

A contribution to the study of crystalluria: significance in the diagnosis of metabolic and renal diseases

ABSTRACT

The study of crystalluria is of great importance for the detection of substances of endogenous or exogenous origin that are present in the urine, to a greater or lesser extent. Urinary sediment crystals can provide valuable answers for the assessment of therapeutic efficacy, as well as congenital and/or acquired pathophysiological conditions. The nature of the observed crystals informs the clinician of the biochemical irregularity of the urine.

Crystalluria is of clinical significance only if it has been studied under good test conditions (sample selection, time and storage conditions). Crystalluria interpretations are performed on the basis of the urinary pH determined with statistically significant reliability. When studying crystalluria by light microscopy, it is necessary to provide light polarization or bidirectional illumination in order to reduce the risk of diagnostic error.

Keywords: Urine pH, crystalline precipitation, promoters and inhibitors of crystalluria

I NTRODUCTION

Crystalluria implies the presence of crystals in the urine and appears due to the over-saturation of certain electrolytes and/or certain substances, which are most often caused by metabolic disorders, hereditary diseases or drugs. (Daudon & Frochot, 2015; Fogazzi, 1996; Fogazzi et al., 2000; Kouri et al., 2000; Okafor et al., 2018; Sikirica et al., 2002).

Crystals of metabolic origin are the result of an imbalance between the two large groups of substances: promoters and inhibitors of crystalluria. The most representative promoters of crystalluria are calcium oxalates, urates and phosphate ions, which are excreted by the kidney, and whose concentration exceeds the ability of urine to maintain them as soluble molecules and ions (Abraham & Smith, 1987; Daudon & Frochot, 2015; Werness et al., 1981; Wilcox et al., 1972). The collecting duct cells produce substances called crystalluria inhibitors. They can delay the formation of crystals in a way, by either stopping their growth and aggregation on the tubular epithelium, or by completely stopping their formation (Daudon & Frochot, 2015; Kok et al., 1986; Ryall et al., 1981). Crystalluria inhibitors include magnesium molecules, citrates and pyrophosphates (Daudon & Frochot, 2015; Kok et al., 1986). Macromolecules such as osteopontin, bikunin, matrix GLA protein, Tamm-Horsfall protein or urinary prothrombin fragment 1 are also able to stop the growth, aggregation and adhesion of crystals on to the collecting duct cells, thus allowing for the removal of crystals from the kidney with urine flow (Daudon & Frochot, 2015; Kok et al., 1986; Ryall et al., 1981).

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Review Article

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Many crystals tend to precipitate in the urine after miction, when the urine temperature becomes lower than the animal's body temperature (Bush, 1998; Daudon, 2015). Crystals are a relatively common microscopic finding in urinary sediment, but they are usually not present in fresh urine. The time consumed during the collection of urine, as well as the conditions and manner of its storage, will contribute to a greater or lesser presence of crystals in the urinary sediment. Some crystals (e.g. struvite, calcium oxalate dihydrate, amorphous crystals) can be found in urinary sediment in healthy animals (Daudon & Frochot, 2015). Oxalate and cystine crystals are less frequently found in alkaline, stale urine (Bush, 1998).

The presence of crystals in urine depends on many factors such as pH, specific urine gravity and salt solubility (Bush, 1998). On the other hand, crystals of ammonium urate and tyrosine are formed during metabolic disorders. Calcium oxalate crystals often appear in poisoning. Crystals of sulfonamides, ampicillin etc., are observed during drug therapy (Rizzi et al., 2017).

Crystalluria is not a specific marker of the pathophysiological condition in veterinary practice, especially not in cats, but it is definitely a very reliable marker in human medicine for predicting the recurrence of uroliths (Daudon & Frochot, 2015; Okafor et al., 2018).

It represents the first stage of urinary calculi formation; however, the significance of crystalluria in diagnosing and predicting urolithiasis has not been fully elucidated (Robert et al., 1998).

