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A Multiple Technique Approach to the Analysis of Urinary Calculi

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Summary. 10 urinary calculi have been qualitatively and quantitatively analysed using X-ray diffraction, infra-red, scanning electron microscopy, X-ray fluorescence, atomic absorption and density gradient procedures. Constituents and compositional features which often go undetected due to limitations in the particular analytical procedure being used, have been identified and a detailed picture of each stone's composition and structure has been obtained. In all cases at least two components were detected suggesting that the multiple technique approach might cast some doubt as to the existence of "pure" stones. Evidence for a continuous, non-sequential deposition mechanism has been detected. In addition, the usefulness of each technique in the analysis of urinary stones has been assessed and the multiple technique approach has been evaluated as a whole.

Key words: Urinary calculi, Analysis, Physico-chemical techniques.

Introduction

Several techniques have been used for the routine analysis of human urinary calculi. These include chemical methods [6, 25], infra-red spectroscopy [2, 12, 23], X-ray powder diffraction [4, 5, 8, 9], and more recently, scanning electron microscopy [18–20]. While each of these has a certain usefulness, they all have significant inherent limitations and shortcomings. Chemical investigation in general is unsatisfactory because the method is destructive and confusion exists as to the exact nature of the reactions which take place in qualitative tests and because only atoms or groups of atoms rather than compounds may be identified. Infra-red studies are limited to minute representative pieces of the stone and cannot easily distinguish between the various phosphates that occur in urinary calculi. Furthermore, the powdering of a stone sample for infra-red analysis is undesirable since certain constituents change composition when subjected to grinding [22]. Unlike infra-red, X-ray powder diffraction procedures can readily distinguish the different urates, oxalates and phosphates with a certainty that cannot be equalled or attempted by any other technique. Nevertheless, here too certain shortcomings have been described [21]. In calculi of mixed composition for example, one component may mask the presence of another if the latter's concentration is less than ~10%–20% of the sample. Apatite in general is awkward to identify by X-ray diffraction and no distinction between the two commonly occurring types (hydroxyl and carbonate) can be made using this technique. As with the preparation of samples for infra-red investigation, powdering is undesirable. Scanning electron microscopy coupled with X-ray micro-analysis, while being a most useful tool for the investigation of urinary calculi, is limited in that micro-analysis of elements lighter than Na cannot be easily accomplished. For accurate quantitative determinations, ground and polished surfaces are necessary, again introducing the risk of compositional changes during sample preparation.

All of the above techniques are thus *individually* inadequate for providing an intimate picture of stone composition and structure. In an attempt to acquire such data we employed a *multiple* technique approach utilising several of the well-established routine procedures mentioned above. In addition we investigated and developed other techniques and incorporated these, where suitable, in the analyses. In such an approach, the effects of the inherent limitations and shortcomings of some procedures are minimised by the inherent advantages of others, yielding structural and compositional data which might prove useful for understanding the physico-chemical factors governing stone initiation and growth.

This paper presents an evaluation of such an approach.

Materials and Methods

10 urinary calculi were washed in distilled water, air dried, weighed, photographed and cut with a low speed cutting saw. Representative pieces for analysis were selected from each stone.

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X-ray Powder Diffraction (XRD)

Diffraction patterns were recorded on KODAK NS-392T film using a PHILIPS powder camera of radius 28.65 mm, mounted on a PHILIPS 1008 X-ray generator with nickel filtered CuK_α radiation. The procedure is described elsewhere [13].

X-ray Fluorescence Spectrometry (XRF)

Stone specimens were prepared by drying (100 °C) and ashing (1,000 °C) as described by Norrish and Hutton [11]. The sample (0.14 g) was added to 1.5 g Johnson Matthey Spectroflux 105 (lithium tetraborate, lithium carbonate and lanthanum oxide [11]), sodium nitrate (0.02 g) and silicon dioxide (0.14 g). This preparation was melted at 930 °C and a 30 mm diameter fusion disc was prepared. The disc was then positioned in a computer controlled SIEMENS SRS 1 automatic X-ray flucrescence spectrometer, the operating conditions for which have been described by Willis et al. [26]. International rock standards were used for calibration.

