A quantitative study on the chemical composition of renal stones and their fluoride content from Anantapur District, Andhra Pradesh, India

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The present study was designed to investigate and arrive at the quantitative chemical composition, inclusive of fluoride content, of 56 kidney stone (renal calculi) samples from 50 patients aged between 20 and 60 years, admitted to Sri Sathyai Sai Institute of Higher Medical Sciences Hospital, Prasanthigram, Anantapur District, Andhra Pradesh. Analysis of the calculi was performed using Fourier Transform Infrared vibrational spectroscopic method along with Differential Scanning Calorimetry and the Fluoride Ion Selective Electrode. These are the preferred instrumental techniques with the inherent advantages of providing fast, sensitive, accurate and detailed data about the stone composition, enabling more reliable and efficient post-operative care of individual patients. The results indicate that calcium oxalate monohydrate (whewellite) and dihydrate (wedellite) stones are predominant in this region of Anantapur, with the binary combination of the two accounting for 90% of the stone composition in nearly 85% of the stones, with traces of carbonate–hydroxyapatite (dahlrite) or uric acid being the ternary component. In the remaining 15% of the stones, uric acid was the major component. The mean fluoride content in renal calculi was determined to be 2.14 mg/g.

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A quantitative understanding of the exact chemical composition of the urinary calculi is of importance not only because of its relationship with dietary and other health factors, but also in prevention of nephrolithiasis.

Keywords: Chemical composition, fluoride, nephrolithiasis, renal calculi.

Nephrolithiasis, the formation of stones in the urogenital system has afflicted humans since antiquity, with the earliest recorded example1 being an urinary calculus found in the pelvic area of a young man in a tomb near El Amrah (Egypt) dating back to 4800 BC. Stone disease in man is as old as mankind. This common disorder of renal stone formation affects up to 15% of the population over a lifetime2, and without proper treatment and preventive measures, approximately 75% of these patients will have at least one recurrence. An extrapolated incidence statistics of 3,915,700 kidney stones has been estimated from a population of 1,065,070,607 persons in India3, an indication of its high prevalence. The diagnostic usefulness in knowing the chemical composition of renal stones has been recognized since 1950s with significant improvement in therapeutic regimen since then, as exact determination of the compositional structure enables the classification of the disease and its subsequent treatment. In addition, the definite role of fluoride in renal stone formation has been arrived at4 after a lot of controversy5.

Anantapur District (lat. 13°14’–15°14’N and long. 76°47’–78°26’E), located in southwestern corner of the Andhra Pradesh (AP) in South India, is the most backward and drought-prone area of Rayalaseema. It is one of the poorest districts in the country, with a population of about 4 million, of which 3 million inhabitants depend on groundwater as a major source of water supply. To add to this plight, the district lies at a twin cross-section of the renal stone belt and fluoride belt of India. High levels of fluoride, beyond 3.0 mg/l, in groundwater as a source of drinking water is common in many of the 964 revenue villages included in 63 mandals of this district6. Fluoride-bearing minerals like apatite, micas and clay minerals present in the country rocks like shales and pyroclastic materials of the study area are the main source of fluoride ion (F−) in groundwater. This is concluded from the positive correlation between fluoride content in the bulk rock and F− content in the nearby groundwater, and also between fluoride content in the soil leachates and F− in the nearby surface water of the district. A good positive correlation between fluoride and lithogenic sodium is obtained, reflecting that weathering activity caused by a semi-arid climate and intensive irrigation practices are responsible for the leaching of F− from mineralogical sources. Hence the fluoride-rich groundwater, used extensively as drinking water in the Anantapur region, causes permanent tooth and skeletal fluorosis due to high body fluoride in the population7. Scanty rainfall, a near-arid dehydrating envi-
ronment and a sliding water table with high incidence of fluoride provide ideal conditions for the high prevalence of nephrolithiasis in Anantapur District. As no detailed literature is available on the chemical composition of renal stones from this region, that would help design an appropriate post-operative treatment to prevent stone recurrence, the current study was undertaken to arrive at a quantitative picture of the renal stone composition, including that of fluoride, with trace amount of the sample in milligram quantity being required for analysis.

Symptomatic renal stones retrieved by surgical operation of 50 patients aged between 20 and 60 years, admitted to the Sri Sathyai Sai Institute of Higher Medical Sciences (S.S.S.I.H.M.S) Hospital, Prasanthigaram during January–December 2005, were collected for this study. For renal calculi of size between 1 and 2.5 cm, Extracorporeal Shock Wave Lithotripsy (ESWL) was the noninvasive method for treating urinary stones, by generating shock waves outside the body and focusing them on the stones. During the treatment, stones were fragmented into pieces and rendered small enough to be excreted. An endoscopic percutaneous nephrolithotomy (PCNL) was used to crush moderate to large stones, with the use of optical instruments introduced into the kidneys through a stab wound in the loin. First time stone formers, with no structural abnormality in the urinary tract, no concurrent infection and no hereditary predisposition to stone formation were the inclusion criteria used for patient recruitment in this study.