This article contributes to the acquisition of a more complete impression of the appropriate crystal analysis protocol, as well as the major categories of urinary crystals, and points to the usefulness of crystalluria assessment for the

diagnosis and monitoring of renal diseases or recurrent uroliths.

Crystal assessment protocol

Crystalluria assessment must be carried out in accordance with the proper methodology (Bader et al., 1994; Daudon & Jungers, 2004; Daudon, 2015; Sturgess et al., 2001). The crystalluria of a first morning urine sample is studied in practice, assuming that the patient has abstained from drinking at night (Daudon, 2015).

The urine sample container should be delivered to the laboratory no later than 2 hours from the time of urine sampling, i.e. between urination and microscopic examination of the crystals, to avoid in vitro formation of crystals during storage. The ideal thing to do would be to do urine analysis immediately, without delay (Daudon, 2015; Katica et al., 2019; Katica, 2020; Sikirica et al., 2002).

The test urine is stored in a clean container (500 ml) at laboratory temperature ($> 15^{\circ}\text{C}$). After taking the sample, the urine is homogenized by inversion.

The urine sample is centrifuged at 300 g for 5 minutes, 1.8 ml of the supernatant is removed and the sediment is resuspended in the remaining 200 μl by slight tapping. 50 μl of stain is added, (Kova stain - Hycor Biochemical) and the sample is stirred by pipetting 4 times.

In practice, crystals are most commonly analyzed using a phase-contrast microscope, which must be equipped with a device capable of polarizing light, which emits bidirectional illumination. The use of such an optical aid enables the correct identification of crystals, especially those with atypical morphology (Daudon & Frochot, 2015). Microscopy of the morphology of the possibly present crystals is done at 400 X magnification (Sturgess et al., 2001). The crystals found are expressed, for example, as the number of crystals found per milliliter of urine sample. In scientific studies,

double microscopy of one sample is performed to obtain more accurate results.

Microscopic examination of crystals involves a comprehensive evaluation of crystals; their identification, quantification and measurement, as well as the measurement of the size of the aggregates. However, there is a wide morphological spectrum of each crystal category, together with their complex chemical composition, which can make identification difficult. In such cases, it is recommended that the reference textbooks that address urinary sediment be consulted (Bader et al., 1994; Daudon et al., 2004; Daudon & Jungers, 2004; Daudon, 2015; Graff, 1982; Sturgess et al., 2001). It is recommended to use infrared spectroscopy for unidentifiable crystals, using their polarizing characteristics as well as prior knowledge of the pH of the urine sample. This is especially important if, in specific cases, there are crystals that indicate hereditary diseases or crystals that have arisen after drug therapy (Daudon et al., 1991; Sturgess et al., 2001).

Automated urine sediment screening devices are also widely used, especially in laboratories where a large number of urine samples are analyzed (Fogazzi & Garigali, 2013).

Most crystalline forms are known to be pH sensitive, with the exception of calcium oxalate and 2,8-dihydroxyadenine crystals. A representative example of the effect of pH on uric acid; for example at a urine pH of 5.0 it can crystallize at a molar concentration of about 2 mmol/L, and at pH 6.0 a concentration \geq 4 mmol/L is required for crystallization to occur (Daudan & Frochot, 2015).

Therefore, accurate measurement of urine pH is required for crystalluria research, where in routine analysis a universal paper indicator is used for this purpose (Sturgess et al., 2001).

Crystals independent of pH concentration

There are not many crystals independent of pH concentration. These are mainly calcium oxalates and cholesterol crystals, which are rarely present in the urine. In reality, even calcium oxalate and cystine are partially pH dependent, but within the normal urine pH range we may consider their pH sensitivity to be low (Daudon, 2015).

There are other approaches to crystal analysis, or slightly different analytical protocols. In the case of omission of centrifugation with homogenization of inverted urine in order to disperse the crystals without excessive cracking, it is possible to preserve all the aggregates of the present crystals. This increases the reliability of the obtained results, as well as their specificity, and thus the clinical interest (Daudon, 2015).

