Urol Res (1995) 23:407–411

ORIGINAL PAPER

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Automated and computer-controlled method for the measurement of the crystallization of calcium oxalate monohydrate in urine

Received: 7 February 1995 / Accepted: 13 July 1995

Abstract Monitoring of crystallization of calcium salts with ion-selective electrodes has turned out to be a very sensitive method. The difficulties of handling these electrodes in native whole urine and other biological fluids have been eliminated by new calcium analyzers, which clean and calibrate the electrodes after each measurement. To study crystallization kinetics, repeated calcium ion measurements have to be performed at regular intervals. For this purpose we have developed a special sampler and software. The sampler brings a thermostat-controlled crystallization chamber to the analyzer at preselected intervals. The computer directs and coordinates the sampler and the analyzer, stores the received results and prints out growth curves. Furthermore it calculates the half-time (h) and the maximum decrease of ionic calcium at infinite incubation time (ΔCa_{∞}^{2+}). Both values are shown to characterize the growth of calcium oxalate monohydrate in urine. Results are obtained within 40 min.

Key words Urolithiasis · calcium oxalate · Crystal growth · Ion-selective electrode · Automated kinetic measurement

Stone formation is generally attributed to crystallization processes in supersaturated urine. The state of supersaturation, which is the driving force for nucleation and crystal growth, can now easily be calculated by computer programs. The latest version of these programs is based on the calculation of 104 complexes of 23 ions influencing the state of urinary saturation with respect to stone-forming salts [4]. Not only are crystallization processes in urine influenced by supersaturation but also by promoting and inhibiting substances of which there is only partial knowledge. Crystallization processes must therefore be studied experimentally.

Many sophisticated crystallization tests have been developed, but the ideal method remains to be found [7]. Most techniques are time consuming and therefore only a few analyses can be performed per day [1]. Results obtained by different systems can often be difficult to compare because they are not expressed in current physicochemistry terms. Most test systems do not tolerate the addition of more than 20% of urine. However, the promoting or inhibiting effect of some substances changes with urinary dilution [8, 9]. Some urinary inhibitors are lost by centrifugation or filtration [11]. Therefore crystallization tests performed in native whole urine are important for stone research.

Recently in our laboratory a rapid method of measuring the growth of calcium oxalate monohydrate in native whole urine was developed [2]. Growth curves of calcium oxalate monohydrate were obtained by continuous measurement of the decrease of calcium concentration in urine with a calcium-ion-selective electrode. As a measure of crystallization, the half-time (h) of the calcium concentration decrease after 20 min incubation was calculated from the growth curve. Will et al. [12] have shown that the growth of calcium oxalate monohydrate is characterized not only by a half-time value, but, in addition, by the maximum calcium uptake into the crystals at infinite time. Both parameters can be extrapolated from the linearization of the growth curve.

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Further evaluation of our method revealed that the need for frequent calibration of the electrode as well as the frequent obstruction of the ion-selective membrane by urinary macromolecules were major problems in clinical use. Commerically available calcium analyzers, cleaning and calibrating the electrode automatically after each measurement, avoid these two problems. Therefore our purpose was to measure crystal growth by such an analyzer performing repeated measurements from the same sample at given intervals and to evaluate the growth curves by a computer program. For these purposes we developed a special sampler and special software for an ordinary PC station. The efficiency of this approach is demonstrated by growth curves of calcium oxalate monohydrate obtained in freshly voided urine.

Material and methods

The calcium analyzer

We chose the calcium analyzer (AVL Medical Instruments), which measures the ion concentrations of calcium, sodium and potassium by ion-selective electrodes as well as pH. An integrated software program allows measurements within 45 s with automatic cleaning and calibration of the electrode between each analysis. Results given as millimoles per liter or pH, respectively, are indicated on a digital display and sent to a PC port (RS 232). For the analysis, the samples have to be brought to an aspiration needle, which is rotated out in front of the instrument (Fig. 1b).

