Are conventional stone analysis techniques reliable for the identification of 2,8-dihydroxyadenine kidney stones? A case series

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Abstract

Objectives: To examine the accuracy of stone analysis for identification of 2,8-dihydroxyadenine (DHA).

Materials and methods: Patients referred to the APRT Deficiency Research Program of the Rare Kidney Stone Consortium from 2010 to 2018 with a presumptive diagnosis of APRT deficiency based on kidney stone analysis were reviewed retrospectively. Measurement of APRT activity in red blood cells or genetic testing was used to verify the diagnosis. Available infrared spectra of patient stone materials were compared with an attenuated total reflection-Fourier transform infrared (ATR-FTIR) reference spectrum generated in our laboratory by analysis of DHA stone sample from a patient with APRT deficiency that matched closely the spectrum of pure crystalline DHA.

Results: Of 17 patients referred to our program with a presumptive diagnosis of APRT deficiency based on kidney stone analysis, 14 had sufficient data available to be included in the study. Analyses of stones from all of these patients had been performed by ATR-FTIR spectroscopy. In 7 cases, we confirmed the diagnosis of APRT deficiency by demonstrating abnormal APRT enzyme activity and/or pathogenic biallelic *APRT* variants. Our own FTIR analysis of a kidney stone from one of these patients revealed a characteristic DHA spectrum. In the 7 remaining cases, the diagnosis of APRT deficiency was rejected. Stone samples from 6 of these patients were reported to contain DHA, ranging from 12-100% (median of 60%), while trace amounts were found in one sample. However, comparison of the available infrared spectra of stone specimens from three of these cases with the ATR-FTIR reference spectrum of a genuine DHA stone specimen showed that none of the stones contained detectable levels of DHA.

Conclusions: Misidentification of DHA as a kidney stone component using gold standard stone analysis techniques appears common among patients referred to our program. Hence,

current clinical protocols used to interpret infrared spectra for stone analysis cannot be considered diagnostic of APRT deficiency, which must be confirmed by other methods.

Introduction

Adenine phosphoribosyltransferase (APRT) deficiency (OMIM 102600) is a rare inherited disorder of purine metabolism that leads to excessive renal excretion of 2,8-dihydroxyadenine (DHA), resulting in kidney stones, crystal nephropathy and chronic kidney disease (CKD). End-stage kidney disease (ESKD) occurs in 15-20% of adult cases (1, 2). In many cases, APRT deficiency is not diagnosed until after kidney transplantation, often in the setting of allograft failure due to disease recurrence (3, 4).

Radiolucent DHA kidney stones are the most common feature of APRT deficiency, reported in up to 60% of those affected (1, 5) and as many as one-third of patients experience recurrent kidney stones despite pharmacotherapy. Treatment with the oxidoreductase (XOR; xanthine dehydrogenase/oxidase) inhibitors, allopurinol and febuxostat, has been shown to halt the progression of CKD and stone formation in patients with APRT deficiency (2, 5). Early diagnosis of the disorder is a prerequisite for timely institution of pharmacotherapy.

The diagnosis of APRT deficiency is confirmed by identification of biallelic pathogenic variants in the *APRT* gene or abolished enzyme function in red blood cell lysates (6, 7). Analysis of urine crystal or kidney stone material has also been considered diagnostic of the disorder, with the recommended techniques of x-ray diffraction crystallography or infrared spectroscopy (6-10). Currently, Fourier-transform infrared spectroscopy (FTIR) is the most widely used technique in clinical stone analysis laboratories (11).

We have recently encountered cases misidentified as DHA stone formers by infrared spectroscopy. The objective of this study was to examine the accuracy of stone analysis for identification of DHA as a kidney stone component.

Methods

Ethical Approval

The study was approved by the National Bioethics Committee of Iceland (NBC 09-072) and the Icelandic Data Protection Authority. The clinical and research activities reported are consistent with the Principles of the Declaration of Helsinki.

Study Population

This was a retrospective study of all patients referred to the APRT Deficiency Research Program of the Rare Kidney Stone Consortium (RKSC, http://www.rarekidneystones.org/) from 2010 to 2018 with a presumptive diagnosis of the disorder based on kidney stone analysis.

