

Compositional analysis of various layers of upper urinary tract stones by infrared spectroscopy

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Abstract. The objective of the present study was to determine the composition of various layers of upper urinary stones and assess the mechanisms of stone nucleation and aggregation. A total of 40 integrated urinary tract stones with a diameter of >0.8 cm were removed from the patients. All of the stones were cut in half perpendicularly to the longitudinal axis. Samples were selected from nuclear, internal and external layers of each stone. Fourier transform infrared spectroscopy (FT-IR) was adopted for qualitative and quantitative analysis of all of the fragments and compositional differences among nuclear, internal and external layers of various types of stone were subsequently investigated. A total of 25 cases of calcium oxalate (CaOx) stones and 10 cases of calcium phosphate (CaP) stones were identified to be mixed stones, while 5 uric acid (UA) calculi were pure stones (purity, >95%). In addition, the contents of CaOx and carbapatite (CA.AP) crystals in various layers of the mixed stones were found to be variable. In CaOx stones, the content of CA.AP in nuclear layers was significantly higher than that of the outer layers (32.0 vs. 6.8%; $P<0.05$), while the content of CaOx was lower in the inner than in the outer layers (57.6 vs. 86.6%; $P<0.05$). In CaP stones, the content of CA.AP in the nuclear layers was higher than that in the outer layers (74.0 vs. 47.3%; $P<0.05$), while the content of CaOx was lower in the inner than in the outer layers (7.0 vs. 40.0%; $P<0.05$). The UA stones showed no significant differences in their composition among different layers. In conclusion, FT-IR analysis of various layers of human upper urinary tract stones revealed that CaOx and CaP stones showed differences in composition between their core and surface, while all of the UA calculi were pure stones. The composition

showed a marked variation among different layers of the stones, indicating that metabolism has an important role in different phases of the evolution of stones. The present study provided novel insight into the pathogenesis of urinary tract stones and may contribute to their prevention and treatment.

Introduction

Urolithiasis, a worldwide endemic condition arising from malnutrition, has significantly impacted health-care systems in previous years due to the growing cost of diagnosis and treatment of stone episodes (1-3). Urolithiasis is a common urologic disease with multiple etiologies and risk factors. Studies have suggested an increase in the incidence of urolithiasis in developed nations over recent decades (4,5). The prevalence is 1-5% in Asia, 5-9% in Europe, 13% in North America and 20% in Saudi Arabia, and increasing throughout the world (4). The increase in incidence of urolithiasis was most likely associated with changes in environmental factors, including dietary habits, fluid intake and obesity (6). Historically, urolithiasis has been indicated to be more common among men than women (7). Early epidemiological studies placed the incidence rate of urolithiasis in men at 2.2 to 3.4 times that of women (8). However, evidence suggests that these gender ratios may be changing (9). Increasing obesity/dietary changes, or decreased fluid intake may be contributing to the rapid increase in urolithiasis in women (10). The removal of urinary stones has been greatly facilitated by percutaneous nephrolithotomy, retrograde ureteroscopy and shock wave lithotripsy, but the recurrence rate was >50% at a mean follow-up time of 10 years (11,12). Urinary calculi are crystals with mainly one or more of the following five components: Uric acid (UA), struvite, cystine, calcium phosphate (CaP) and calcium oxalate (CaOx) (13,14). Knowledge of the chemical composition of a patient's stones is important for determining a treatment plan, understanding their etiology and preventing recurrences. A number of methods have been reported for the analysis of urinary stones (13,15-17). Fourier transform infrared spectroscopy (FT-IR) is now considered a standard technique for the analysis of urinary stones, since it is sensitive, reliable and accurate, and requires <1 mg of sample (18,19). However, studies on the application of the FT-IR technique for capturing details of the composition of individual areas of stones are scarce. In addition, to understand the influence of etiological factors

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on the growth of a given stone, it is essential to determine its composition at different phases of stone development (20).

In the present study, taking in consideration the above statements, evaluated the variations in composition of different layers of human urinary tract stones by using FT-IR in order to provide basic compositional data giving insight into the mechanism of formation of urolithiasis and propose an appropriate means of clinical management for this condition.

Materials and methods

Samples. A total of 40 mixed human urinary stones with a minimum diameter of 8 mm were retrieved by percutaneous nephrolithotomy procedures, open surgeries or spontaneous stone passage. Samples of stones were washed once with distilled water, dried in a drying oven at a constant temperature of 60°C for 24 h, put into adapted pre-drilled wood grooves for fixation and cut perpendicularly to the longitudinal axis into two approximate halves using a fretsaw machine, which was 1.5 mm in diameter (Fig. 1). One half was retained for further inspection. A total of 120 fragments were collected from the central core and the internal and external regions of the stones. These fragments were pulverized into 2- μ m particles by grinding using a pestle and mortar. The resulting powdered specimens were mixed with dried potassium bromide of spectroscopic purity at a ratio of 1:20 and converted into pellets by exerting a standard pressure of 10 KPa.