The sampler

To study crystallization kinetics, a series of measurements within exactly defined intervals have to be performed from the same sample. Since crystallization rate decreases with decreasing supersaturation and crystallization persists during calcium measurement, the analyses have to be done directly out of the crystallization chamber. These problems were solved by the construction of the sampler, which is shown in two positions in Fig. 1a, b. In short this sampler consists of a sliding carriage which for each measurement transports a thermostat-controlled (= 37 °C) and magnetically stirred crystallization chamber from its vertical position (Fig. 1a) to the outwardly rotated aspiration needle of the analyzer (Fig. 1b).

The software

The evaluation of different PC programming languages resulted in the choice of MODULA-2. This computer language was developed by Prof. N. Wirth, ETH, Zürich [5, 13]. Using this program the PC controls the sampler and the analyzer. For the coordination of the two devices, the computer has to check whether the analyzer is calibrated and is thus ready for a new measurement. At time intervals which can be selected in the range of minutes to hours, the computer starts the analyzer to perform a calcium measurement and directs the sampler to elevate the crystallization chamber to the aspiration needle. After aspiration and before the retraction of the needle, the chamber is brought back to its vertical starting position. The results of the calcium measurements (Ca^{2+}) are transmitted to the computer and stored together with the corresponding time (t) of the analysis. The course of the experiment can be followed directly on a monitor showing a plot of Ca^{2+} versus t. This plot and the underlying data are printed out. Finally all data are stored for evaluation by a supplementary calculation program.

Crystallization experiments

Twenty milliliters of freshly voided urine of a healthy person was placed in the thermostat-controlled (37 °C) crystallization chamber with continuous magnetic stirring. The urine was seeded by the addition of a slurry of calcium oxalate monohydrate crystals to a final concentration of 1 mg/ml. The reliability of the electrode and the metastability of the seeded urine were checked by repeated

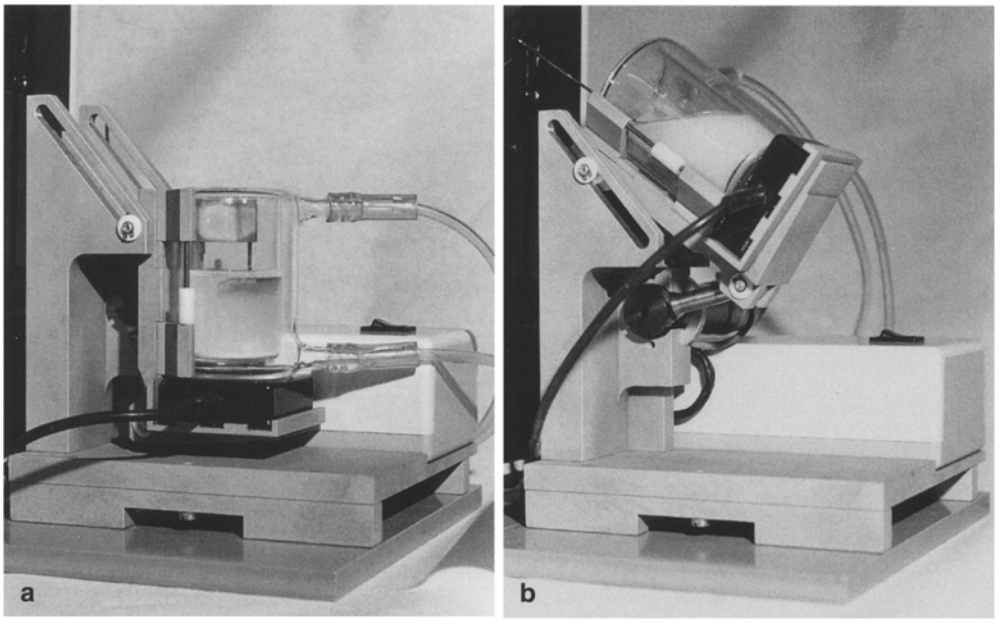

Fig. 1 a Sampler with thermostat-controlled and magnetically stirred crystallization chamber. Working position between the probe aspiration procedure: **b** Elevated position for the probe aspiration by the AVL for calcium measurement (aspirated volume = 120 µl)