Clinical Data and Diagnostic Testing

The APRT Deficiency Registry was established in 2010 to collect observational data from patients with the disease worldwide. Registry variables included age at presentation, first kidney stone event and number of clinical kidney stone episodes; results of laboratory studies, including serum creatinine (SCr) measurements, urine microscopy, renal imaging studies and kidney stone analysis; and XOR inhibitor treatment. Urinary DHA excretion was measured using an ultra-high performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) assay as previously described (12). APRT enzyme activity measurement and/or DNA mutation analysis, was performed to confirm or rule out the diagnosis of APRT deficiency obtained by kidney stone analysis.

Evaluation of Kidney Stone Spectra

To facilitate the study of individual patient stone analysis results, ATR-FTIR reference spectra were generated in the Glynn Laboratory of Bioenergetics at University College London by one of the authors (PRR). First a reference spectrum of pure crystalline DHA, supplied by Santa Cruz Biotechnology (cat. no. sc-498575), was produced. To ensure its crystalline state and purity, the DHA was solubilised in alkali and recrystallised after neutralisation. ATR-FTIR reference spectra were also generated for solid urea, sodium hydrogen urate, uric acid, hydroxyapatite (Sigma H-0252; calcium hydroxide phosphate, dried at pH 6.8 with 1mM phosphate) and ammonium acid urate. All these chemicals were purchased from Sigma Aldrich Chemical Company except ammonium acid urate which was synthesised by addition of 0.6 mL of 0.2 M ammonium sulphate to 1 mL of 11 mM sodium hydrogen urate at pH 6.5–7 and 80°C. Ammonium acid urate precipitated on cooling to 4°C and was washed with water and dried. The ATR-FTIR spectra were recorded with a Bruker IFS 66/S spectrometer, fitted with a liquid nitrogen-cooled MCT-A detector and a silicon ATR microprism (3 mm diameter; 3 reflections; DuraSampl*IR* II, SensIR/Smith Detection). Frequencies quoted have an accuracy to ±1 cm^{-1}. Sample spectra were recorded *versus* a background spectrum of the clean prism surface. Reference materials were either loaded onto the prism surface as a suspension in several microliters of distilled water, followed by thorough drying with a gentle stream of dry argon gas or placed on the prism as solids and pressed to maximise surface contact.

In addition, an ATR-FTIR spectrum of a stone sample obtained from one of the patients (case 13; Table 1) was recorded The original infrared spectra of kidney stone specimens from three patients (cases 4, 5 and 8; Table 1) were available for examination and these were compared to the recorded DHA spectra.

Statistical Analysis

Descriptive statistics were carried out using SPSS (IBM SPSS Statistics, version 21.0; 2012).

Data are presented as number, percentage, and median (range).

Results

Seventeen patients were referred to the APRT Deficiency Research Program at Landspitali–The National University Hospital in Reykjavik, Iceland, with the presumptive diagnosis of APRT deficiency based on analysis of kidney stone composition demonstrating DHA. Information on the stone analysis method used in 2 patients who subsequently had the diagnosis of APRT deficiency confirmed was not available. In one case, stone analysis carried out by X-ray diffraction indicated DHA as a stone component, but the diagnosis of APRT deficiency was excluded by genetic testing and measurement of APRT activity. The details of stone analysis results were not available. These 3 cases were excluded from further analysis because of incomplete data. In the remaining 14 cases, kidney stone analysis had been carried out using infrared spectroscopy (Table 1). Their median age was 29 years (range, 2–58 years) and 7 were females. The diagnosis of APRT deficiency was confirmed by analysis of APRT enzyme activity and/or genetic testing in 7 out of 14 patients, all of whom had kidney stones composed of 100% DHA. The stone analysis spectra could not be retrieved for any of these 7 cases. However, analysis of a kidney stone sample obtained from Case 13 (Table 1) revealed an FTIR spectrum which corresponded closely to that of the pure crystalline DHA, indicating that it was indeed an essentially pure (100%) DHA stone (Figure 1). This spectrum was used as reference in the evaluation of available infrared spectra.

In 7 cases, the diagnosis of APRT deficiency made by stone analysis was rejected by confirmatory testing. The first case was a 37-year-old woman from the United States who experienced her second kidney stone episode with infrared-based stone analysis reported as revealing 100% DHA. Treatment with allopurinol was initiated. However, no DHA crystals were observed on urine microscopy and analysis of a 24-hour urine collection showed DHA levels below the level of quantification. The diagnosis of APRT deficiency was excluded by

demonstration of normal APRT enzyme function. Subsequently, allopurinol was discontinued.

