Analytical study of renal calculi. A new insight

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SUMMARY

In this paper, the most appropriate procedure for the study and analysis of renal calculi, its importance in the determination of the aetiology of the disease and the various techniques used for that study, are presented. First of all, the importance of the methodology of analysis of uroliths is commented. Then, a brief description of the most useful techniques for the study of renal calculi, including stereoscopic microscopy, infrared spectroscopy and scanning electron microscopy coupled with X ray dispersive energy microanalysis, is performed. The complementary nature of those techniques to obtain a good result is also indicated. Finally, a summary of the process of routine analysis and study of renal calculi is presented.

INTRODUCTION

Investigation of urinary calculi has been an important part of clinical chemistry for more than a century (1). The chemical characterization of urinary calculi was initiated by J. F. Heller in the nineteenth century who, in 1860, proposed a scheme for chemical investigation of urinary calculi based on the colour, hardness and chemical reactions performed directly on the dry material. Since then, study of renal stones has advanced considerably and a lot of chemical and physical methods have been applied to the identification of their majoritary and minoritary components and to the elucidation of their internal structure (2-10). However, little attention has been given to the adaptation of all this information to the clinical practice, therefore the complete analysis has been used exclusively for scientific purposes (11).

During the first three decades of the century, qualitative methods to establish the chemical composition of the stone had
dominated the investigation. These analysis implied wet chemistry qualitative reactions in order to identify the different anions and cations present in the calculus. This examination is still often carried out in the routine clinical laboratory using specifically designed kits (12). Recently, dissolution of the calculus in acidic solution and quantitative measurement of different ions is performed by atomic absorption spectroscopy or atomic emission spectroscopy with inductively coupled plasma (13). The quantified ions are later combined into the original salts by calculations, although some compounds and hydration degrees are difficult to elucidate.

The well recognized inadequacies of elemental chemical methods of calculi analysis and the obvious similarity between calculi and minerals provided, at the beginning of the 40, stimulus for the use of compositional physical techniques, like X ray diffraction. The powder diffraction method consists in irradiating a finely powdered sample of a substance with a beam of monochromatic X ray and recording the angles at which diffraction peaks occur and their intensities. The rays are reflected from planes of atoms forming a pattern of the irradiated substance, which is unique and characteristic offering a rigorous means of identification. Despite the fact of the specificity of the described technique, it presents a series of complications that make it incompatible with clinical purposes. X ray diffraction equipment is expensive, time consuming, entails extensive theoretical knowledge, is not sensitive enough when dealing with mixtures and is unable to detect amorphous substances (not only high-molecular-weight substances such as proteins but also hydroxyapatite and carbonate apatite) which are also normally present in the calculi.

At late, optical methods of analysis have imposed on diffraction techniques as a consequence of the mentioned problems. Thus, infrared spectroscopy is an easy and rapid procedure able to identify all crystalline and amorphous compounds, with more sensitivity when dealing with mixtures.

Despite the fact that accurate knowledge of stone composition is of great value when setting up therapeutic advice to prevent stone recurrence, it was not demonstrated until the beginning of 1950 that the structure and internal arrangement of calculi, which is impossible to elucidate using chemical methods, is crucial in determining
the mechanism of formation of the different kind of stones.

Since then, several methods have been used to study the crystalline form and general structure of the various types of urinary calculi. In the beginning, the most powerful methods were those in which a suitable cross-section of the stone was studied using the polarizing microscope (14). These observations provide a glimpse of their basic structure: lamination, discernible nucleus located in the centre of the stone or near the periphery, anarchic structure, sequence of deposition... The disadvantages of the thin section technique, apart from being bothersome, are obvious. As the term indicates, only thin plain sections are observed, implying that a high number of sections must be studied in order to obtain a tridimensional general idea of the studied stone, moreover, important portions of a calculus would be destroyed on cutting, besides the maximum resolution of a light microscope being 2000 Å. These facts have stimulated the use throughout the last two decades of the scanning electron microscopy in order to study the tridimensional inner structure of calculi and demonstrate the morphology of small crystals present in calculi (14-15).