Scanning Electron Microscopy (SEM)

Stone or stone fragments were fractured with a sharp blade to expose a cleaved surface prior to examination in the scanning electron microscope. Samples were mounted on aluminium stubs and were coated with approximately 80 nm of carbon at a pressure of about 1.3 mPa in a Balzer's vacuum coater equipped with a planetary sample rotator. Specimens tilted at 35° to the collector were examined using a Cambridge S180 Scanning Electron Microscope operating in the secondary electron collection mode at a nominal beam potential of 20 kV and beam current of 500 μA. Condenser lenses 1 and 2 were each excited at 0.9 A; lens 3 (the objective lens) was operated with a 200 μm final aperture and a working distance of approximately 13 mm. Typically, images were recorded on Ilford FP4 roll film at 120 second frame period and 800 lines per frame on a carefully focussed camera. The scanning electron microscope was equiped with an energy dispersive X-ray analyser system which was used for the qualitative determination of Ca, Mg and P.

Density Gradient Analysis

A detailed description of the application of this technique in the analysis of urinary calculi appears elsewhere [14]. Initially a column of carbon tetrachloride (ρ = 1.60) and methylene iodide (ρ = 3.32 kg/dm^3) was constructed as this covers the entire density range of commonly occurring urinary calculus constituents. Subsequently a column of carbon tetrachloride and tetrabromoethane (ρ = 2.96 kg/dm^3) was used for more sensitive determinations. The columns were calibrated by allowing immiscible solutions or crystals of known density to descend through the gradient until their equilibrium positions were reached. A small quantity of dried stone sample was ground to a fine powder and introduced into the column. By reference to the calibration curve the sample density was determined and the percentage composition calculated using the appropriate equation [14].

Infra-red Spectroscopy (IR)

Mull preparation of samples using nujol and hexachlorobutadiene proved to be undesirable due to solvent absorption bands obscuring sample bands. The KBr disc method was used instead: 10 mg of sample were added to 90 mg of spectroscopic grade KBr and placed in a 12 mm diameter die (Perkin Elmer) in which a disc was pressed at 10.0 kg/cm^{-2} pressure. The disc was placed in a holder and spectra were recorded on a Perkin Elmer 180 grating infra-red spectrophotometer.

All samples were scanned from 4,000 cm^{-1} – 250 cm^{-1}. Pure samples of analar grade uric acid, hydroxyapatite and L-cystine (Sigma Corp.) and struvite, whewellite, sodium urate and ammonium urate (Merck Co.), were all first identified by X-ray diffraction and were then used for the recording of standard infra-red spectra.

Atomic Absorption Spectrophotometry (AA)

A search for possible interference effects showed that while Ca absorbances were unaffected by the presence of struvite and phosphate, Mg absorbance values were enhanced by the presence of calcium oxalate. A "standard additions" method was thus used for the determination of Ca and Mg in the stones: 10 mg stone sample were dissolved in 10% HCl and made up to 20 ml in a volumetric flask. For the determination of the calcium content, a 10 ml aliquot was diluted by a factor of 20 and divided into five equal portions. To these were added 0, 25, 50, 75 and 100 ppm calcium as calcium oxalate, from a stock solution, and the volume in each case made up to 20 ml. These solutions were then analysed and a "standard additions" calibration curve was plotted. Similarly, for magnesium determinations, a 5 ml aliquot was diluted by a factor of 80 and divided into six equal portions. To these were added 0, 2, 4, 6, 8, and 10 ppm Mg, as struvite. The solution was then made up to volume (20 ml) and analysed.

All analyses were carried out on a Varian Techtron Model 1000 atomic absorption spectrophotometer using an air acetylene burner and calcium/magnesium hollow cathode lamp.

Results

X-ray Powder Diffraction

The constituents present in the various representative regions of each stone were identified by comparing the measured "d" and "I" values with the published reference standards of Sutor and Scheidt [22]. In all cases, at least two components were detected (Table 1).

X-ray Fluorescence Spectrometry

Values for the percentage of Mg, Ca and P were determined and these were used in simple stoichiometric calculations to give the relative mass of each component present (Table 1). The technique was applied to only those stones in which sufficient material was available for analysis.