The second case was referred to the same physician as the second case for evaluation of suspected APRT deficiency based on reported stone composition of 100% DHA. This 58-year-old male with a family history of gout had passed three kidney stones during a 16-month period, the last of which was sent for analysis of stone composition. Metabolic evaluation showed a low urinary pH and high 24-hour urinary uric acid excretion, which is highly inconsistent with APRT deficiency. Sequencing of the *APRT* gene did not disclose pathogenic variants. Repeat stone analysis revealed calcium oxalate as the principal stone constituent.

Two patients were referred from the United Kingdom. The first patient (Case 3) was a 45-year-old female with a history of recurrent kidney stones and high serum uric acid levels who passed an 8 mm stone that was reported to be composed of 64% DHA, 26% calcium oxalate and 10% uric acid using infrared spectroscopy. However, replotting of the fingerprint region of the supplied FTIR transmittance spectrum with the equivalent spectrum of the confirmed DHA stone failed to reveal any features consistent with DHA (Figure 2). The majority of dominant features of the spectrum appeared to most closely resemble urea, suggesting that significant soluble urinary contaminants were present in the analysed sample. The second case (Case 4) was a 28-year-old male with a first kidney stone reported to contain 12% DHA, 69% calcium phosphate and 18% calcium oxalate by infrared analysis. Again, replotting of the fingerprint region of the supplied FTIR transmittance spectrum with the equivalent spectrum of the validated DHA stone failed to reveal any features consistent with DHA (Figure 3), and the majority of the stone composition could instead be assigned to a hydroxyapatite (calcium phosphate hydroxide)-like material plus residual water (Figure 3). Urinary DHA was not detected in either of these patients, while the female patient reportedly

had elevated 24-hour urinary uric acid excretion. Both patients had normal APRT activity and genetic testing did not disclose a pathogenic variant in the *APRT* gene. The results of the stone analysis for the female patient were re-evaluated by the original laboratory, again using FTIR, revealing a purine component that was not DHA.

Two brothers from South Africa, 22 and 26 years of age (cases 5 and 6) were referred to our program after infrared analysis of stone material was reported as 60% and 30% DHA, respectively. Both had unquantifiable urine DHA and the APRT enzyme function was normal. Genetic analysis did not reveal pathogenic variants in the *APRT* gene.

The final patient (Case 7), was a female from Australia who presented with hematuria and multiple bilateral kidney stones at age 11 years. Kidney stone composition, analyzed using infrared spectroscopy, was reported as calcium phosphate with trace amounts of DHA. The patient was placed on treatment with allopurinol and increased fluid intake was recommended. However, the fingerprint region of the supplied FTIR transmission spectrum again showed no correspondence to the spectrum of the DHA stone (Figure 4). Instead, a close match was found to the infrared spectrum of ammonium acid urate (13-15). APRT enzyme activity proved to be normal on two separate occasions. Urine testing was negative for DHA, but metabolic screening performed at the referring institution showed slightly elevated urinary cystine evels, possibly consistent with heterozygous mutation in *SLC7A9*. Treatment with allopurinol was subsequently discontinued.

Discussion

This study shows that 7 out of 14 patients who had analysis of kidney stone composition performed, using infrared spectroscopy, were erroneously diagnosed with APRT deficiency. This finding highlights shortcomings of the clinical infrared protocols for stone analysis techniques in reliably identifying DHA as a stone constituent. It emphasizes the need for confirming the diagnosis either by demonstrating absence of APRT enzyme activity or a pathogenic variant in both alleles of the *APRT* gene. In addition, measurement of urinary DHA excretion appears to be a reliable method for diagnosing APRT deficiency.

Analysis of stone composition is an essential component of the clinical evaluation of nephrolithiasis, providing clues regarding the pathophysiology and facilitating the selection of targeted treatment for prevention of recurrent stone episodes (16, 17). Infrared spectroscopy and X-ray diffraction crystallography are the recommended techniques for analysis of kidney stone composition, assumed to reliably identify all constituents and providing semi-quantitative determination of their proportions (18, 19). X-ray crystallography is generally considered the gold standard method (9, 19). However, Fourier transform infrared spectroscopy (FTIR), first introduced as a stone analysis procedure in 1955 (20) is currently preferred in most laboratories worldwide as the analysis time is short and the cost of the equipment is significantly less than for X-ray crystallography (19, 21).