As mentioned before, in spite of the great progress achieved in the field of the study of renal stones, all these advances have been used exclusively for scientific purposes. In fact, the great majority of routine clinical laboratories only performs a qualitative analysis of the calculus by means of wet chemistry kits or infrared spectroscopy of the whole pulverized calculi, omitting details concerning minoritary components, percentages of different compounds or internal structure. The information obtained with wet methods or infrared spectroscopy is valuable on setting up therapeutic advice but is not totally valid for guidance on the concrete etiological causes of the lithiasis (16).

At present it is accepted that no single method provides total information on the structure and composition of the stone, and at least two different methods have to be combined for accurate study of calculi. The aim of this paper is to present a standarized protocol to study renal stones with clinical purposes. We attempt to show that the use of a combination of light microscopic examination, infrared spectroscopy and
scanning electron microscopy provides a simple and useful method to inform on the aetiology of all kind of renal stones.

**BINOCULAR STEREOSCOPIC MICROSCOPE**

Since first publications, all authors agree that the first step in analysis of a calculus consists of the examination of the specimen features under a binocular stereoscopic microscope (12-13, 17-18).

Binocular stereoscopic microscope entails examination of specimens using visible light to provide a magnified image of the sample with neither preparation nor destruction. The most useful magnification is about 20x, although lower and higher magnifications may be reached, between 4x and 100x. Many of these instruments are available on the market, the most useful ones being those equipped with a photographic camera.

In practice, intact calculi are first externally observed with a low power magnification (10x to 20x) in order to

Figure 1.- Image of a calculus composed by calcium oxalate monohydrate presenting calcium oxalate dihydrate crystallization on its surface.
observe several superficial features like shape and dimensions, colour, existence of a stone area adhered to the kidney walls, presence of superficial deposits... This large-scale external characterization permits to carry out some hypothesis on the composition and possible structural arrangement of the calculus and on the renal and urinary characteristics. For instance, the presence of superficial deposits with different composition from the bulk of the calculus may indicate an ultimate growth of the calculus due to a change in the chemical composition of the urine, as observed in some calcium oxalate monohydrate renal calculi which exhibit superficial deposits of calcium oxalate dihydrate crystals (Figure 1).

The observation of a large calculus with no point of attachment to the papillae suggests that this calculus has probably an anarchic internal structure as a result of its development in a renal cavity with deficient urodynamic conditions (Figure 2).

On the other hand, this external examination allows to select the optimal

![Figure 2.- Image of a calcium oxalate monohydrate calculus with no point of attachment to the papillae, mulberry shaped and probably developed in a renal cavity with low urodynamic efficacy.](image)
fracture plane to obtain the maximum information of the internal arrangement of the stone. The sectioning of a lithiasic sample must be performed carefully to avoid alterations or destruction of the structure of interest.

Many constituents of renal calculi may be recognized on sight when examining the fractured surfaces under a binocular stereoscopic microscope, permitting a guess as to the probable majority composition of the stone. In practice, the method permits to distinguish between calcium oxalate and calcium phosphate stones. Moreover, what is even more important is that internal inspection of such sections informs on several structural features, such as the degree of internal organization, the location and size of the nucleus of the stone, the presence of lamination and/or radial structure in the bulk of the stone, the order of deposition of the components when lamination is present and other structural details, if present. Likewise, it is possible to distinguish between a sedimentary calcium oxalate monohydrate stone, which shows little or no regularity of the central structure but an outer layer of perfectly developed columnar crystals, and a calculus of the same composition developed by crystal growth which shows a perfectly arranged internal structure.

This last step is critical to the success of any subsequent study. In fact, macrostructural study under binocular stereoscopic microscope entails selecting the regions of the sample that are of interest and deciding the most appropriate technique to apply in order to obtain the maximum information.

In conclusion, optical macroscopic external and internal examination of the stone reveals features that will help to establish the history of the calculus and thereby, to give an idea of the cause and/or causes of stone formation.

**INFRARED SPECTROSCOPY**

A sample can transmit, scatter or absorb the incident radiation when irradiated with infrared light. Absorbed infrared radiation can excite molecules into higher-energy vibrational states when the light matches the energy difference between two vibrational states. Absorbance is then recorded versus wavelength thus originating the spectrum of the sample which has been
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temed the finger-print of the compound.

