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Urological Research

How to Measure Crystallization Conditions in Urine: A Comparison of 7 Methods

Report from a Workshop Held on November 28, 1987 in Basle

At this workshop organized by J. M. Baumann, Biel, together with F. Hering and G. Rutishauser, Basle, seven authors, mentioned below, presented and compared their methods compiled and commented in this report. The authors also answered a questionnaire from which technical and scientific information was taken to characterize the different methods. Some explanation of terminology and general problems are given in the introductory editorial.

The Gel Crystallization Method

W. Achilles (1985) Fortschritte in Urologie und Nephrologie 23:252–260

The Gel Crystallization Method (GCM) represents a highly efficient optical microprocedure for the determination of relative growth rates of crystal phases.

Predominantly, it has been applied to the crystallization of calcium oxalate hydrates (CaOx).

Principle: From two ions which may form a solid phase, one is dissolved as a soluble compound (e.g. sodium oxalate) in a gel (e.g. 0.5% agar-agar) containing seed crystals. The gel is located in multiple wells of a 96-well microtitre plate (100 µl gel/well). Solutions containing the counterion (e.g. Ca^{2+}) are pipetted onto the gel. The subsequent crystal formation in the gel matrix is followed by vertical-light-path photometry as a function of time.

High efficiency (about 120 complete crystal growth kinetics/hour) and high precision (RSD <2% for standard measuring values) is achieved by multiple sample handling of small volumes (200 µl/well), automated quasi-simultaneous kinetic determinations in 48(–96) positions of microplates and current reference to a kinetic standard taking into account blank values.

Measuring Device: Automated microphotometric system for transmitted light (Zeiss, Oberkochen, FRG) comprising the following parts.

1. Inverted microscope IM35 equipped with power-stabilized light source, rapid (50 µm-step) scanning stage for microtitre plates, photometer SFD and camera Contax RTS;

2. Electronic control unit MPC64 and

3. On-line computer HP9816S with double disk drive, matrix printer and plotter (Hewlett-Packard) for acquisition, processing and display of data.

The method may be used for large scale determinations of crystal growth rates in artificial solutions as well as in natural and undiluted urine samples.

The measuring parameter Vcr reflects the effect of all urinary constituents, except oxalate, on crystal growth, including complex formation and inhibition.

The GCM has been proved to be of high value with respect to basic research, diagnosis and control of efficacy of medical treatment of urinary stone formation.

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Technical Specifications

- Optical measurement of growth of calcium oxalate seeds in agar-agar gel at fixed time intervals (variation coefficient: 1–2%).
- Special equipment: Computer controlled microscopic photometer equipped with scanning stage for microplates (approx. costs: 100,000 DM).

- Allows 120 analyses per hour.
- Results are given as relative crystal growth parameter (Vcr), which is the ratio between the growth rate with the test and with a standard solution.

Remarks for Stone Research

- Measures in gel with relative high oxalate concentration (2 mmol/l) global effect of urinary calcium, inhibitors and chelators on the growth rate of calcium oxalate.
- Has discriminated 24 h urines of stone formers and controls with $p < 0.01$ and is used for basic research as well as clinical routine.

The Direct Measurement of Inhibitor Capacity in Urine

J. M. Baumann, K. Lauber, F. X. Lusterberger, M. Wacker, and E. J. Zingg (1980) Urological Research 8:171–175; (1985) Urological Research 13:169–174

The critical (Ca) · (Ox) or (Ca) · (P) concentration products for seeded crystallization of calcium oxalate and calcium phosphate are determined in urine as a measure of inhibitor activity. Urine portions are gradually supersaturated by the addition of oxalate or calcium and are seeded with 0.02 mg/ml calcium oxalate monohydrate or 0.2 mg/ml hydroxy apatite. Crystallization is monitored by a decrease of calcium in the supernatant after constant incubation time and centrifugation. The critical concentration products for crystallization can be extrapolated graphically or calculated. In order to eliminate the effects of chelators and of ionic strength, activity products can be calculated from the critical concentration products and from urine chemistry by a computer program. In an other approach, urinary solubility of calcium oxalate and calcium phosphate are directly determined by equilibration experiments and from the critical concentration product and the apparent solubility product a concentration product ratio is calculated.

Clinically, the method has proved to be useful for the discrimination of urine between stone formers and controls, for the definition of the relative importance of low molecular inhibitors and for the evaluation of the risk of crystallization in urine under different dietary manipulations.