In FTIR spectroscopy, the sample is irradiated with a broadband infrared beam. Absorption occurs at frequencies associated with specific infrared-active molecular vibrational normal modes, which increase in number as the number of atoms increases. Hence, most molecules of more than a few atoms have characteristic absorption spectra with multiple bands. Infrared absorption spectra of the stone samples can be analyzed by comparison to reference spectra of pure materials to determine the chemical composition of the stone (17). However, infrared spectra in the 'fingerprint' region below 1800 cm^{-1} tend to

be complex, and mixtures of components may have overlapping bands from the different constituents. In general, published works on stone analyses employing infrared spectroscopy have not included details of the reference infrared spectra that were used, and so the robustness of the deconvolution methods cannot be independently verified. A number of additional factors can complicate the analyses. Pure compounds may exist in different ionic, crystalline or hydration states, and these states can result in significant differences in their infrared signatures. Furthermore, the relative intensities of bands of a pure material are different when spectra are recorded in transmission *versus* ATR mode and this must be considered when comparing spectra measured in these modes. In the case of ATR spectra, care must be taken to make sure that material representative of the whole sample is present in the very thin (several microns) infrared-active volume. In many instances, spectra are presented in transmittance form; although useful in highlighting minor bands, transmittance (as opposed to absorbance) band intensities of a pure compound will not remain at a constant ratio to each other when bands are strongly absorbing. When fitting reference spectra to stone spectra, care must also be taken to account for possible artefacts caused by broad baseline drifts by beamline water vapour interference. All of these factors will complicate the accurate deconvolution of component mixtures in dried stone samples, making automated analysis of spectra difficult without additional expert scrutiny of data. Indeed, several reports have highlighted the potential inaccuracies in current automated methods of infrared stone analyses (10, 19, 22), as was clearly the case in the three examples reported here.

Morphologic examination coupled to FTIR is considered an important technique in the diagnosis of rare types of kidney stones, including cystine, DHA, xanthine, struvite, ammonium urate or drug-containing calculi (9, 23). The same technique is useful for analysis of crystal composition when no stones are available, including in kidney biopsies when crystal nephropathy is present, for example due to APRT deficiency.

Wet chemical methods, which were commonly used for analysis of stone composition in the past, are inaccurate and often lead to erroneous diagnosis. Furthermore, these methods fail to identify rare purine stones resulting from genetic disorders, including DHA stones which are confused with uric acid stones (9). Consequently, only physical methods such as X-ray diffraction crystallography and FTIR are currently considered acceptable (24).

The misidentification of kidney stone components as DHA in our study is striking in view of the reported accuracy of FTIR stone analysis. Although FTIR is currently the most widely used stone analysis method, it is limited by the quality of the reference library available and the choice of computer algorithm chosen for matching sample spectral data with the reference library data (10, 19, 24). Indeed, erroneous results are known to occur and are more common when stones contain a mixture of constituents (24, 25). It is noteworthy that the majority of misidentified stone specimens from the patients included in the current study reportedly contained less than 70% DHA. Stones from patients with APRT deficiency are typically composed of pure DHA (26-28), although occasional mixed stones containing calcium salts have been reported (29, 30). Thus, stone analysis reports of mixed stones containing DHA should raise a suspicion of erroneous interpretation. Our findings also raise the concern that true DHA stones could be missed due to erroneous interpretation of the spectra.

The misdiagnosis of the cases reported herein resulted from incorrect assignment of the infrared spectra in most cases. Although, the infrared spectrum of DHA is very specific and the identification of DHA from infrared spectra can be definitive when performed by trained laboratory personnel (9), untrained operators can erroneously attribute spectra of other materials such as uric acid and its salts to DHA. Clinicians caring for patients with rare kidney stone disorders must be familiar with the potential misidentification of DHA stones from infrared spectra and should invariably confirm the diagnosis of APRT deficiency using

APRT enzyme function analysis or sequencing of the *APRT* gene to search for pathogenic mutations affecting both alleles. Indeed, due to increasing availability and markedly reduced cost, genetic testing is becoming a favoured diagnostic method for APRT deficiency. Furthermore, the *APRT* gene should be included in high-throughput next-generation sequencing panels for rare types of CKD and kidney stone disease (31). Finally, our recently described UPLC-MS/MS for measurement of urinary DHA is a promising alternative method for the diagnosis of APRT deficiency (12).

