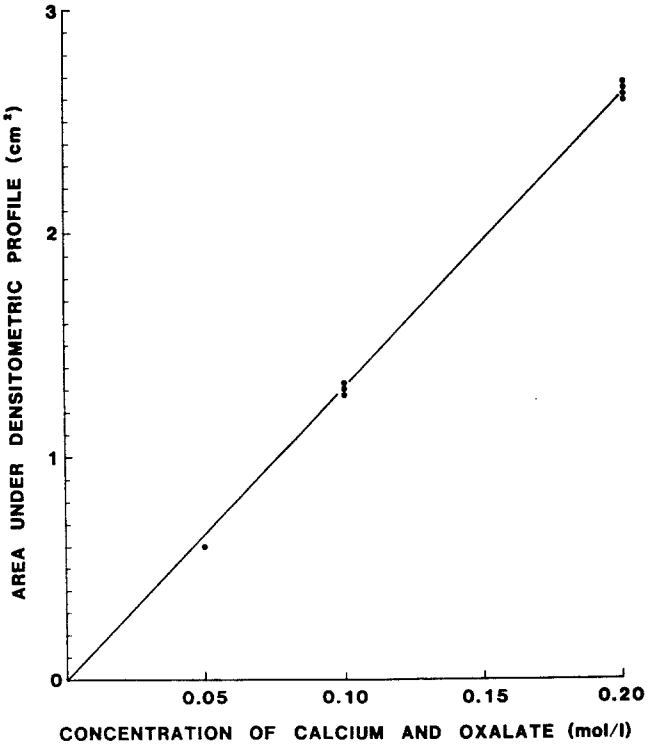

Fig. 1. Relationship between the area under the densitometric profile and the concentration of calcium/oxalate ions applied into the respective wells

of less than 1%. Furthermore, the area under the profile was directly proportional to the concentration of calcium and oxalate originally applied (Fig. 1). This relationship can thus be exploited to characterize the density of the crystal zone. In order to compare the density of crystal zones formed in the presence and absence of inhibitors of crystallization, the concentration of calcium and oxalate solutions provided was fixed at 0.2 M to give crystal zones that were within the measurable range of the densitometer. The inhibitory activity is represented by an index calculated as the relative difference in the area under the densitometric profile of crystal zones formed in the presence of urine sample (Au) in relation to that formed in the presence of distilled water as control (Ac):

$$\text{Inhibitory index of urine (I)} = 1 - \frac{Au}{Ac}$$

A inhibitory index of 0 denotes no inhibition while 1 means complete inhibition. Negative values indicate enhancement by the urine.

Preliminary experiments performed with the same samples of urine but different stocks of agar gel showed inhibitory indices with errors of more than 30%, while the "within-stock" results showed errors of 10%. All subsequent statistical analysis was therefore made among "within-stock" results using the Mann-Whitney method.

Results

The inhibitory indices of both groups were shown in Fig. 2. The normal controls showed a mean inhibitory index of 0.01 ± 0.073 (mean ± SEM) with no significant difference among the three periods.

Among the stone formers, the inhibitory indices of urine in the morning and afternoon were not significantly different, yielding an average of 0.155 ± 0.055. The evening samples showed a significantly lower inhibitory index of −0.139 ± 0.166 compared to both morning ($P < 0.01$) and afternoon ($P < 0.01$) samples indicating enhanced crystallization activity in the evening urine.

Urine from stone formers showed inhibitory indices higher than normal controls in both the morning and afternoon periods. The evening samples of stone formers were lower than the evening samples of normal controls.