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Technical Specifications

- Determines in urine, seeded with calcium oxalate monohydrate or hydroxy apatite, critical oxalate or calcium addition for a decrease of calcium after constant incubation time (Variation coefficient: 4–6%).
- No special equipment is required.
- Allows 8 analyses per day.
- Results are given in concentration products ($mmol/l^2$) from which together with urine chemistry thermodynamic activity products can be calculated.

Remarks for Stone Research

- Can measure in native whole urine inhibitor and chelator effects on seeded nucleation and growth of calcium oxalate and calcium phosphate.
- Has distinguished stone formers and controls with $p < 0.001$, and was used in basic and clinical research, but is not recommended for clinical routine.

Quantitative Determination of Inhibitors of Calcium-Phosphate Precipitation in Whole Urine

S. Bisaz, R. Felix, W. F. Neuman, and H. Fleisch (1978) Mineral and Electrolyte Metabolism 1:74–83

The technique is based on the determination of the amount of apatite needed to induce a precipitation from whole urine brought to a constant supersaturation.

For this purpose, a urine sample is first equilibrated with brushite ($CaHPO_4 \cdot 2H_2O$) at pH 6.2 and the Ca and Pi concentrations are then determined. Another batch of urine is brought to the CaxPi product of the equilibrated one by adding $CaCl_2$ or if necessary EGTA. In this way all urines are brought to a standard CaxPi activity product, that is to the solubility product of brushite, which is supersaturated towards apatite.

Various amounts of apatite are then added to such adjusted urine samples, and the mixture incubated for 24 h at 37°. The urines are centrifuged and the Ca and Pi measured in the supernatant. It is possible, using probit paper, to determine the amount of apatite necessary to induce a 50% precipitation. This amount is related to the amount of inhibitors present in the urine.

The method is reproducible and precise, the coefficient of variation being 4.8%.

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Technical Specifications

- Determines in urine, presaturated with respect to brushite, critical hydroxyapatite addition to induce a 50% drop of (Ca) · (P) concentration product. (Variation coefficient: 4,8%.)
- No special equipment is required.
- Allows 10 analyses per day.
- Results are given in mg/ml apatite.

Remarks for Stone Research

- Measures in pretreated whole urine inhibition of the growth of relative high concentrations of hydroxyapatite (2–8 mg/ml).
- Was used in basic research to evaluate the relative importance of low molecular inhibitors in normal urine, but was not applied in urolithiasis research until now.

The Oxalate-Tolerance Method

Th. Briellmann, H. Seiler, F. Hering, and G. Rutishauser 1985) Urological Research 13:291–295

Undiluted urine portions of stone formers and other persons are titrated with sodiumoxalate and the beginning precipitation detected by turbidimetry. The oxalate-concentration required for this precipitation of calciumoxalate in the urine is called oxalate-tolerance.

In an other portion of the same urine the calcium-concentration is determined by AAS. This calcium-concentration is plotted against the oxalate-tolerance-value of the corresponding urine. With regard to a standard-curve made by measurements in synthetic urine the plotted urine values of stone-formers and non stone-formers are compared. Points above the standard-curve are considered as results of urines with little stone-forming risk, points on or below the standard-curve as results of urines with tendency to form stones. Between the two groups of stones-formers and others no significant difference can be found.

Further investigations with urines equalized in their conductivity before the titration do not show a better significance between the urines of stone-formers and non stone-formers.

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Technical Specifications

- Turbidimetric measurement of critical oxalate addition to calcium containing solutions and to urine for the precipitation of calcium oxalate.
- Special equipment: Dive photometer, automatic microburette, registration (approx. costs: 15,000 sFr.).
- Allows 10–12 analyses per day.
- Results are given as oxalate tolerance (Δ mmol/l).

Remarks for Stone Research

- Measures in native whole urine with relative high oxalate addition (1–6 mmol/l) global effect of promoters, inhibitors and supersaturation on the nucleation and growth of calcium oxalate.
- Needs further evaluation to define clinical application.

Rapid Evaporation Technique

P. C. Hallson and G. A. Rose (1978) British Journal of Urology 50:442–448

Fresh urine is passed into Dewar flasks with no preservatives and studied within two hours. Osmolarity and pH are measured. Sufficient water is removed in vacuo on a rotary evaporator at 37 °C to obtain a pre-dicted osmolarity of 1,000–1,450 mosmol/kg. This takes about 5–10 minutes. The concentrated urine is incubated at 37 °C for a pre-determined period of time. Zero time corresponds to urine in the renal pelvis and two hours corresponds to retention in the bladder. After incubation, the crystals are separated by centrifuging and studied in one ore more of the following ways:

1. Microscopy: The deposit is suspended in a known small volume of fluid and examined under the microscope. Size, shape, aggregation and frequency of crystals can be assessed semiquantitatively.