FT-IR. Pellets prepared as described above were placed in a scanning chamber for FT-IR analysis using a Shimadzu Fourier Transform Infrared Spectrophotometer 8300 (Shimadzu Corp., Kyoto, Japan). The spectral range was fixed from 4,000-400/cm and the resolution was adjusted at 4/cm. A total of 32 scans were implemented for infrared data acquisition. In principle, the IR spectrum creates a molecular fingerprint representing the molecular absorption and transmission. The peak size of the fingerprint is directly correlated with the quality and the quantity of a specific chemical. To obtain precise data on various stone components, the FT-IR spectrum was matched against a computer library of reference spectra.

Statistical analysis. Data was presented as the mean \pm standard deviation. Differences in categorical variables and continuous variables between the groups were analyzed using the χ^2 test and the Student's t-test, respectively, using SPSS statistical software 17. (SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a significant difference.

Results

Relative fraction of urinary stone components determined using FT-IR. By FT-IR analysis of the stones, almost 87.5% of the 120 fragments of the 40 stone samples analyzed were identified as mixed stones (Table I). The major component (percentage of content, $>50\%$) detected was CaOx monohydrate (COM, 52.5%), followed by CaOx dihydrate (COD, 10%) and carbapatite (CA.AP) (25%). The remaining 12.5% were pure UA stones.

Composition of different layers of CaOx stones. A total of 25 stones were identified to be mainly composed of CaOx. The major component in the core, middle and outer regions of these stones was CaOx, with the exception of 7 cases in which the cores were constituted of CaP. Furthermore, CaOx was found to be abundant in the periphery of CaOx stones, while CaP was predominant in the inner part (Fig. 2). Only 1 stone was found to contain 30% magnesium ammonium phosphate (struvite) in the core layers.

Composition in different layers of CaP calculi. In the 10 CaP calculi, the main components of the three different layers were CaP. From the interior to the exterior, the content of CaP was decreased, while CaOx increased gradually. The content of UA and struvite among different samples did not display any significant differences (Fig. 3).

Composition in different layers of UA calculi. A total of 5 pure UA stones (purity, $>95\%$) existing mainly in the form of anhydrous UA (UAA) and UA dihydrate (UAD). The content in different layers was not significantly different. UA typically crystallizes into the anhydrous compound UAA, the dihydrate UAD or a mixture of both. The formation of a monohydrate form is rare (21).

Discussion

Urolithiasis is a common and frequently-occurring condition and various effective ways are available for its treatment; however, its recurrence rate at a follow-up after 10 years is up to 50%. Although urinary calculi have been reported as early as 4,800 BC, the pathological mechanism has remained to be fully clarified (22). In order to understand the influence of external factors on the process of stone formation, it is essential to know its constituents. Accurate analysis of the composition of urinary calculi may provide clues on the mechanisms involved in their formation as well as a scientific basis for prevention measures and the best choice of clinical management (23).

Generally speaking, urinary stones are composed of minerals and/or organic compounds. The same chemical components may crystallize in different forms. Proper stone analysis is necessary for determining not only the molecular composition of the calculus, but also the crystalline forms of chemical constituents. At present, numerous methods are available for stone analysis, but apart from FT-IR, none of these techniques is able to provide a reliable quantitative analysis. Moreover, with a computerized reference library match of the closest IR spectrum, FT-IR can virtually characterize any stone sample (24).

FT-IR usually captures only one fragment from each region of the whole stone and thus, single captures may not be representative of the whole samples. In order to understand the stone composition as a whole, it is obligatory to perform segmental analysis of larger stones (25). Quantitative stone analysis by FT-IR also warrants separate and accurate analysis of the stone nucleus, including external and internal layers without making errors of judgment.

The most common component of urinary calculi in industrialized countries is CaOx, which represents 75-90%

Table I. Relative fraction of urinary stone components obtained using Fourier transform infrared spectroscopy analysis.

Main component	N (%)
COM + CA.AP	16 (40.0)
COD + CA.AP	7 (17.5)
COM + COD	2 (5.0)
COM + UA	5 (12.5)
CA.AP + UA+struvite	5 (12.5)
UA	5 (12.5)
Total	40 (100)

COM, calcium oxalate monohydrate; COD, calcium oxalate dehydrate; CA.AP, carbapapite; UA, uric acid.