The quantitative chemical composition of the pre-treated stone sample was then arrived at using FTIR vibrational spectroscopic technique along with differential scanning calorimetry (DSC) and the fluoride ion selective electrode (FISE). A combination of these three techniques provides fast, sensitive, accurate and detailed data about the stone composition, enabling more reliable and efficient treatment of individual patients during post-operative care to prevent recurrence of stones.

The renal stones were washed with distilled water, dried in an oven for 2 hr at 100°C, then powdered in a pestle and mortar and used for quantitative chemical analysis.

The stone samples for FTIR analysis were prepared using the KBr pellet technique, where 0.5 mg of the pre-treated stone powder was mixed with 50 mg of analytical grade KBr using an agate pestle and mortar. The homogenized mixture was then prepared as moisture-free, transparent KBr pellet at 6 × 10^5 Pa pressure for 2 min. The digital spectrum was then scanned in the mid-infrared region from 4000 to 400 cm^-1 (with resolution of 4 cm^-1) at room temperature with 64 scans after the background correction in a Nicolet Avatar-360 spectrometer equipped with a KBr beam splitter, dtGS/KBr detector and an OMNIC 7.0 software (from Thermo Electron Corporation).

The quantitative composition of each stone sample was estimated by comparison of the recorded spectra with the KBr reference spectra that were stored in a computer library (Kidney Stone Library – Basic, NICODOM). This spectral library contains data of pure components of urinary calculi and their binary and ternary combinations, providing a huge database with 1115 FTIR spectra. The library was then used as the standard spectral library with OMNIC search to identify the major components of an unknown stone. After obtaining the final composition of the renal stone from the library search, the result was verified with standard qualitative wet chemical analysis to confirm the presence or absence of carbonate, calcium oxalate, non-oxalate calcium, phosphate, uric acid and ammonium ion.

DSC of the pretreated stone powder was performed on the Universal V2.6D TA instruments, DSC 2010 module. The stone sample was placed in a crimped but vented aluminium sample pan for the DSC experiment. Sample size was 3–5 mg and in each case was heated from 30 to 500°C at a rate of 10°C/min. The samples were purged with a flow of dry nitrogen at 150 ml/min for the DSC run.

Fluoride ion concentration in renal stone samples was measured with FISE in combination with a single-junction silver/silver chloride reference electrode by a potentiometric method using an Orion potentiometer, Model SA 720. This method requires the use of a calibration curve prepared with standardized solutions and provides for fluoride determination in the range 0.01–100 mg/l. The renal stone solution for fluoride determination was prepared according to the modification of a previously published procedure, where 40 mg of the pretreated stone powder was taken in 2 ml of 0.5 N HCl and the final volume was made up to 10 ml with distilled water. The tubes were then kept in a boiling water bath for 1 hr and left undisturbed overnight. The supernatant solution was then collected and used for quantitative fluoride determination using FISE, for which the pH of the solution was adjusted to 5.0–5.5 using total ionic strength adjustment buffer (TISAB II with (CDTA) trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid monohydrate).

The TISAB II solution was prepared by addition of 57 ml glacial acetic acid, 58 g NaCl and 4 g CDTA into a 1000 ml beaker, containing approximately 500 ml of distilled water and stirred to dissolve. The beaker was then placed in a water bath for cooling and 5 N NaOH solution was added slowly until the pH was between 5.0 and 5.5. When the solution reached room temperature it was poured into a 1000 ml volumetric flask and made up to the mark with distilled water. The buffer not only assures constant ionic strength, but also ensures that complexing cations like Al(III) and Fe(III) do not cause interference in fluoride measurement.