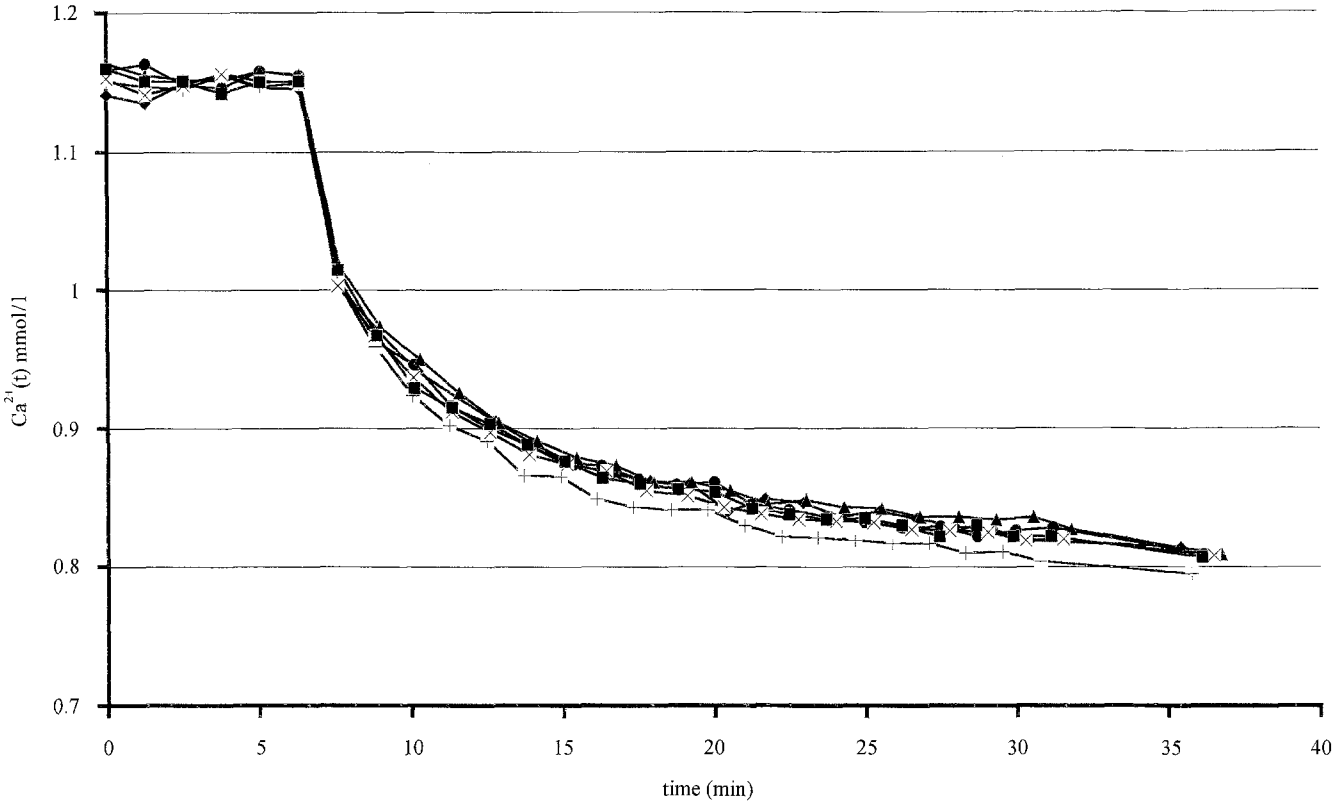

Fig. 2 Growth curves (plot of ionic calcium vs time) of calcium oxalate monohydrate in freshly voided urine. Sixfold repetition of the experiment from the same urine probe

calcium measurements before beginning the crystallization experiment. Crystallization was induced by the addition of sodium oxalate to a final concentration of 1 mmol/l in urine in which the oxalate concentration had previously been determined.