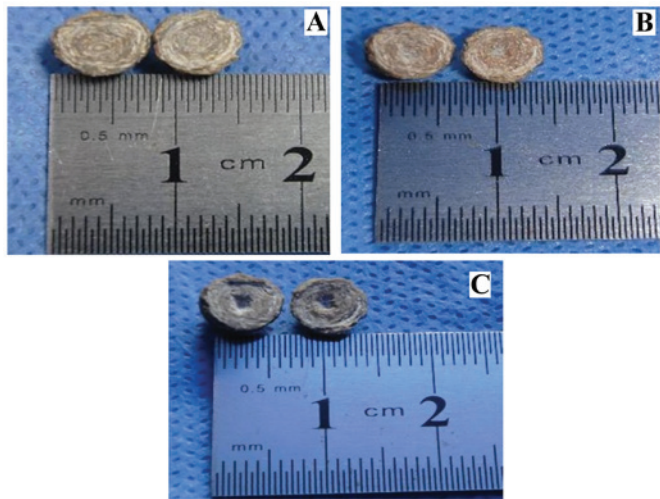


Figure 1. Sections of stones obtained by cutting with a fretsaw machine. (A) Cross-section of a stone, (B) after obtaining a fragment from the core of the stone and (C) after obtaining a fragment from the middle of the stone.

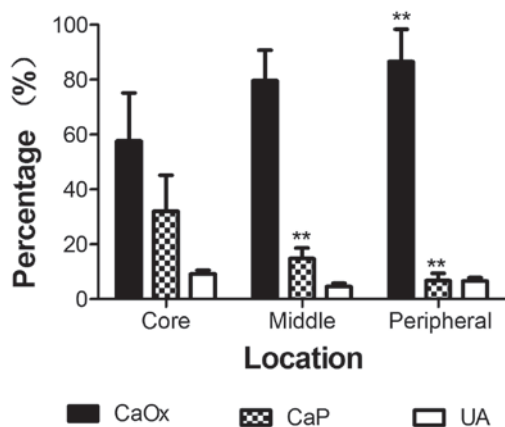


Figure 2. Composition of various layers of 25 CaOx stones. **P<0.05 compared with the core group. CaOx, calcium oxalate; CaP, calcium phosphate; UA, uric acid.

of urinary calculi (26), but the etiology of CaOx calculus formation has remained poorly understood until recently. In

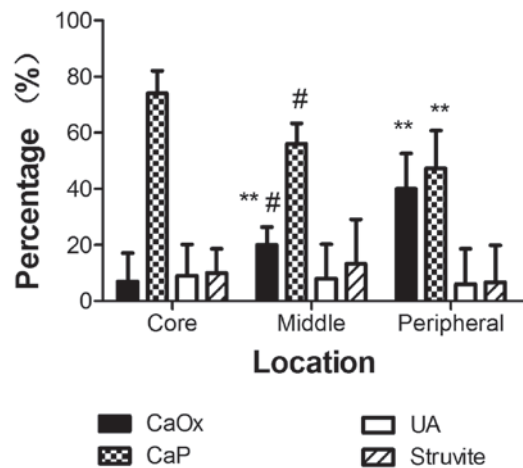


Figure 3. Composition of various layers of 10 CaP stones. **P<0.05 compared with the core group; #P<0.05 vs. peripheral group. CaOx, calcium oxalate; CaP, calcium phosphate; UA, uric acid.

the present study, CaOx was detected in 25 stones as the main component (62.5%). Most of the calcium CaOx stones were composed of mixture of COM + COD and COM + CA.AP. According to Marickar *et al* (25), the composition of COM and UA differs among different layers of the stone. The UA component was more abundant in the inner layers by comparison with the surface layers, but COM was the predominant external component in most mixed stones. The outer portion of the CaOx calculi was rich in COM + COD, while CA.AP appeared mostly in the inner portion. The analysis of sequential samples from the core to the surface revealed that CaOx stone formation occurs in different stages, which are separated by intervening periods of inactivity. It may be inferred from the present findings that there is a close association between the metabolism of oxalate and that of phosphates. The exact knowledge of renal stone composition may therefore be useful for predicting underlying clinical disorders. The abundance of CA.AP in the nucleus of the stone indicated that chronic urinary tract infection may be associated with the initiation period of the formation of CaOx calculi. As early as 1937, Randall's plaque, which is defined as interstitial papillary and medullary deposits of apatite, a common crystalline phase of CaP, was described in patients with stones, revealing that stones can grow attached to the plaque (24). Randall's plaque is detected in almost 100% of CaOx stone formers and 43% of other stone formers (27). It is considered as an ideal site for CaOx and CaP overgrowth, which may develop into calculi (28). In line with this, studies have reported that COM and apatite are usually concurrently encountered in the CaOx calculus (29,30). In particular, in the core of the calculi, it is hard to find pure COM. Convincing studies have shown that epitaxial mechanisms may be of considerable importance in causing the stone to grow larger, once a suitable nucleus has been deposited. The present study revealed that the CaOx stone potentially originates from the heterogeneous nucleation of CA.AP, which subsequently develops into pathological calculi. In addition, certain stones were mixtures of UA and CaOx. The information provided by the present study may aid in selecting a more appropriate prophylaxis for patients with urolithiasis.