Scanning Electron Microscopy

All SEM studies were undertaken only after the components in each stone had been identified by X-ray diffraction analysis. Qualitative assignments were based on characteristic morphologies and energy dispersive X-ray analysis.

Apatite, identified by XRD in seven stones, was frequently observed as spherular clusters of diameter less than 10 μm similar to those reported by Spector [18, 19]. In some cases the apatite appeared to be growing on a substrate (weddelite in Fig. 1), while in others it completely engulfed a crystal of another component (whewhellite in Fig. 2).

Table 1. Qualitative and quantitative results as determined by all six techniques

Stone No.	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
XRD	Str HA	Str HA	Whew Wedd HA	Whew Wedd HA	Str HA	HA Whew Wedd	NAUM UA	Whew Wedd	Whew Wedd HA	Whew Wedd
XRF	Str-91 HA-9	Str-59 HA-41	–	–	Str-49 HA-25 Whew-11 Wedd-15	HA-74 Whew-7 Wedd-19	–	–	Str-19 HA-52 Whew-25 Wedd-4	Whew-63 Wedd-37
SEM	Str HA	Str HA	–	Whew Wedd HA	–	HA Whew Wedd	NAUM UA Str	–	Whew Wedd Str HA	Whew
Density	Str-94 HA-6 (1.80 g/ml	Str-59 HA-41 (2.30 g/ml)	HA-17 Whew-83 HA-38 Wedd-62 (2.40 g/ml)	HA-23 Whew-77 HA-43 Wedd-57 (2.46 g/ml)	Str-47 HA-27 (2.19 g/ml)	HA-78 Whew-4 (2.90 g/ml)	NAUM-42 UA-58 (1.88 g/ml)	Whew-67 Wedd-33 (2.15 g/ml)	Str-31 HA-40 (2.44 g/ml)	Whew-64 Wedd-36 (2.14 g/ml)
I.R.	Str HA	Str HA	Whew Wedd HA Str	Whew Wedd HA	HA Str CaOx	HA Whew Wedd	NAUM UA	Whew Wedd	Whew Wedd HA Str	Whew Wedd HA
A.A.	Str-92 HA-8	Str-59 HA-41	Str-4		Str-52		NAUM-70 UA-30		Str-14	

Str: struvite; *HA*: hydroxyapatite; *Whew*: whewhellite; *Wedd*: weddellite; *NAUM*: sodium acid urate monohydrate; *UA*: uric acid; *CaOx*: calcium oxalate. All figures are quoted as percentages. Where no percentages are quoted, analyses were not performed due to insufficient sample