Misdiagnosis of the patients in this case series as having APRT deficiency could have led to lifelong XOR inhibitor therapy. These medications, particularly allopurinol, are associated with adverse effects. Erroneous stone analysis results could also lead to missed cases of APRT deficiency, thereby precluding the institution of appropriate therapy. Incorrect analysis or failure to identify a stone substance may also result in inadequate therapy of other stone types.

In conclusion, false identification of DHA as a kidney stone component using gold standard stone analysis techniques appears to be common. The determination of kidney stone composition is based on interpretation of automated analysis of varying spectra by laboratory technicians, and thus is subject to error. The diagnosis of APRT deficiency should always be confirmed by enzyme activity measurement, genetic testing or detection of DHA in urine samples.

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Legends to figures

Figure 1. Comparison of the fingerprint region of FTIR spectra of a DHA stone and pure crystalline DHA. Spectra were recorded in ATR mode and are plotted as transmittance spectra. All have been scaled to roughly the same signal intensities for ease of comparison. The stone sample was from a confirmed APRT deficiency patient. Wavenumbers of features of the stone spectrum that closely match those of crystalline DHA are labelled. Abbreviations: DHA, 2,8-dihydroxyadenine; FTIR, Fourier transform infrared.

Figure 2. Comparison of the fingerprint region of FTIR spectrum from case 4 with reference compounds. Spectra were recorded in ATR mode and are plotted as transmittance spectra. All have been scaled to roughly the same signal intensities for ease of comparison. Reference spectra are of the DHA stone and crystalline urea. Abbreviations: ATR, attenuated total reflection; DHA, 2,8-dihydroxyadenine; FTIR, Fourier transform infrared.

Figure 3. Comparison of the fingerprint region of FTIR spectrum from case 5 with reference compounds. Spectra were recorded in ATR mode and are plotted as transmittance spectra. All have been scaled to roughly the same signal intensities for ease of comparison. Reference spectra are of the DHA stone, hydroxyapatite (calcium phosphate hydroxide) and liquid water. Abbreviations: ATR, attenuated total reflection; DHA, 2,8-dihydroxyadenine; FTIR, Fourier transform infrared.

Figure 4. Comparison of the fingerprint region of FTIR spectrum from case 8 with reference compounds. Spectra were recorded in ATR mode and are plotted as transmittance spectra. All have been scaled to roughly the same signal intensities for ease of comparison. Reference spectra are of the DHA stone, ammonium acid urate, sodium hydrogen urate and

uric acid. Abbreviations: ATR, attenuated total reflection; DHA, 2,8-dihydroxyadenine; FTIR, Fourier transform infrared.

Table 1. Clinical characteristics of patients with (FTIR) stone analysis

Case	Sex	Country	Age at stone analysis (years)	Stone events (number)	SCr at last follow-up (µmol/L)	Stone analysis method	Proportion of DHA in stone material	Urinary DHA/Cr ratio (mg/mmol)	Genetic testing	APRT enzyme analysis
1	Female	US	34	1	NA	FTIR	100%	BLQ	NA	Normal
2	Male	US	58	4	88	FTIR	100%	NA	Normal	NA
3	Male	UK	28	1	70	FTIR	12%	BLQ	NA	Normal
4	Female	UK	45	1	95	FTIR	64%	BLQ	Benign variant	NA
5	Male	S-Africa	22	1	88	FTIR	60%	BLQ	Normal	Normal
6	Male	S-Africa	26	1	80	FTIR	30%	BLQ	Normal	Normal
7	Female	Australia	11	NA	NA	FTIR	Trace	BLQ	Normal	Normal
8	Male	US	37	5	153	FTIR	100%	NA	Pathogenic biallelic variants	No enzyme function
9	Female	US	22	7	106	FTIR	100%	14.9	Pathogenic biallelic variants	NA
10	Female	US	47	6	72	FTIR	100%	NA	Pathogenic biallelic variants	No enzyme function
11	Male	US	30	2	71	FTIR	100%	BLQ*	NA	No enzyme function
12	Female	India	2	2	35	FTIR	100%	26.3	Pathogenic biallelic variants	No enzyme function
13	Male	Italy	2	2	49	FTIR	100%	2.5*	Pathogenic biallelic variants	No enzyme function
14	Female	UK	55	4	69	FTIR	100%	37.2	Pathogenic biallelic variants	NA

*On treatment with an XOR inhibitor. Abbreviations: BLQ, below level of quantification; FTIR, Fourier transform infrared; SCr, serum creatinine; NA, not available.