In our investigation, the quantitative picture of the composition of 56 kidney stone samples from 50 patients was arrived at using FTIR technique. Detailed literature survey revealed that using FTIR analysis, the renal calculi have been divided into several groups depending on
Table 1. Quantitative composition of renal calculi. The number of stone samples is given in parenthesis

<table>
<thead>
<tr>
<th>Biochemical parameters</th>
<th>Mean content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxalate (g/100 g)</td>
<td>39.2 (52)</td>
</tr>
<tr>
<td>Calcium (g/100 g)</td>
<td>24.4 (52)</td>
</tr>
<tr>
<td>Phosphate (g/100 g)</td>
<td>12.6 (47)</td>
</tr>
<tr>
<td>Carbonate (g/100 g)</td>
<td>2.6 (47)</td>
</tr>
<tr>
<td>Uric acid (g/100 g)</td>
<td>62.0 (10)</td>
</tr>
<tr>
<td>Ammonium (g/100 g)</td>
<td>2.7 (5)</td>
</tr>
<tr>
<td>Fluoride (mg/g)</td>
<td>2.1 (56)</td>
</tr>
</tbody>
</table>

*Fifty-six kidney stones from 50 patients were analysed in this study.

Their chemical composition. The current study showed that there are two different groups with the calcium oxalate (monohydrate and dihydrate) stones being predominant in this region of Anantapur, as in other parts of India. The binary combination of wheewellite (Figure 1a) and wedellite accounted for 90% of the stone composition in nearly 85% of the stones, with traces of dahllite or uric acid being the ternary component. This is in agreement with the result obtained in the analysis of 1050 upper urinary tract calculi from northern India, where 977 (93.04%) of the renal calculi were calcium oxalate stones, of which wheewellite predominates with 782 (80.00%) over wedellite with 195 (20%) 11.

The quantitative study of the remaining 15% of stones from this region revealed uric acid (Figure 1b) as the major component, with traces of dahllite, uric acid dihydrate, ammonium urate, ammonium hydrogen urate, wedellite or wheewellite. Table 1 summarizes the mean content of each of the components present in the kidney stone samples. One of the unusual and rare findings in this study was a renal stone of silicon dioxide (alpha-quartz) chemical composition (Figure 1c), as confirmed in a previous study 13, which is responsible for piezoelectric behaviour (electrical and dielectrical properties) of such materials. Further, on studying the cross-section of certain renal stones with different layers, an understanding into the already well-known mechanism of calculi formation was attained. An increased urinary supersaturation with calcium salts and oxalates resulted in crystallization of wheewellite and/or wedellite on the nidus of dead epithelial cells, followed by traces of dahllite or uric acid. The details available from the literature also reveal that the rate of precipitation of calcium oxalate monohydrate is linearly dependent on uric acid, as the calculi growth is mediated through a surface diffusion controlled mechanism 14. A tentative medical interpretation has also shown that such calculi with different layers consisting of a wheewellite kernel, uric acid inner layer and a wedellite outer layer, originated in the kidney and passed to the bladder, where the final growth occurred 15.

Confirmation on the presence of oxalate, uric acid and/ or hydroxyapatite groups in the urinary calculi was arrived at from studying the IR absorptions ($\nu_{\text{max}}$) of the sample. The IR spectrum of pure synthethic calcium oxalate monohydrate is characterized by strong bands at 1622 and 1315 cm$^{-1}$ that correspond to the asymmetric and symmetric C–O stretching respectively, of coordinated oxalate groups 14. The symmetric and asymmetric stretching above 3000 cm$^{-1}$, shown by the broad spectrum of peaks, are by the water molecules coordinated with calcium oxalate and the two peaks between 850 and 950 cm$^{-1}$ are
characteristic of whewellite\textsuperscript{15}. In comparison to this, the
calciuli have oxalate stretching frequency at 1619 and
1316 cm\textsuperscript{-1} (C–O) and stretching bands above 3000 cm\textsuperscript{-1}
due to the water of crystallization. The two peaks at 884
and 950 cm\textsuperscript{-1} (Figure 1\textsuperscript{a}) confirm the renal stone as an
oxalate, with whewellite in predominance. The purine
ring vibrations account for the many bands seen in the IR
spectrum of pure uric acid substrate\textsuperscript{11}. The absorption
bands at 3139, 3011 and 2819 cm\textsuperscript{-1} are due to the N–H
stretching vibrations and there are additional bands at the
frequencies 1360, 1130, 1020 and 880 cm\textsuperscript{-1}. The N–H
stretching vibrations in the renal stone (Figure 1\textsuperscript{b}) appear
at 3142, 3015 and 2826 cm\textsuperscript{-1}, and there are IR absorptions
at 1349, 1123, 1026 and 877 cm\textsuperscript{-1}, confirming the presen-
ce of uric acid in the calculi.