Infrared spectroscopy was first applied to stone analysis by Beischer in the mid fifties (19) and spread quickly (8-10). It is a useful technique for identifying organic and inorganic compounds. In fact, it is particularly useful for determining functional groups present in a molecule, because they vibrate at nearly the same frequencies independently on their molecular environment. Like X ray diffraction, infrared spectroscopy provides results on the actual salts, including the different degree of hydration, with an additional advantage of identifying non-crystalline compounds, whereas X ray diffraction can not. Moreover, recent advances in computerized infrared spectroscopy, particularly Fourier transform infrared (FT-IR) spectroscopy, have allowed to obtain infrared spectra in less than a minute, whereas in a conventional X ray apparatus each run requires some hours. Finally, the quantity of sample needed for Fourier transform infrared spectroscopy can be less than one microgram.

If inspection of the superficial and cross-section of the stone reveals an homogeneous appearance, the identification of calculus composition can be performed by powdering the whole stone and taking an average sample for infrared study. But if inspection reveals an heterogeneous appearance such as areas of differing colours or textures, lamination or other structural details, it may become necessary to scrape off bits of calculus material from the different regions with a scalpel and perform several identifications by infrared spectroscopy.

| TABLE 1 |
| More common majoritary components of renal calculi |

**PURE RENAL CALCULI**
1. Calcium oxalate monohydrate (whewellite)
2. Calcium oxalate dihydrate (weddelite)
3. Ammonium-magnesium phosphate (struvite)
4. Calcium phosphate, carbonated form (calciumphosphate)
5. Calcium phosphate, hydroxyl form (hydroxyapatite)
6. Calcium monohydrogen phosphate dihydrate (brushtite)
7. Uric acid anhydrous
8. Uric acid dihydrate
9. Sodium urate monohydrate
10. Ammonium urate
11. Cystine

**MIXED CALCULI (more frequently found binary associations)**
1. Calcium oxalate monohydrate + calcium oxalate dihydrate
2. Calcium oxalate dihydrate + calcium phosphate
3. Uric acid anhydrous + uric acid dihydrate
4. Struvite + calcium phosphate
5. Calcium oxalate monohydrate + uric acid anhydrous
6. Calcium oxalate monohydrate + calcium phosphate
7. Brushtite + hydroxyapatite
When dealing with solid samples, as renal stones, preparation requires dispersing 0.4 to 1.0 mg of the sample in a KBr matrix and pressing into a transparent pellet.

Between 40 (12) and 65 (13) different molecules, including several of exogenous origin, have been identified in urinary calculi, although only 8 of them are most frequently found (Table 1). Identification is very simple if a reference spectrum that matches that of the unknown material is found. When an exact reference spectrum match cannot be found, a band by band assignment is necessary to determine the composition of the solid.

Infrared spectroscopy permits to

![Infrared spectra of (a) calcium oxalate monohydrate and (b) calcium oxalate dihydrate.](image)

Figure 3.- Infrared spectra of (a) calcium oxalate monohydrate and (b) calcium oxalate dihydrate.
clearly distinguish between a calcium oxalate monohydrate renal calculus and a calcium oxalate dihydrate renal calculus. Thus, absorption bands comprised between 3500 cm\(^{-1}\) and 750 cm\(^{-1}\) are clearly different for both compounds (see Figure 3). Although to distinguish both types of calculi can be easy when they are intact, such distinction must be difficult when the calculi are fragmented or in sand form.

All phosphate containing calculi (calcium, magnesium or ammonium-magnesium phosphates) show an intense absorption band around 1000 cm\(^{-1}\). This band permits its easy identification even in mixtures with calcium oxalate monohydrate or dihydrate. Pure brushite calculi are not frequent, but they exhibit a characteristic IR spectra that allow to clearly distinguish them from hydroxyapatite or ammonium-magnesium phosphate calculi (see Figure 4).