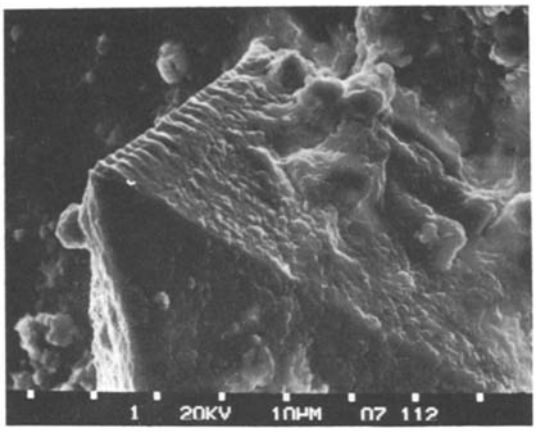

Fig. 1. Apatite crystals growing on weddellite substrate

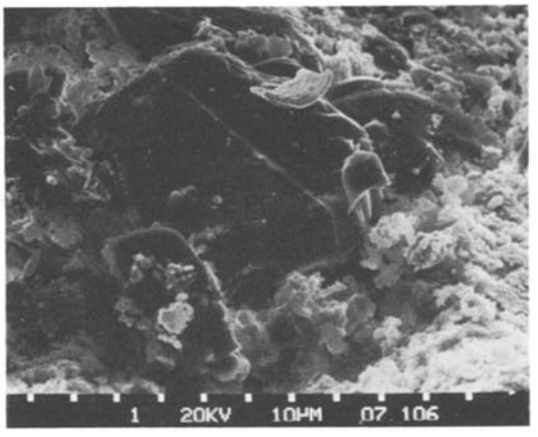

Fig. 2. Apatite deposits engulfing a crystal of whewhellite

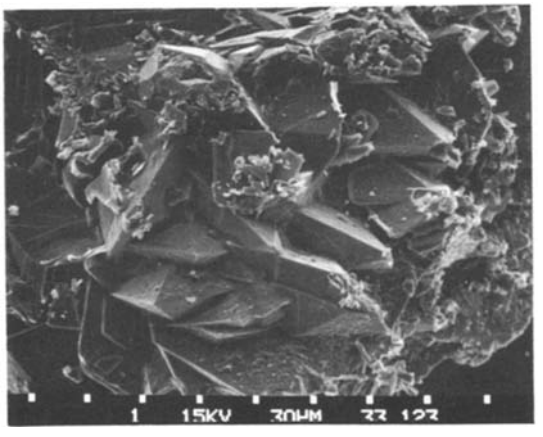

Fig. 3. Bipyramidal crystals of weddellite on exterior of stone

The characteristic bipyramidal morphology of calcium oxalate dihydrate [15] was a frequent observation, particularly on the exterior of the stones (Fig. 3). The coffin-shape (Fig. 2) and fan-like array (Fig. 4) of the monohydrate crystals [15] were easily recognised.

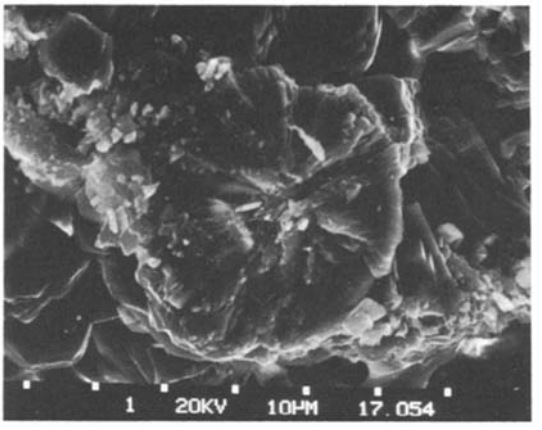

Fig. 4. Fan-like array of whewhellite crystals

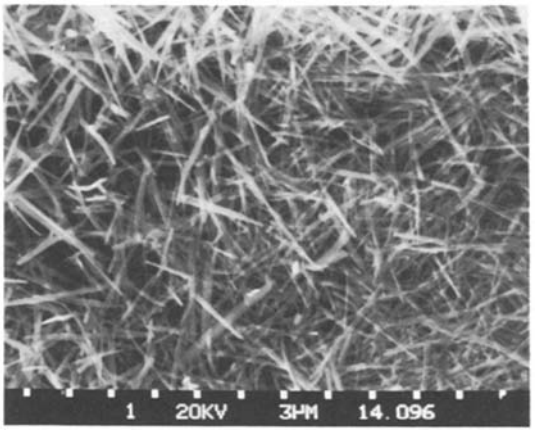

Fig. 5. Needle-like crystals of uric acid (cross-sections ~ 0.4 μm)

In stone 7 both sodium acid urate monohydrate and uric acid were observed and characterised on the basis of energy dispersive analysis with respect to sodium. Uric acid was present as fine, minute, needle-like crystals of approximately 0.3 – 0.5 μm in cross-section (Fig. 5). Sodium urate, however, was observed as oblong rod-like crystals approximately 1 μm in cross-section (Fig. 6). In addition, other crystals of different morphology were observed within this stone (Fig. 7). Microanalysis revealed the presence of Mg and P only, thereby suggesting struvite. Similar crystals were observed in stone 9.