2. Isotopically: ^{14}C-oxalate is added to the urine before evaporation. After counting ^{14}C-oxalate in urine and crystals, and measuring urinary oxalate, the oxalate in the crystals can be calculated.

3. Chemical: By dissolving the crystals in diluted acid and measuring oxalate and phosphate in the crystals one can calculate the concentration in the original urine of calcium oxalate and calcium phosphate crystals.

The role of inhibitors in whole urine can be studied by dividing urine samples into three aliquots. The test substance is added to one, subtracted from a second by appropriate means, and the third used as a control. All are evaporated to the same degree and the crystals formed compared.

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Technical Specifications

- Studies in urine, concentrated by evaporation, crystallization of stone forming salts by chemical analyses and microscopy of deposit after fixed incubation time.
- Special equipment: Rotary evaporator (approx. costs: £ 750).
- Allows 10 analyses per day.
- Results are given in μmol/l precipitates.

Remarks for Stone Research

- Measures in whole urine at more or less defined supersaturation (1,000–1,450 mosmol/kg) global effect of promoters and inhibitors on the crystallization of calcium oxalate, calcium phosphate and other crystals.
- Allows by variation of incubation time and urinary concentration kinetic considerations and by microscopy a qualitative estimate of aggregation.
- Was used in basic research (study of promotors) and is also recommended for clinical routine.

A Method for Determination of the Risk of Calcium Oxalate Crystallization in Urine

H. G. Tiselius (1985) Urological Research 13:297–300

This method was developed in an attempt to measure the net effect of supersaturation with respect to calcium oxalate (CaOx) as well as promotion and inhibition of crystallization.

Method: Urine samples were heated to 37 °C, carefully mixed and centrifuged. The crystals in the sediment were dissolved in a small amount of hydrochloric acid and pH adjusted to 5.8. The supernatant was also adjusted to pH 5.8 and the combined fractions passed through filters of Millipore, diamter 0.22 μm or centrifuged. Urine was diluted to a final concentration of 80–90 per cent.

During continuous stirring 100 μl portions of a 0.08 mol/l sodium oxalate solution were added in a standardized way. After each increment in the oxalate concentration the number of crystals in the size range 3.5 to 5 μm was determined in a Coulter counter. It was hereby possible to establish the increase in oxalate concentration necessary for the formation of 100 crystals in the size range 3.5 to 5 μm.

An estimate of the crystallization risk was obtained by the reciprocal of this concentration (CaOx-CR).

Result: Comparison of CaOx-CR between normal subjects and stone formers showed slightly higher values for the latter group, but the difference did not reach statistical significance either in 24 h or 4 h urine collections. These findings are very similar to analysis of AP(CaOx)-index in 24 h urine. Correction for variations in urine flow resulted in a better separation of the two groups.

Comments: The presented method measures the nucleation and crystal growth and most probably reflects the combined effects of supersaturation, promotion, and inhibition, whereas in this design agglomeration is not accounted for.

The draw-back of the method is that the analytical procedure is very time-consuming and at least so far has to be performed manually. It is therefore unsuitable for large series of urine samples.

However, it can advantageously be used for evaluation and follow-up of specific therapeutic regimens as well as for determination of the effect on crystallization of different manipulations of urine composition.

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Technical Specifications

- Determines in urine critical oxalate addition for the formation of 100 calcium oxalate crystals in the size range of 3.5–5 μm (variation coefficient: 3%).
- Special equipment: Coulter counter (approx. costs: 30,000 DM).
- Allows 7–8 analyses per day.
- Results are given as calcium oxalate crystallization risk (CaOx-CR) which is the reciprocal value of the critical oxalate addition.

Remarks for Stone Research

- Can measure in whole urine global effect of inhibitors promoters and supersaturation on nucleation and growth of calcium oxalate.
- Results correlated well with an index of supersaturation.
- Was used in clinical research (therapeutic follow-up), but is not recommended for clinical routine.

A Seeded Crystal Growth Method for Measuring the Effects of Compounds or Urine on the Solubility, the Growth and the Agglomeration of Calcium Oxalate Monohydrate Crystals

E. J. Will, O. L. M. Bijvoet, L. J. M. J. Blomen, and H. Van der Linden (1983) Journal of Crystal Growth 64:297–305; 306–315; 316–325

A seeded crystal growth method in which the solubility, the growth and the agglomeration of calcium oxalate

monohydrate crystals (C.O.M.) are measured as three separate and system-independent parameters has been developed. Solubility is measured in mM and is expressed as the squareroot of the equilibrium concentration product. The crystal growth inhibition is the actual growth constant in the presence of an additive in relation to the growth constant without additive and is expressed as a percentage of the latter. Agglomeration, [tm], is measured in minutes.