The most frequent phosphate calculi are those with infectious origin, generally constituted by mixtures of ammonium-magnesium phosphates and hydroxyapatite. Calculi formed exclusively by hydroxyapatite are less common and due to their external appearance can be easily confused with calcium oxalate monohydrate or even uric acid. Calculi with infectious origin (ammonium-magnesium phosphate + hydroxyapatite) are normally large, consequently, the determination of their composition requires the obtention of the infrared spectra of different zones of the calculus, due to the irregular distribution of hydroxyapatite and ammonium-magnesium phosphate crystals. Even following such
cautious, it is possible in some cases to miss one of such components, although to be present, consequently a wrong classification of the calculus, if only the infrared spectrum is considered, would be performed.

Although uric acid calculi are easily identified in most cases due to their characteristic reddish colour. Nevertheless, uric acid is probably one of the cases where a wider variety of sizes and colours can be found, and consequently important mistakes can be produced if the identification is exclusively performed visually. The infrared spectra of such calculi are, nevertheless, characteristic and permit their easy identification without any difficulty and also allow their clear differentiation from the infrared spectrum corresponding to ammonium urate calculi due to the different absorption bands comprised between 1300 cm$^{-1}$ and 500 cm$^{-1}$ (see Figure 5).

![Infrared spectra of (a) uric acid and (b) ammonium urate.](image)
Cystine calculi, that in some cases are easily identified by their waxen aspect, exhibit a characteristic infrared spectrum that permits their unequivocal identification (see Figure 6).

The main advantages of the infrared spectrometry use in the identification of calculi components are the speediness, simplicity and specificity for the identification of rare components as calcium carbonate, calcium urate, drugs (triamterene, indinavir, ...), organic matter, etc. (see Figure 7).

SCANNING ELECTRON MICROSCOPE

The first commercial scanning electron microscope became available in 1965, although until 1985 it was not widely used in stone research. The scanning electron microscope provides three outstanding improvements over the optical microscope: it extends the resolution limits so that picture magnifications may be increased up to 30000x to 60000x, it improves the depth-of-field resolution more dramatically, by a factor of approximately 300, and finally, it entails the observation of several surfaces of a sample because of the possibility of rotating the sample in several directions.

The image is produced by focusing a beam of electrons, coming from an electron gun at one end of a vacuum column, to a small spot. A primary use of scanning electron microscope is to produce high resolution and depth-of-field images. But in

Figure 6.- Infrared spectra of cystine.
addition to image formation, this instrument may provide elemental analysis of micron-sized areas of the specimen observed when coupled with a special device for energy dispersive X-ray microanalysis. Non-conducting materials as renal stones should be sputter-coated with a thin layer of gold after being mounted on an aluminium stub.

Microstructural observation of calculi emphasizes on several fine structure features (14-15). First, it informs on the composition of the core if present. In this sense, it is possible to locate inducer components of the calculus development as apatite nests in calcium oxalate calculi core (Figure 8), such a finding would indicate the utility of

![Figure 7. Infrared spectra of (a) calcium carbonate, (b) calcium urate](image-url)
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Figure 7.- Infrared spectra of (c) silica, (d) triamterene and (e) organic matter

Figure 8.- Scanning electron microscope image of the core of a calcium oxalate monohydrate papillary calculus containing hydroxyapatite spherulites.
acidification therapy to prevent recurrence. By contrast, the finding of uric acid mixed with calcium oxalate would suggest the utility of alkalinization therapy (Figure 9). On the other hand, the presence of tissue and/or urine proteins which act as inducer of calcium salts would suggest a high proteinuria as a consequence of intense severe epithelial renal lesions or important renal dysfunction that deal with increased urinary protein.

Figure 9.- Scanning electron microscope image of a calculus zone with calcium oxalate monohydrate (interior) and uric acid anhidrous (exterior).

Figure 10.- Scanning electron microscope image of the core of a calcium oxalate monohydrate papillary calculus containing high amounts of organic matter.
Analytical study of renal calculi excretion (Figure 10).

The degree of twinning of the crystals in the nucleus may inform on the severity of the renal lesion causing the development of a papillary calculus. Thus, calculi with a central core integrated by calcium oxalate monohydrate crystals developed by twinning and intergrown are generated on no severe epithelial lesions (Figure 11), whereas

Figure 11.- Papillary calcium oxalate monohydrate calculus core containing twinned and intergrown calcium oxalate monohydrate crystals.