Density Gradient Analysis

In this investigation complete separation of components did not occur. Samples from all 10 stones were introduced into density columns and their densities determined. Using these together with the density values of pure constituents [14] the percentage composition for each stone was calculated. For the 2-component stone systems (stones 1, 2, 7, 8 and 10) the equation

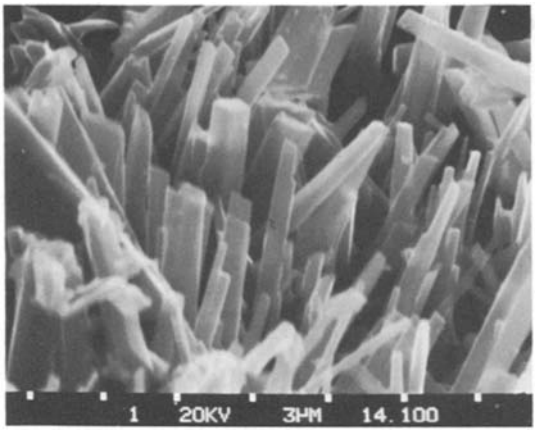

Fig. 6. Oblong rod-like crystals of sodium acid urate nonohydrate (cross-section ~1 μm)

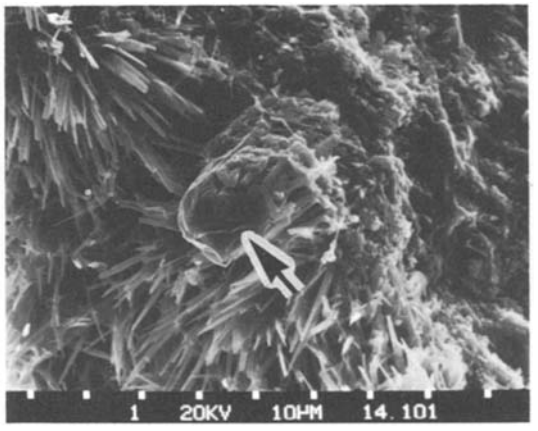

Fig. 7. Struvite crystal observed in stone 7

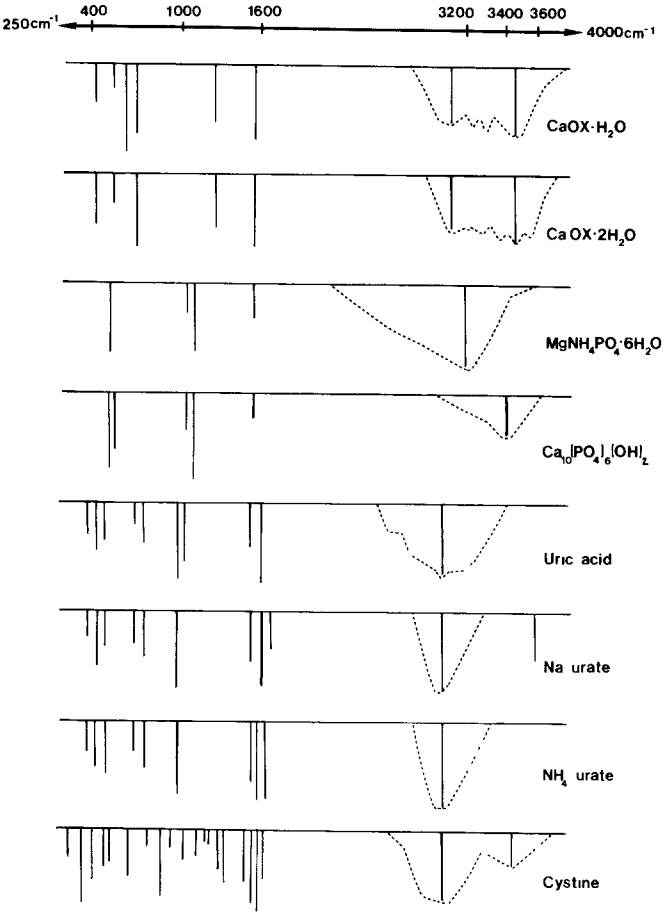

Fig. 8. Schematic representation of major infra-red absorption bands recorded for the standard substances

$$\left(\frac{x}{100}\right)\rho_1 + \left(\frac{100-x}{100}\right)\rho_2 = \rho_m$$

was used [14]. For the 3- (stone 6) and 4-component systems (stones 5 and 9), quantitative data from the XRF analysis were incorporated into the calculations and the system in each case was reduced to a 2-component one. In stone 5 for example, where struvite, hydroxyapatite, whewhellite and weddelite were known to be present, the above equation was modified to read:

$$\left(\frac{x}{100}\right)1.71 + \left(\frac{100-26.5-x}{100}\right)3.15 + \left(\frac{11.54}{100}\right)2.25 + \left(\frac{14.96}{100}\right)1.94 = 2.19$$

(x = unknown percentage struvite; 26.5 = combined percentage calcium oxalate mono- and dihydrate (from XRF); density struvite = 1.71; density hydroxyapatite = 3.15; density whewhellite = 2.25; density weddelite = 1.94; density sample = 2.19 g cm^{-3}).