Figure 1

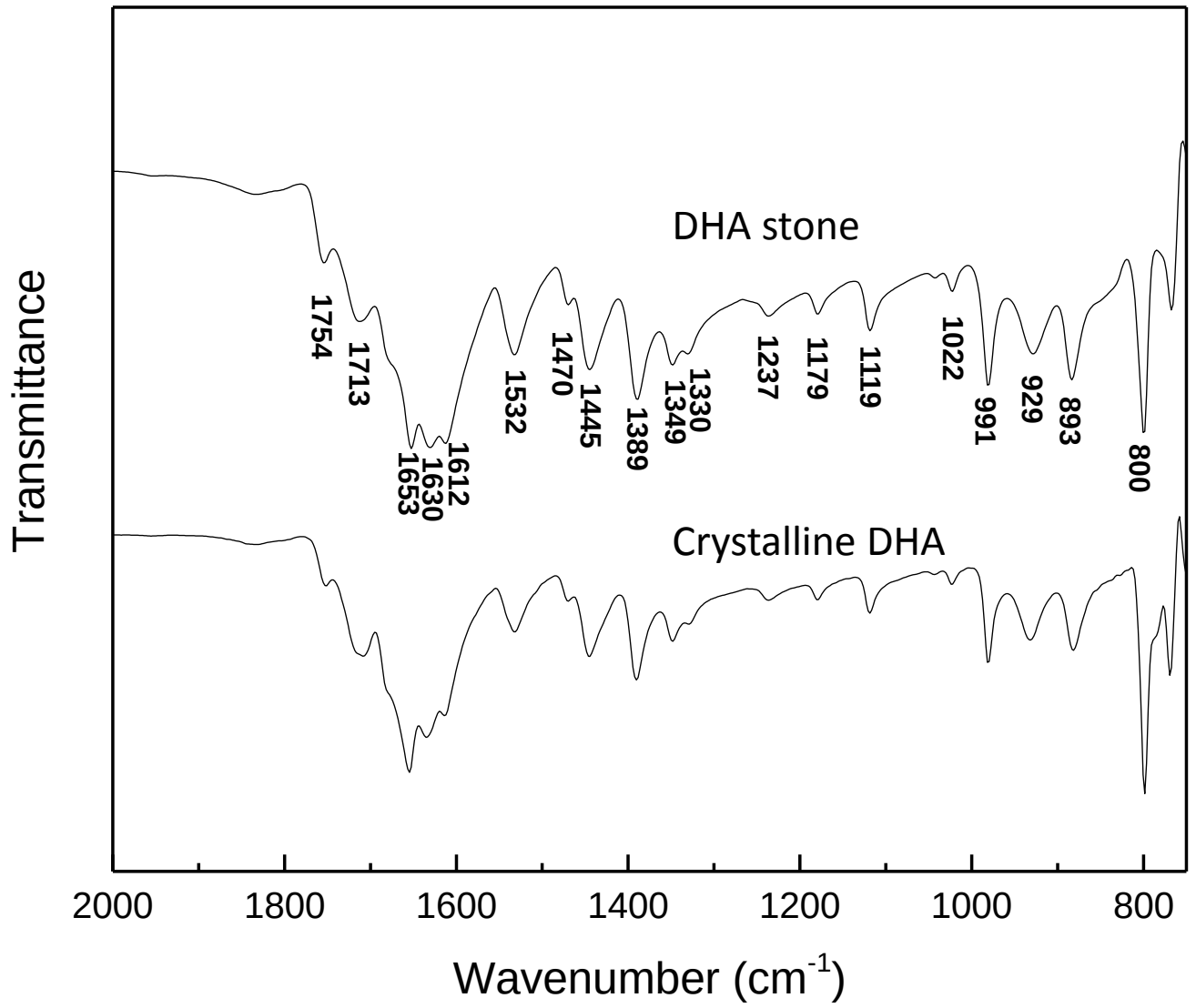

DHA stone
Crystalline DHA
Transmittance
1754
1713
1653
1630
1612
1532
1470
1445
1389
1349
1330
1237
1179
1119
1022
991
929
893
800
2000
1800
1600
1400
1200
1000
800
Wavenumber (cm-1)