Figure 12.- Scanning electron microscope image of the section of a calcium oxalate monohydrate papillary calculus. It can be observed that the striated columnar body of the calculus starts on the calculus basis in a very thin line, composed by calcified organic matter.
superficial plain cores rich in organic matrix suggest intense lesions even necrosis (Figure 12).

Scanning electron microscopy may emphasize crystalline conversions which sometimes are not evident under a binocular stereoscopic microscope. Thus, the presence of etching on the surface of calcium oxalate dihydrate calculi and big crystals of calcium oxalate monohydrate, suggest a high degree of transformation of calcium oxalate dihydrate into calcium oxalate monohydrate as a consequence of a long time of permanence of the calculus in the kidney (Figure 13).

On the other hand, the observation of external details may be crucial in determining the history of the calculus. In this way, the observation of an external cavity coated with tubular apical cells suggests the presence of a point of attachment to the renal papilla and hence, the existence of an epithelial microlesion as happens in papillary calcium oxalate monohydrate stones. Besides, lamination due to the development of crystals with a different composition indicates a change in the urinary composition, for example, if magnesium ammonium phosphate is found only in the outer layers of a stone and not in the centre, it is inferred that urea-splitting infection was not the original cause, although it has been responsible for continued stone growth after alkaline urinary infection.

Figure 13.- Scanning electron microscope image of the surface of a originally formed calcium oxalate dihydrate crystal, that has suffered a typical etching process, transforming into the monohydrated form.
Figure 14.- Scanning electron microscope images of different crystalline compounds present in renal calculi. (a) Hydroxyapatite in spherule form, (b) columnar brushite crystals, (c) anhidrous uric acid crystals and (d) anhidrous uric acid resulting from dehydration of initially formed dihydrate uric acid.
supervened.

Finally, such a method provides information about the nature of crystalline compounds, shape of the crystals, internal structure, location of components, crystalline conversions, crystallite size distribution, characteristics of the aggregates and some data about intimate relations between crystals and organic matrix or relationships between different crystalline species, as can be seen in Figure 14.

**RECOMMENDED PROCEDURE TO STUDY RENAL CALCULI**

Superficial and cross-sectional composition and morphology of stones may inform not only on urinary composition but also on anatomic conditions such as pyelocaliceal dilatation, cavities which exhibit urinary stasis, that is on the aetiology of the pathology (13, 20). Routine analysis must be able to join all these aspects and give comprehensive information for clinical purposes.

The used procedure to analyze and study renal calculi requires an appropriate combination of observation by means of macroscopic and microscopie conventional techniques with physical techniques as IR spectrometry and scanning electron microscopy coupled with X ray microanalysis (21). In the Figure 15 a scheme of the proposed recommended procedure to study renal calculi is shown.

The study of the calculus begin through the direct observation of its external aspect and IR surface analysis. Afterwards, the calculus is sectioned in two parts along a plane as near as possible to its geometric centre, to be able to establish the internal structure. This step will indicate, in most cases, what is the more adequate process to further apply. This must imply:

a) IR spectrometry analysis of one or several parts of the calculus. If in the fragmented calculus several parts with different appearance appear, it is necessary to perform an IR analysis of each one. The recommended technique to perform such analysis consists on the well known KBr method (it only requires 1 mg or even less of sample).

b) The deep study of the fine inner structure of the calculus and the detection
and identification of microcomponents requires the use of scanning electron microscopy coupled with X ray microanalysis. It must be considered that the presence of a substance in minute amounts, not identifiable using conventional IR spectrometry, could be decisive to establish the aetiology of the calculus formation. Precisely, to be able to determine the importance of a given microcomponent, the accurate knowledge of the calculus fine structure is fundamental, in such a manner that the initial zone of the calculus development should be established. It must be considered that this zone contains the keys of the calculus origin.

The used methodology to carry out such study consists on locating calculus fragments on a microscope slide fixed with silver stuff. Afterwards the sample is gold covered (300 Å thickness) using an
spattering. The observation of calculus fragments is performed between 30 and 20000 magnification. The combined use of X ray microanalysis is of great importance in the identification of some components.

It must be emphasized that the experience of the specialized staff devoted to such analysis is fundamental to obtain the major number of aetiological data from the calculus study.

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