Effect of prolonged urine analysis and storage temperature on the presence of crystals in urine

Another very important criterion is the urine storing time period. Ideally, urine should be analyzed without delay, but a number of technical limiting factors prevent this. It is desirable to store urine at room temperature (Sikirica et al., 2002).

It has been found that if the sample is stored for less than 3 hours, at a temperature of above 20 °C, the crystal formation process in such a case is negligible and the results could be interpreted in the same way as the results obtained from fresh urine (Daudon, 2015; Elliot & Rabinowitz, 1980).

The third very important criterion is the temperature at which the urine is being stored. The lower the environment temperature, the greater the tendency to form crystals. Accordingly, it is not indicated to analyze urine crystals if it is stored at +4 °C. The exception in this case is the monitoring of cystinuria (Daudon, 2015).

A variety of crystal forms

Calcium can form calcium oxalate with oxalate ions, calcium phosphate with phosphate ions, or even urate with urate ions. Likewise, phosphate ions can bind to calcium and form calcium phosphates. Most of the molecular varieties formed in that way are able to crystallize in various forms (Daudon, 2015).

Classification of urinary crystals

Calcium oxalates

Calcium oxalate dihydrate (weddellite) and monohydrate (whewellite) crystals can occur in the urine of seemingly normal dogs and cats (Bush, 1998). Dihydrate crystals are the most common crystals of calcium oxalate. They come in a variety of sizes and shapes; they can be in the shape of a double pyramid, a letter (Figure 1) or an hourglass, rarely oval (Sikirica et al., 2002).

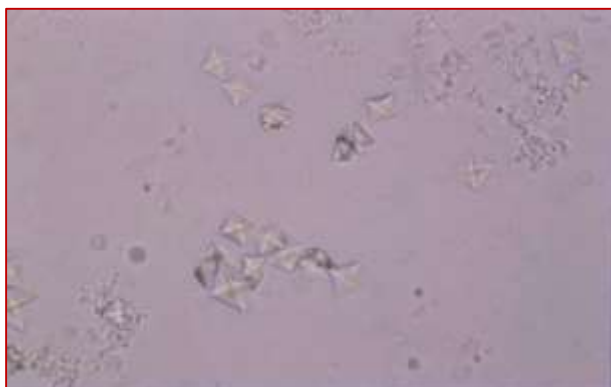


Figure 1 Calcium oxalate dihydrate (200X)



Figure 2 Calcium oxalate monohydrate (200X)

(F1&2.Rizzi et al., 2017)

Calcium oxalate monohydrate - whewellite (Figure 2) is closely related to high concentrations of oxalate in the presence of normal or low calcium (Azoury et al., 1987; Doudan, 2015). In contrast, calcium oxalate dihydrate - weddellite (Figure 1) is usually associated with hypercalciuria. Calcium monohydrate crystals - whewellites (Figure 2) are in the form of elongated and narrow hexagons, and are found in excess in urine after ethylene glycol poisoning (Azoury et al., 1987; Elliot & Rabinowitz, 1980).