CaP stones account for ~15% of all urinary stones, with a marked female preponderance, and reflect a wide diversity of etiologies (31). CA.AP is the most frequent crystalline phase (74.0%) of CaP and stones composed thereof have a number of possible causes, including metabolic disorder (such as hypercalciuria, hypocitraturia, tubular acidosis, primary hyperparathyroidism and medullary sponge kidney) and chronic urinary tract infection, while the latter etiology remains under debate. The carbonate content of Ca.AP is of clinical interest, since carbonate rates above 15% are commonly associated with urinary tract infection with urea-splitting bacteria. Conversely, the carbonate rate is frequently <10% if calculi are induced by metabolic disorders (32). Infection stones caused by urea-splitting bacteria are composed of CA.AP admixed with struvite and exhibit a high carbonation rate (the ratio of carbonate or phosphate; $\text{CO}_3^{2-}/\text{PO}_4^{3-}$ ratio) (33). Recent studies have shown that nanobacteria are also responsible for the pathogenesis of intra- and extracellular calcification and urinary stones with phosphate components. The nanobacteria form a coating of carbonate apatite, which promotes epitactic deposition of more crystals (34). Infection is not a prerequisite for the formation of carbonate apatite stones, but infective conditions favor carbonate apatite formation. Hence, characteristic infection stones (struvite) are often composed of a mixture containing carbonate apatite. The association of a high carbonation rate with urinary tract infection showed a positive correlation between the carbonation rate of struvite-free CaP stones and the number of bacterial imprints, which was revealed by scanning electron microscopy at the surface of CaP stones (35). The present study revealed that CA.AP is the major component of the nucleus as well as external and internal layers of the CaP calculus. In addition, the percentage of CA.AP decreased with the increase in the percentage of CaOx from the nucleus to the external layer. Therefore, the active prevention and treatment of urinary tract infection is key for preventing the recurrence of CaOx and CaP stones.

Calculi predominantly composed of UA represent 13% of human kidney stones (36). The trend in UA stone formation appears to be on the rise throughout China, but their precise pathogenesis has remained elusive. UA calculi can be prevented and they are one of the few types of urinary tract stones that can be dissolved successfully. The three main determinants of UA stone formation are low pH, low urine volume and hyperuricosuria. The most significant etiological factor is a low urine pH, as most patients with UA stones have normal UA excretion but invariably demonstrate a persistent low urine pH (37). UA calculi may be associated with insulin resistance, molecular and genetic factors that influence the urinary pH, and UA metabolism (38). The mechanistic association between insulin resistance and low urinary pH is currently elusive; however, it appears to occur secondary to defective ammonia synthesis by proximal tubule cells as well as ammonium transport into the renal tubular lumen (39). Furthermore, UA crystals can be classified into UAA, UAD or a mixture of the two modifications, and UAD easily transforms into the anhydrous salt. UA occurs most frequently in calculi in a pure state and is rarely accompanied by urates, phosphates or CaOx. In the present study, all of the UA stones were pure stones and UA crystallization in calculi was found to occur mostly as UAA, occasionally with an admixture of a small amount of UAD.

The mechanism of formation of these calculi is suspected to be similar to that, giving rise to COM concretions. According to a previous study, UA calculi can be divided into two types, differing in outer appearance and inner structure (40). The first type includes stones with a little core and a compact columnar UAA shell and stones with an interior structure of alternating densely non-columnar layers developed around a core, while the second type includes porous stones without inner structure and stones formed by a well-developed outermost layer with an inner central cavity. Energy-dispersive X-ray spectroscopy is needed to provide full information for identifying the structure and crystalline composition. Future research by our group will aim to apply this technique.

In conclusion, analysis of various layers of upper urinary tract stones using FT-IR provided significant results on stone composition. It was revealed that CaOx and CaP stones showed differences in their individual composition between the core and the surface, while all UA calculi were pure stones. The composition showed marked variation in different layers of the stone, showing that the metabolism has an important role in different phases of the evolution of stones. The separate analysis of the stone nucleus as well as external and internal layers provided novel insight into the pathogenesis of urinary tract stones, and may pave the road for novel therapeutic strategies.

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