For stones 3 and 4 (both three component systems), quantitative data from another source were not available. However, by considering the extreme cases 0% whewhellite (100% weddellite) and 0% weddellite (100% whewhellite) the limits of hydroxyapatite/calcium oxalate composition were calculated. These values, together with those for the other calculi are presented in Table 1.

Infra-red Spectroscopy

The major infra-red absorption bands of the standards are schematically presented in Fig. 8. These were used in conjunction with the stepwise system of analysis devised by Oliver and Sweet [12] for the assignment of the major bands recorded for the samples. The infra-red spectra of some of the stones are shown in Fig. 9 while Table 2 summarises the data obtained from one of these (stone 7) and indicates the various assignments. The spectra of all the other samples were interpreted in similar fashion and assigned as shown in Table 1.

Table 2. Assignment of major Ir bands of stone 7

Major Bands (cm^{-1})	Intensity	Assignment
3730	medium	NAUM
2930	medium	NAUM/uric acid
2660	medium	uric acid
1780	medium	NAUM
1680	strong	NAUM/uric acid
1640	strong	NAUM/uric acid
1592	strong	NAUM/uric acid
1020	medium	uric acid
994	strong	NAUM/uric acid
785	medium	uric acid
478	medium	uric acid

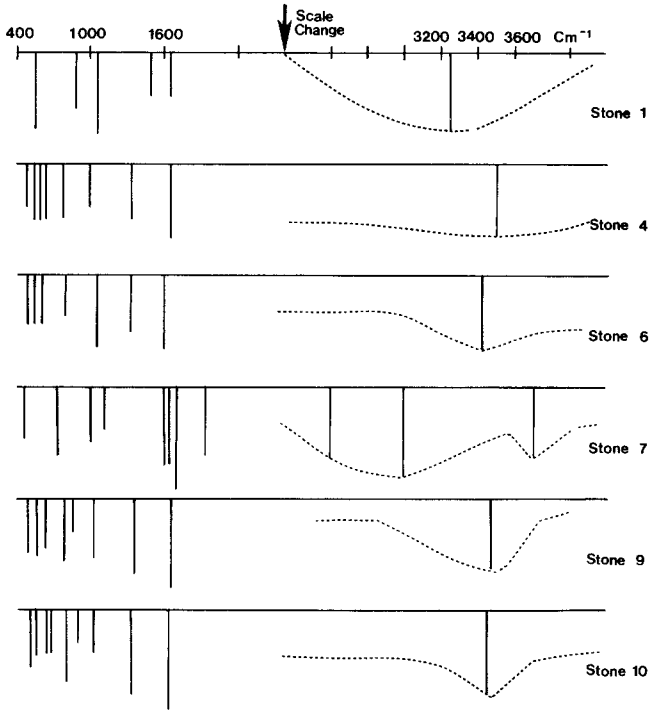

Fig. 9. Major infra red bands of some of the calculi

Atomic Absorption Spectrophotometry

Standard additions absorption data were collected for samples from 9 calculi and calibration curves were constructed (Figs. 10 and 11). These were used to determine the masses of Mg and Ca (and Na in stone 7) when present. In stones 3, 4, 6 and 10, in which only Ca containing constituents are present, no differentiation between these can be made using AA.