Calcium phosphate in the mineralogical form of
hydroxyapatite has stretching frequencies at 568, 603, 985,
1037 and 1128 cm\textsuperscript{-1} with a strong and broad absorption
between 1100 and 1000 cm\textsuperscript{-1} that characterizes the phos-
phate ion\textsuperscript{12}. In contrast, the mixed kidney stone with dahlite
has its vibrational modes at 567, 600 and 985 cm\textsuperscript{-1} with a
strong and broad absorption with a maximum at 1099 cm\textsuperscript{-1}.
The IR spectrum of silicon dioxide of a renal calculus\textsuperscript{16}
has a weak absorption at 975 cm\textsuperscript{-1} and a strong band at
1080 cm\textsuperscript{-1}.

The same absorption bands were obtained in our study
(Figure 1\textsuperscript{c}). The DSC of pure calcium oxalate monohy-
drate\textsuperscript{9} has an endothermic peak at 230°C with a range
from 140°C to 250°C. DSC run (Figure 2\textsuperscript{a}) shows the
sample peak at 228.06°C. The same sample was identi-
fied as a whewellite stone from the FTIR data, in conso-
nance with the DSC, establishing the chemical composi-
tion of the renal stone sample. In Figure 2\textsuperscript{b}, the DSC of the
oxalate stone (as revealed by FTIR) composed of both
whewellite and wedellite, shows three different peaks.
The first at 94.86°C, corresponds to the loss of the loosely
bound water of crystallization of the dihydrate (wedellite)
to yield the monohydrate (whewellite) in the tempera-
ture range 90–160°C.

The water of crystallization of the monohydrate in the
crystal lattice is removed at a relatively higher temperature
around 170–300°C to produce the second peak at 180.03°C.
The third peak at 229.51°C, is the endothermic peak of a
stone containing both whewellite and wedellite, indicating
maximum dehydration at 230°C. This peak shows over-
lapping of two dehydration processes: dehydration of
wedellite to whewellite which is converted into anhydrous
calcium oxalate\textsuperscript{9}. A predominantly oxalate renal stone
with dahlite as the ternary component is encountered in
Figure 2\textsuperscript{c}, with three peaks at 199.46°C, 382.65°C and
433.80°C. The peak at 199.46°C results from the loss of
the water of crystallization from calcium oxalate mono-
hydrate. An exothermic peak at 433.80°C is due to the
decomposition of anhydrous calcium oxalate with the
evolution of CO and cracking of the remaining products
in the organic matter. The presence of calcium phos-
phate\textsuperscript{9}, dahlite is indicated by an endothermic peak at
382.65°C. Hence DSC and FTIR techniques complement
each other in arriving at an accurate quantitative under-
standing of the chemical composition of kidney stones.

The electrochemical measurement with the lanthanum
trifluoride crystal electrode or FISE is a sensitive and
selective tool for the determination of fluoride ion ac-
activity. The dynamic response of the FISE results from four
processes, namely ion diffusion, reaction, LaF\textsubscript{3} dissolution
and calibration drift\textsuperscript{17}. This method has been ex-
ploded to study the fluoride ion concentration in our renal

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{DSC curves of kidney stones from Anantapur District of
India.}
\end{figure}
calculi. The sensitivity, accuracy and reproducibility of the procedure was assessed by (a) spiking of test samples (urinary calculi and their pure primary components) with four different aqueous sodium fluoride standards (0.1, 1.0, 10.0 100.0 mg/l) inclusive of the blank sample, (b) determining the fluoride ion contribution of the primary components of urinary calculi such as calcium oxalate monohydrate, di-calcium phosphate and calcium carbonate, (c) replication of samples having extreme mg/l fluoride content values. The mean fluoride content of renal calculi was determined to be 2.14 mg/l in our study. The amount of fluoride in stones is mainly dependent on the geographic area under study, Anantapur District in this case, with a fluorois menace problem at large. An earlier study\textsuperscript{18} has revealed that among all the geological formations, the groundwater in younger granites has a very high incidence of fluoride. The fluoride content in 87.5% of the samples was more than 1.5 mg/l, out of which 57% of the samples had >3 mg/l.

In case of a stone with different layers consisting of a whewellite kernel, uric acid inner layer and a wedellite outer layer, the fluoride concentration increased from the kernel to the outer layer. The fluoride concentration was 0.04 mg/g in the kernel, became 1.23 mg/g in the inner layer and increased to a maximum of 5.39 mg/g in the outer layer. This can be better understood by introducing the concept of adsorption. Initially when the foundation of the renal stone is laid, the size and in turn the surface area of the stone are small, but as the stone grows in size, the surface area for fluoride adsorption from the surrounding urine increases and so does adsorbed fluoride concentration, as found in the study. Further, the contact of the kernel and the inner layer are cut-off on formation of the outer stone layer, which alone is exposed to the urinary fluoride now and hence has maximum fluoride uptake.