Calculation of crystallization parameters

Will et al. [12] have shown that growth curves of calcium oxalate monohydrate can be described as fractional calcium uptake (U) into crystals versus time (t) by the following equation:

$$U_t = U_\infty \times t \times (t + t_m)^{-1} \quad (1)$$

(U_∞ denotes U at infinite time and t_m the half-time of U_∞)

U_∞ and t_m are easily extrapolated from a plot of t/U_t versus t, this plot being linear. Subtracting calcium activity at time t from the activity at the beginning of our crystallization experiment, the calcium decrease at time t (ΔCa_t^{2+}) can be calculated. This ΔCa_t^{2+} proportionates to the U_t of Will et al., both parameters reflecting the calcium depletion of the solution, ΔCa_t^{2+} with respect to ionic calcium, and U_t with respect to total calcium. Therefore we tried to describe our crystallization experiments by a new equation similar to Eq. 1:

$$\Delta Ca_t^{2+} = \Delta Ca_\infty^{2+} \times t \times (t + h)^{-1} \quad (2)$$

(ΔCa_∞^{2+} denotes ionic calcium decrease at infinite time and h the half-time of ΔCa_∞^{2+})

Based on this new equation, the engineering school of Biel developed a supplementary software program for the calculation of h and ΔCa_∞^{2+}.

Results

Typical growth curves obtained from six crystallization experiments repeated with the same urine are shown in Fig. 2. The reliability of the electrode was checked by six measurements of the initial calcium activity before inducing crystallization by the oxalate load. Over a 30-min period, measurements were taken at first every 75 s and then spaced at every 5 min after the calcium decrease had leveled off. Figure 2 shows that before the oxalate load no nucleation or growth could be observed. With the oxalate load crystallization started without a detectable induction time. Microscopic analyses at the end of the test (>45 min) revealed exclusively calcium oxalate monohydrate crystals. However, scanning electron microscope (SEM) analyses were not performed in this study. All data of the growth curve measured 75 s after the oxalate load were treated by Eq. 2 and linearized by plotting $t/\Delta Ca_t^{2+}$ versus t (Fig. 3). The slope of the linear curve represents $1/\Delta Ca_\infty^{2+}$ and the intersection with the y-axis $h/\Delta Ca_\infty^{2+}$. The two important characteristics for the growth curve, the half-time (h) and the calcium decrease at infinite