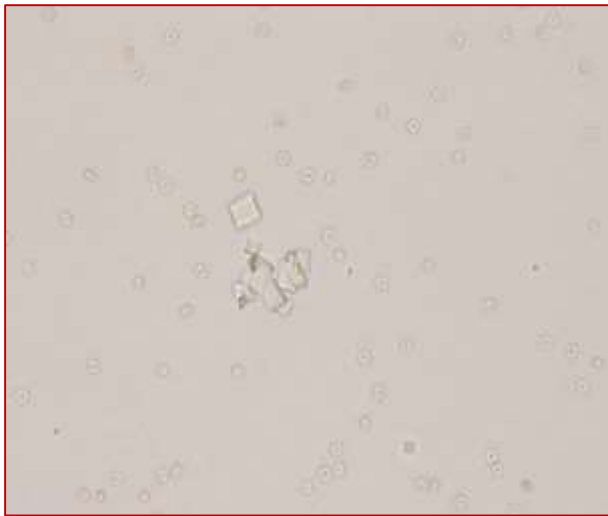
Phosphate crystals

Phosphate crystals - struvite crystals being most common (ammonium magnesium phosphate) - can occur in some healthy dogs and cats, especially in alkaline urine. In dogs they are often associated with some apatite crystals (calcium phosphate). In combination, they are known as triple phosphate (Bush, 1998). As already mentioned, they are precipitated in urine only in the presence of high urine pH associated with high ammonia content. This condition is only found in the case of urinary tract infection by microorganisms (Daudon, 2015).

Struvite crystals are colorless prisms, which most often take the shape of gold bars or resemble the lid of a coffin (Figures 3A and 3B), although in practice other atypical forms of struvite crystals can be observed, such as the fern (Rizzi et al., 2017). Struvite crystals (magnesium ammonium phosphate hexahydrate) are often found in cat urine in diseases of the lower urinary tract (Okafor et al., 2018; Osborne et al., 2009).



A



B

Figure 3 (A and B) struvite crystals with surrounding erythrocytes (500X) (Rizzi, et al., 2017)

Calcium phosphate crystals are formed depending on different biochemical conditions (Daudon, 2015). Calcium phosphate forms are mainly dependent on urine pH, i.e. when urine pH is above 6.5. The formation of this crystal usually requires a high concentration of calcium and phosphate, although it may also be favored by a low concentration of citrate (Figure 4). Calcium phosphate crystals can also be found in aggregates with calcium oxalate monohydrate crystals, which are often associated with hypercalciuria (Fogazzi, 1996).



Figure 4 Calcium phosphate crystal, dog urine sediment (500X) (Rizzi et al., 2017)

Calcium phosphate crystals can appear in the form of colorless plates or needles. The plates are thin and flat, while the needles are long and thin. The diagnostic significance of calcium phosphate crystals is seen under conditions of hypercalciuria and/or hyperphosphaturia. Calcium phosphate uroliths rarely occur in dogs or cats (Rizzi et al., 2017).

Uric acids and urates

Uric acid crystals tend to form in the urine with an acidic electrochemical reaction (Daudon, 2015). The presence of uric acid in urinary sediment of healthy animals is very rare. Under *in vitro* conditions, uric acid crystals can easily form if 10% acetic acid is added to the analyzed urine, which contains urate crystals or amorphous urates (Bush, 1998).

Uric acid crystals are usually yellow-brown in color. Aggregates of uric acid crystals can sometimes form in acidic urine. Occasionally, uric acid crystals are hexagonal and resemble a cystine crystal (Figure 5) (Rizzi et al., 2017). Uric acid precipitates that can be found in urine are: amorphous uric acid and anhydrous uric acid (uricite), as well as two of its hydrated forms (monohydrate and dihydrate). Uric acid dihydrate and amorphous uric acid are the most common forms found in urine. Uric acid dihydrate is mainly dependent on low urine pH (Daudon, 2015).



Figure 5 Uric acid crystals (500X)



Figure 6 Ammonium urate crystals (400X)
(F5& 6.Rizzi et al., 2017)

Urate crystals (ammonium urate)

In the literature (Rizzi et al., 2017), they are also referred to as the ammonium biurate crystals. They are most commonly brown-yellow in color, with irregular protrusions (Figure 6). Sometimes ammonium urate crystals with a smooth surface without visible protrusions occur in the urinary sediment and can be mistaken for calcium carbonate crystals, which are often present in equine urine and are not found in dog and/or cat urine (Meichner et al., 2015).