Food habits and dietary constituents are of paramount importance and concern in the management of nephrolithiasis, as they influence the biochemical parameters such as oxalate, uric acid and calcium\textsuperscript{15}. The main diet of people in the Anantapur region constitutes spinach, groundnuts, tomatoes, animal proteins and fluoride-rich, hard groundwater (carbonate hardness of 912 mg/l as against the permissible limit of 600 mg/l)\textsuperscript{25}. Consumption of these food items rich in oxalate content, enhances the risk of renal stone formation by an increase in the increase in uric oxalate excretion is brought about by spinach with a mean oxalate content\textsuperscript{21} of 774 mg/100 g, peanuts with 187 mg/100 g, and tomatoes with 6.5 mg/100 g. Uric acid, a by-product of purine metabolism, is excreted on excess dietary intake of animal protein, resulting in supersaturation of uric acid in urine and in turn uric acid stones. High dietary protein is also associated with increased urinary calcium. Thus, there is a link between meat consumption and both uric acid and calcium stone formation. In fact, in vegetarians stones form at one-third the rate of those eating a mixed diet\textsuperscript{22}. Further, the intake of hard water is associated with a higher risk of recurrence of calcium stones. Hard water is associated with a significant increase in urinary calcium concentration in the absence of changes of oxalate excretion. The calcium-citrate index revealed a significant threefold increase during ingestion of hard water compared to soft water\textsuperscript{23}.

Information gathered from the literature\textsuperscript{24} on the overall mechanismic pathway for renal stone formation can be presented in the light of our study, based on the patient’s diet. An aggregation of calcium oxalate crystals present in urine by van der Waals forces results in viscous binding of crystals by macromolecules. Chemisorption of these macromolecules, such as glycosaminoglycans, Tamm Horsfall proteins or nephrocalcin causes the electropositive charge on these crystal surfaces to become negative. This leads to the further accumulation of Ca(II) on these particles with negative surface potential. High urinary oxalate concentration working together with this phenomenon creates an ideal condition for the formation and aggregation of new calcium oxalate crystals\textsuperscript{25}. The presence of high urinary fluoride concentration may then increase oxaluria indirectly, by enhancing oxalate absorption from the intestine due to low availability of calcium, as part of the intestinal calcium is precipitated as calcium fluoride\textsuperscript{25}. In addition, the presence of high uric acid and/ or hydroxyapatite concentrations provide a competitive environment for crystallization and sedimentation, leading to stone formation.

A low-calcium and low-oxalate diet, accompanied with an intake of large amounts of fluoride-free water, especially in the near-arid summer environment at Anantapur can dramatically reduce the risk of making more kidney stones, by reducing urinary calcium and oxalate concentrations. Protein from plant sources (beans and legumes) can be substituted as a dietary alternative to animal protein without negative consequences. Alkali therapy too can be invoked in the case of calcium stone metaphylaxis\textsuperscript{24}. Thus knowing the exact chemical composition of the urinary calculi is of great importance not only because of its relationship with dietary and other health factors, but also in the prevention of recurrent urolithiasis.

Diversity of nifH gene amplified from rhizosphere soil DNA

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Rhizosphere soils of chickpea (Cicer arietinum) and wheat (Triticum aestivum) were analysed for the diversity of diazotrophic microorganisms by sequencing the nifH gene. A clone library of soil DNA amplified using highly degenerated nifH primers targeting the prokaryotic nitrogenase was constructed and subsequent RFLP analysis of nifH gene from clone libraries identified 28 and 25 genotypes in chickpea and wheat rhizosphere respectively. A total of 41 nifH genotypes were identified, out of which 12 were common in both rhizosphere soils. Sequencing of 41 nifH genotypes showed similarity to the corresponding genera of diazotrophs belonging to alpha, beta, gamma and delta proteobacteria. The prominent diazotrophs were Azospirillum brasilense, Bradyrhizobium spp., Azoto bacter chroococcum, Methyllococcus capsulatus, M. sylvestris, Sinorhizobium mellotii and Burkholderia vietnemensis. A. brasilense was the dominant diazotroph present in the both rhizosphere soils. S. mellotii was only present in the chickpea rhizosphere, while B. vietnemensis and Azorarcus toluateus were observed only in the wheat rhizosphere. The chickpea and wheat rhizospheres were diverse in diazotrophic bacterial community, as indicated by the occurrence of the nifH genotypes.

Keywords: Arid zone soils, diazotrophs, genetic diversity, nifH gene, soil DNA.

Nitrogenous fertilizers are one of the most widely used chemical fertilizers, as deficiency of nitrogen in the soil often limits crop yields. Consumption of nitrogen fertilizer

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