Supersaturated solutions with known initial total calcium and oxalate concentrations, buffered at pH 6.0 with 7.5 mM sodium dimethylarsinate and brought to 0.15 M ionic strength with NaCl, are prepared. A tracer amount of ^{45}Ca is added. The experiment, performed at 37 °C, starts by adding C.O.M. crystals in a known seed concentration. The crystals are aged, dried and pregrown before each experiment to ensure reproducibility in crystal surface properties and are kept suspended by shaking. After various incubation times, the crystals are collected on a millipore filter and uptake of ^{45}Ca into the crystals is measured. Uptake due to exchange is less than 1%.

Two types of experiments are performed. In the first, growth time is fixed and initial concentrations of calcium and oxalate vary. The concentration product at which net growth of added crystals starts to occur (equilibrium product) is measured.

In the second experiment initial calcium and oxalate concentrations are kept constant but growth time varies. Variation of incubation time yields a hyperbolic relationship between uptake and growth time which can be described by two parameters; U_∞ denoting the extrapolated uptake at infinite growth time and tm, the time where uptake equals $U_\infty/2$. Thermodynamically U_∞ must equal the uptake required to reach equilibrium, U_{eq}, which can be calculated from the measured solubility and the starting conditions. In practice, however, the measured U_∞ is always lower than the U_{eq}. This difference was found to be due to the process of crystal agglomeration and is described by the parameter [tm].

Knowing the contributions of agglomeration and of changes in solubility to the measured growth rate of crystals, the true growth constant can then be calculated. In this way the three processes can be independently assessed.

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Technical Specifications

- Determines in solutions with variable calcium and oxalate concentration ^{45}Ca uptake of calcium oxalate seeds at fixed time intervals (variation coefficient: $< 8\%$).
- Special equipment: Scintillation counter.
- Allows 30 analyses per week.
- Results can be given in current physico-chemical units, namely thermodynamic solubility product (mol/l^2) and growth rate constant ($l \cdot g^{-1} \cdot min^{-1}$) and as agglomeration parameter (min).

Remarks for Stone Research

- Measures inhibitor or chelator effects of diluted urine on solubility, growth and aggregation of calcium oxalate monohydrate.
- Results can be described by physico-chemical equations but the test system tolerates only 20% of urine.
- Was used in basic and clinical research but is not recommended for clinical routine.

Editorial Comment

The 7 test methods presented can roughly be divided into 3 groups:

a) Systems measuring crystallization in initially crystal-free urine (Briellmann et al., Hallson and Rose, Tiselius).

b) Seed tests with endpoint measurement (Baumann et al., Bisaz et al.).

c) Kinetic seed tests (Achilles, Will et al.).

Systems measuring nucleation and growth in initially crystal-free urine give an estimate of the general risk of crystallization which in the studies of Briellmann et al. and of Tiselius was not significantly higher in urines of stone formers than in urine of healthy controls. Tiselius recommends his method for the evaluation and follow-up of stone therapies. The method of Hallson and Rose is not limited to one stone forming salt and is characterized by its adaptability. Supersaturation and incubation time can be independently interchanged to some extent. The system has proved to be useful for the study of crystallization promoters in whole urine.

Experiments with the seed test of Baumann et al. and Bisaz et al. selectively measuring inhibitor effects revealed informations about low molecular inhibitors in whole urine. The methods of Baumann et al. distinguished stone formers and controls with respect to urinary inhibitor activity of calcium phosphate crystallization. Some differences between patients and controls were found in the calcium oxalate system by the kinetic tests of Achilles and Will et al. The system of Will et al. has the advantage that results can be described and predicted by physico-chemical equations. Furthermore, this seems to be the only method allowing an exact measurement of aggregation. The main drawback is that the test system cannot be used for whole urine, an approach being more and more required in stone research. Achilles has developed a kinetic test which, with

respect to its astonishing efficiency, seems to be the method of choice for clinical routine. However, some theoretical reservation have been made, because the method implies that stone formation would be the result of diffusion and precipitation of stone forming ions in a gel-like stone matrix. Even if this hypothesis is acceptable, the author has to prove that his agar-agar gel with the high oxalate concentration is an adequate model for stone matrix.

The different test systems presented in this paper clearly show that almost every method not only gives the results in special units but also has its special application in stone research. An ideal method is yet to be found. It should allow to study all the important crystallization processes of stone formation in native whole urine and to describe them by physico-chemical equations as in the approach of Will et al. For the moment the most challenging problem is trying to find a system of units which allows to compare the results obtained by different methods.

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