Figure 2

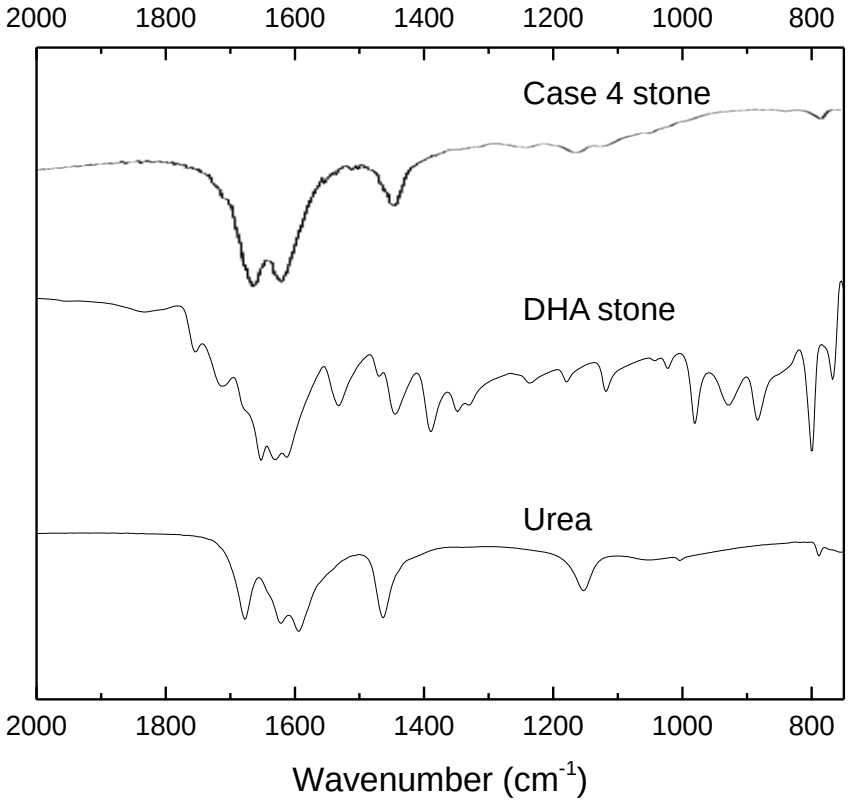

2000
1800
1600
1400
1200
1000
800
Case 4 stone
DHA stone
Urea
2000
1800
1600
1400
1200
1000
800
Wavenumber (cm-1)