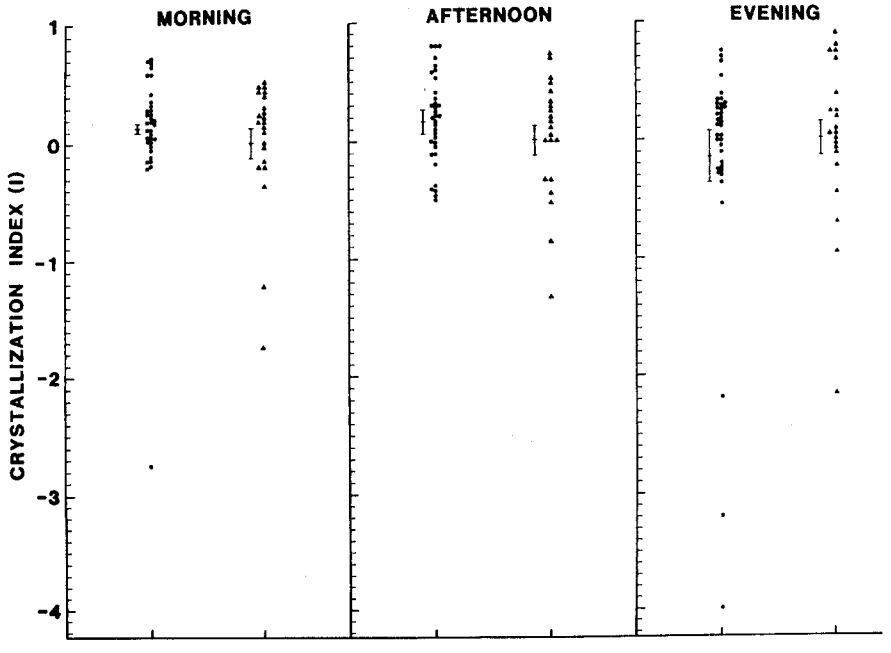

Fig. 2. Inhibitory indices of urine from stone formers ($n = 36$) (*dots*) and normal controls ($n = 21$) (*triangles*). *Lines* denote mean ± SEM

Discussion

Agar gels have been widely used as the supporting medium for the migration of ions and molecules of different sizes. Where the ions meet at a saturating concentration, a visible zone of crystals is formed and the zone can be measured photometrically by a densitometer. Schneider et al. [8] observed that the crystal zone, formed in the presence of inhibitors, described a densitometric profile that was decreased in height and increased in breadth. On the basis of this observation, there is a densitometric profile calculated by the ratio of the height of the crystal zone to the breadth at half of its height. In the present study, decrease in height and increase in breadth of the zone was observed even in the absence of inhibitors, which might have been caused by differences in wetness of the gel media for crystallization. Despite the differences in height and breadth, the area under the profile remained the same. The constancy of area despite variations in height/breadth and the specificity of the area to the effective concentration of calcium and oxalate ions in the gel (Fig. 1) led us use the area under the densitometric profile of the crystal zone to quantify the effect of urine on calcium oxalate crystallization.

The influence of urinary calcium, oxalate and other inorganic ions on crystallization of calcium oxalate in the gel medium cannot be ignored. While calcium and oxalate concentrations were similar in the three periods in both controls and stone formers, phosphate concentrations were found to be higher in the evening samples of both normal controls and stone formers [4]. To observe the inhibitory effect of whole urine we may decrease the effect of these urinary stone-forming ions by the use of high and non-physiological concentrations of calcium and oxalate in the gel; crystallization in the gel may not be applicable to the renal environment. One of the limiations in the experiment is that the crystal measured may not be purely calcium oxalate.

This method showed significantly lower inhibitory activity in the evening urine of stone formers compared to those of morning and afternoon samples of stone formers and samples of the three periods from normal controls. Polyanionic materials in the urine – glycoprotein and glycosaminoglycans [9] have been identified as inhibitors of calcium oxalate crystallization [1]. The level of these substances in the urine of normal subjects was found to follow a cardian rhythm with a trough in the evening [5]. Despite circadian variation in inhibitor level, the inhibitory activity we measured in urine from normal subjects remained similar throughout the day. This indicates that the urinary polyanion level was sufficiently active to maintain inhibition of crystallization in the gel at the observed level. The excretion of these polyanionic materials in the 24-h urine of stone formers was found to be less than normal [6]. If the excretion pattern of polyanions in stone formers follows a circadian rhythm similar to that of normals as suggested by Robertson [7], the low inhibitory activity of evening samples of stone formers in the present study may thus be explained.

The gel method can be applied to the evening urine samples to reflect differences in inhibitory activity of urine from normal subjects and stone formers at risk. In general, a negative inhibitory index suggests risk. In order to obtain accurate and reproducible results, multiple period-defined urine samples, especially in the evening, should be collected from an individual and tested by the gel method for a statistically significant mean inhibitory index. Therefore, it is a relatively inconvenient method to detect patients at risk and the practicality in an ordinary laboratory is doubtful.

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