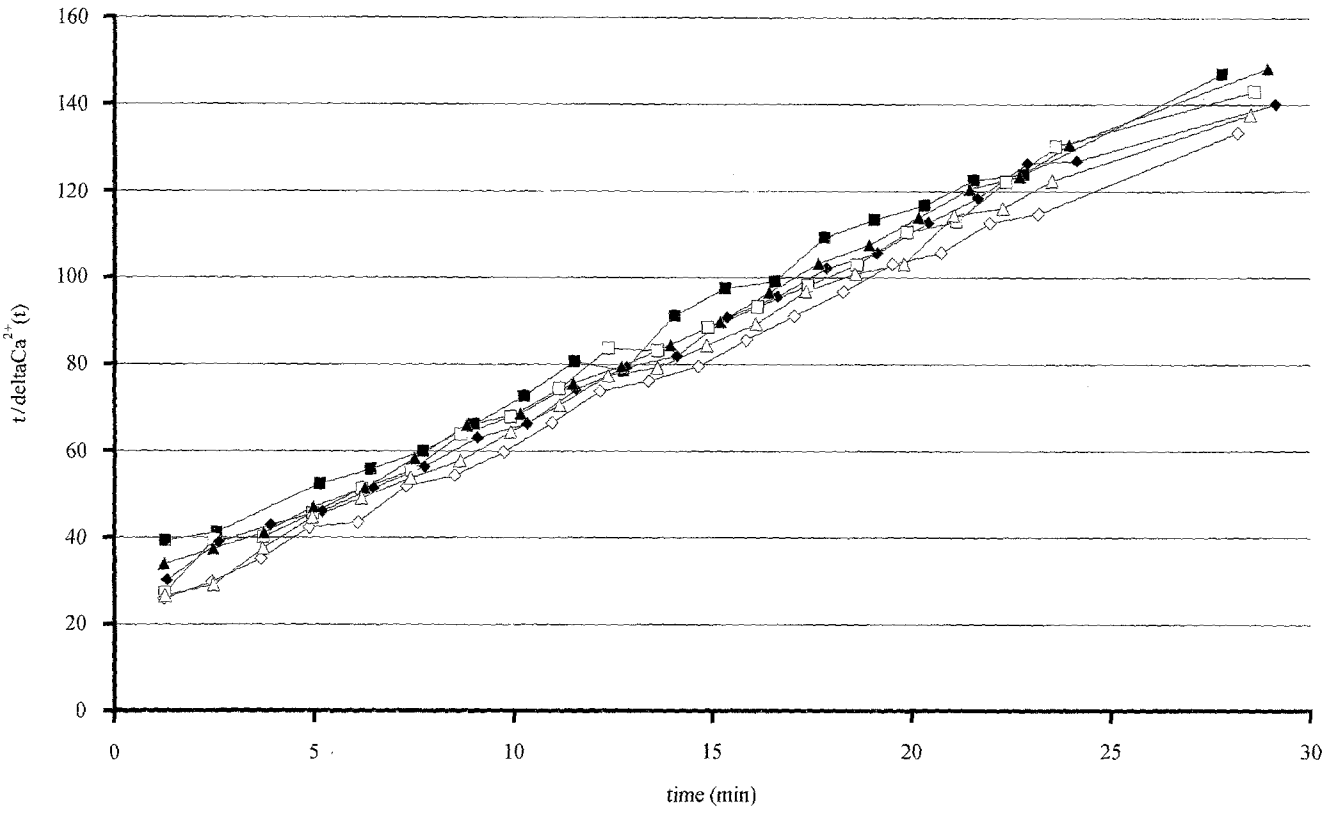

Fig. 3 Linearization of the six growth curves of Fig. 2 by plotting $t/\Delta Ca_i^{2+}$ vs time (Eq. 2 in the text)

Table 1 Half-time (h) and calcium decrease at infinite time (ΔCa_∞^{2+}) of the six experiments (Fig. 2) (CV coefficient of variation)

	h (min)	ΔCa_∞^{2+} (mmol/l)
	5.97	0.24
	6.02	0.24
	6.13	0.24
	5.08	0.24
	6.09	0.23
	5.31	0.24
Mean (c)	5.77	0.24
CV (%)	7.82%	1.09%

time (ΔCa_∞^{2+}), as well as their coefficient of variation, are shown in Table 1.

Discussion

By the development of a special sampler and software we were able to perform crystallization tests on calcium oxalate monohydrate in native whole urine with a commercially available calcium analyzer. Since the original instrument (AVL) was not modified, it can still be used between two crystallization tests in a conventional manner. In contrast to other whole urine tests [7], our system allows the measurement of the crystallization of calcium oxalate at any supersaturation and any seed concentration at which crystallization can be observed. Therefore for clinical purposes the oxalate load can be adopted to a standard initial supersaturation, as proposed by Ryall et al. [10]. The individual calcium measurements can be programmed within intervals of minutes to hours. The technician only has to prepare the crystallization experiment, choose and start the appropriate computer program and induce the crystallization by the oxalate load. Further steps of the program proceed automatically. The course of the experiment can be followed directly on the monitor. At the end of the experiment, the whole growth curve or a selected part of it can be transmitted to the special calculation program, which linearizes the data. An excellent linearization as shown in Fig. 3 is a measure of the quality of the experiment. Furthermore, it demonstrates that Eq. 2 is valid to describe the growth of calcium oxalate monohydrate crystals in urine.

In this series of experiments, secondary nucleation and growth of calcium oxalate monohydrate were examined. Previous studies have shown that inhibitor activity in whole urine is very high and seeded growth of calcium oxalate only occurs at high urinary supersaturation [3]. Stone formation has often been attributed to fixed particle growth [6], where kidney

calcifications are in contact with urine and grow during phases of increased supersaturation. Our test system, which begins by seeding urine with crystals and inducing crystal growth by an oxalate load, attempts to imitate this model of stone formation.

Growth tests performed in urine (Table 1) as well as in artificial solutions (not shown) revealed an adequate reproducibility with respect to (ΔCa_{∞}^{2+}) and a fair one with respect to *h*. In our hands, the technique described here has proved to be a suitable and time saving method for the study of crystallization phenomena of calcium oxalate in urine and artificial solutions. Descriptions of these further applications are in preparation.

Acknowledgements I thank M. Barrer for his advice concerning the software MODULA- 2, R. Joehri, A. Sterren and Ph. Walden from the engineering school of Biel for perfecting the sampler and the software, and E. Fink from the Regionalspital Biel for the excellent photographs.

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