Figure 3

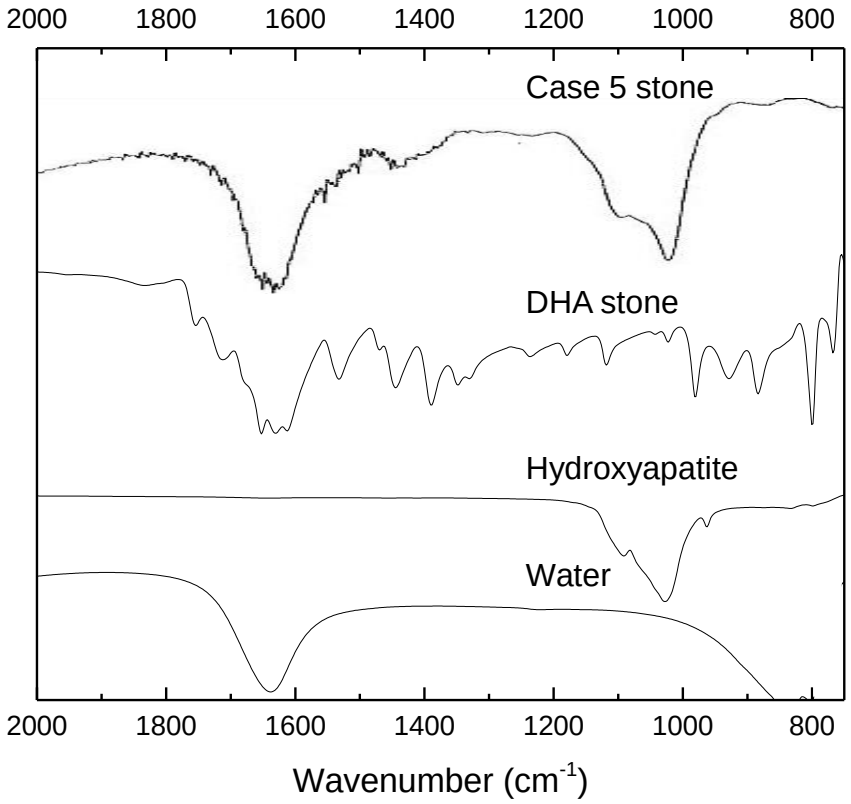
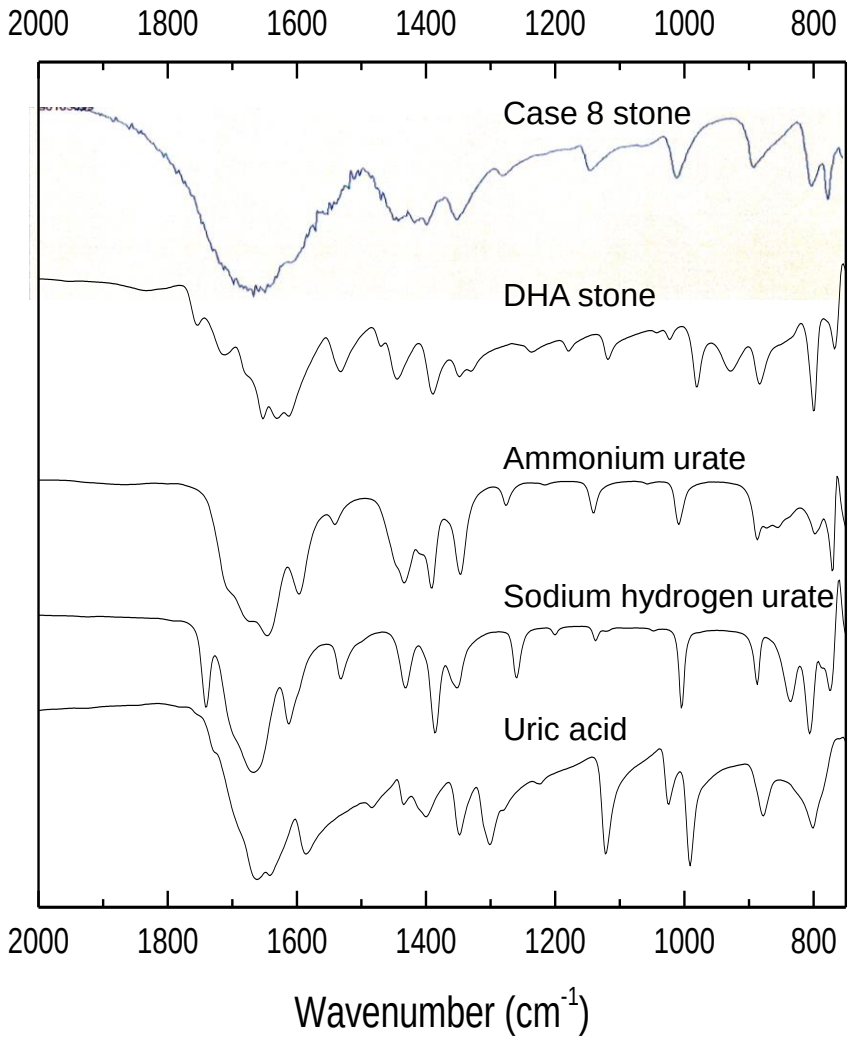

2000
1800
1600
1400
1200
1000
800
Case 5 stone
DHA stone
Hydroxyapatite
Water
2000
1800
1600
1400
1200
1000
800
Wavenumber (cm-1)

Figure 4


2000
1800
1600
1400
1200
1000
800
Case 8 stone
DHA stone
Ammonium urate
Sodium hydrogen urate
Uric acid
2000
1800
1600
1400
1200
1000
800
Wavenumber (cm-1)