Ammonium urate crystals rarely appear in the urine of healthy dogs and cats. The exceptions are English bulldogs. Moderate or high presence of ammonium urate crystals

indicates a high concentration of ammonia and/or uric acid in the blood. In addition, urate crystals are a regular companion to urate uroliths, especially in portal vascular anomalies, in dogs and cats. It is necessary to emphasize that ammonium urate crystals are not always present in portal vascular anomalies (Rizzi et al., 2017).

Ammonium urate crystals (ammonium biurate) most commonly occur in Dalmatians who have reduced hepatic uricase activity and hyperuricosuria as hereditary diseases, and in dogs with hepatic impairment where blood ammonia levels are high (portosystemic shunts and liver failure) (Bush, 1998).

Cystine crystals

Cystinecrystalluria is never considered a normal finding and it suggests an inherited defect in cystine metabolism which can lead to the development of cystineuroliths. Acidic urine is a factor that contributes to the formation of these crystals. Cystine uroliths are unusual occurrence in dogs and cats (Rizzi et al., 2017). Cystine crystals are hexagonal plates in most cases, often irregular, appearing individually or in large aggregates (Figure 7) (Daudon & Frochet, 2015).

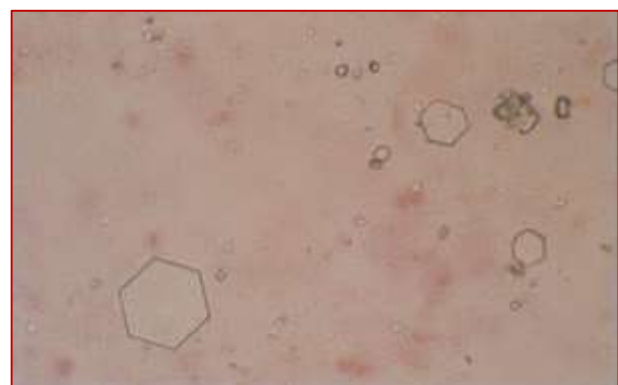


Figure 7 Cystine crystals (400X) (Rizzi et al., 2017)

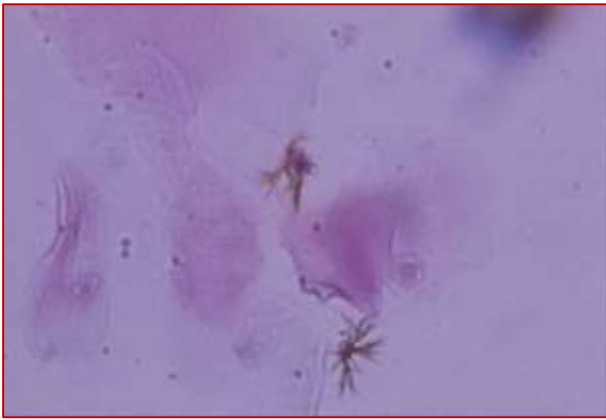


Figure 8 Bilirubin crystals with surrounding squamous cells (400X) (Rizzi et al., 2017)

Xanthine crystals

Xanthine is a product of purine metabolism and is converted to uric acid by the action of xanthine oxidase enzyme. It is impossible to distinguish xanthine crystals from ammonium or amorphous urates using light microscopy (Cairo & Bishop, 2004; Meichner et al, 2015).

All these crystals are yellow-brown and usually look round (Davis & Grindem, 2015; Osborne & Stevens, 1999). Infrared spectroscopy or high pressure liquid chromatography can be used to confirm xanthine crystalluria. Uroliths with 70% xanthine have been observed, which account for less than 0.1% of all canine uroliths (Davis & Grindem, 2015; Osborne et al, 2008).

There are reports describing allantoin in dogs, which is similar to xanthine and uric acid. It is excreted by the kidney as the terminal metabolite of purine metabolism from the corresponding nucleic acid (Cairo & Bishop, 2004; Meichner et al, 2015). Humans and higher primates do not have functional urate oxidase, an enzyme that converts uric acid into allantoin (Meichner et al., 2015; Rivara et al., 2013). Allantoin is 5-10 times more soluble than uric acid and the reason why the dog developed crystalluria is not known (Meichner et al., 2015). Xanthine crystals are associated with rare metabolic diseases and as such are an

unusual finding in urinary sediment (Daudon & Frochot, 2015).

Bilirubin crystals

Conjugated bilirubin, which is filtered through the glomerular membrane in urine, most commonly crystallizes in the form of yellow needles (Figure 8) (Rizzi et al., 2017).

Bilirubin crystals may be present in the concentrated urine of dogs with impaired bilirubin metabolism (Bush, 1998). The large presence of bilirubin crystals indicates hepatic disease or post hepatic jaundice.

Cholesterol crystals

Cholesterol crystals are rarely observed in animal urine and therefore have no diagnostic significance. Although they can be observed in clinically healthy dogs (Bush, 1998) the presence of cholesterol crystals in urine may be associated with excessive cell degeneration, different types of nephropathy and protein loss (Rizzi et al., 2017).

Cholesterol crystals look like transparent rectangular plates with one damaged corner in most cases (Figure 9).

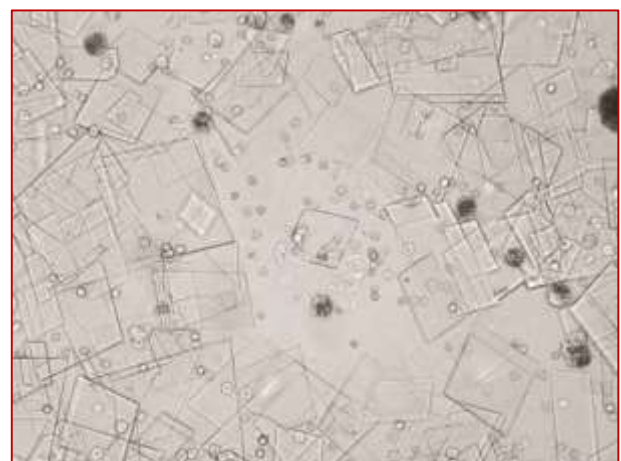


Figure 9 Cholesterol crystals (600X) (Rizzi et al., 2017)

Tyrosine and leucine crystals

Tyrosine and leucine crystals are rare in the urine of dogs and cats; their presence indicates severe liver disease. Tyrosine crystals appear as

refractile needles. They can be present individually, in clusters or in bundles. Leucine crystals are yellowish-brown in color, round in structure, and have radial grooves (Bush, 1998; Rizzi, et al., 2017).

Drug-induced crystals

Atypical crystalline forms are often observed during the microscopy of the urinary tract. In such circumstances, it is necessary to review the patient's history, including known drug use or potential drug exposure.

Sulfonamide crystals

Sulfonamide crystals can take various forms. They usually appear as brown needle crystals arranged in bundles. They can also appear as beads with radial lines, but also as wedge-shaped structures with a single sharp serrated edge. Their presence in the urine indicates long-term sulfonamide therapy or overdose (Rizzi et al., 2017).

Ampicillin crystals

Ampicillin crystals appear as long, thin needles or prisms resembling wheat sheaves (Osborne & Stevens, 1999; Rizzi et al., 2017).

CONCLUSIONS

Analysis of the inorganic part of urine sediment is an important part of the microscopic analysis of urine. It is useful for obtaining information suggestive of certain kidney or urinary tract diseases, as well as diseases of other vital organs, such as the liver. Crystalluria research is an inexpensive and valuable tool for diagnosing inherited lithogenic diseases, identifying atypical crystals due to drug consumption, as well as metabolic disorders associated with urolite formation.

Strict adherence to the crystal testing protocol, as well as the knowledge of the forms in which the most common crystals crystallize in urinary sediment, is a basic prerequisite for correctly performed microscopic analysis of

inorganic sediment, and the key to success of a good diagnosis.

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- **Conflict of interest**, Authors have no conflicts of interest to declare.

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