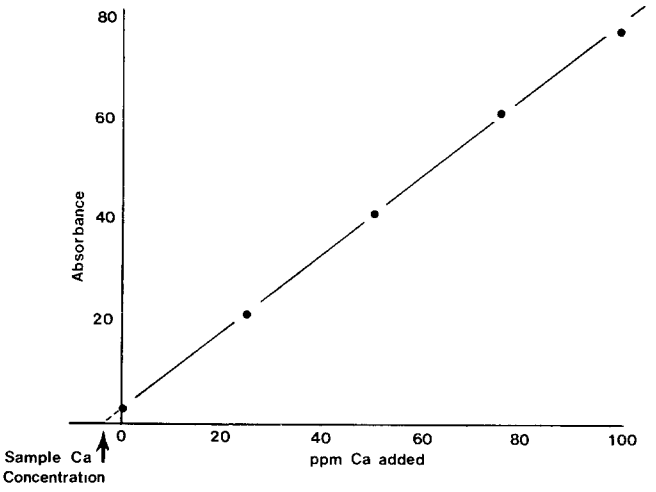

Fig. 10. Ca atomic absorbance for stone 2

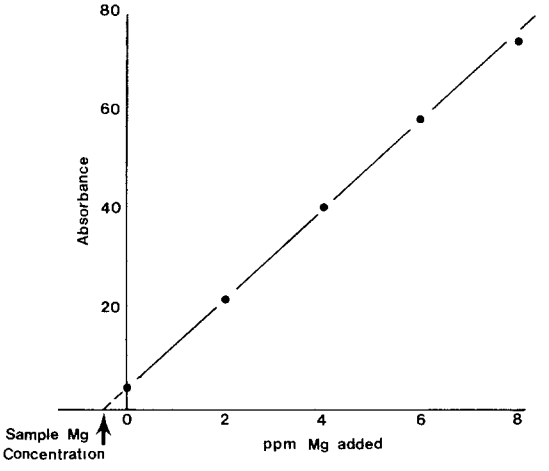

Fig. 11. Mg atomic absorbance for stone 2

Discussion

The techniques used in this study may be divided into two groups – those yielding qualitative results on the one hand (XRD, IR and SEM) and those yielding quantitative data on the other (XRF, density gradient and AA). Application of the three former procedures collectively permitted the identification of several constituents, some of which would otherwise have gone undetected. For example, minor components not detected by XRD because of their low concentrations were identified by Ir (stones 3, 5, 9 and 10) and SEM (stones 7 and 9).

SEM studies yielded much useful data. In the first instance the presence of components detected by XRD and IR was confirmed on the basis of characteristic morphologies and energy dispersive X-ray analysis. In the second instance, interconstituent relationships were clearly observed. This is evident in stone 1 for example where struvite and hydroxyapatite were observed freely interspersed with one another

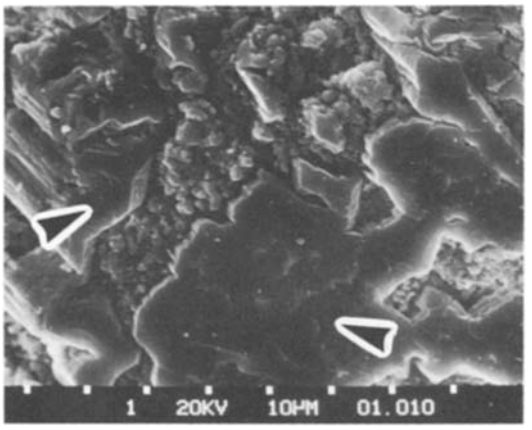

Fig. 12. Struvite and hydroxyapatite deposits in stone1. *Arrows* indicate struvite deposits

(Fig. 12), suggesting that sequential deposition of pure components from the urine was not the major means of crystal aggregation.

Although XRF has long been used for the analysis of geological specimens [26], it has not been widely reported as a tool for urinary stone analysis. We however found it to be very useful, its only limitation being the large sample size required (1.0 g). Four stone systems – struvite/apatite (stones 1 and 2), whewhellite/weddellite (stone 10), apatite/whewhellite/weddellite (stone 6) and struvite/apatite/whewhellite/weddellite (stones 5 and 9) were analysed successfully. Sodium can of course also be determined by this technique thus permitting a component mixture of the type identified in stone 7 to be analysed as well. A pure organic stone, however, cannot be routinely determined by XRF.

The low concentrations measured for whewhellite (11%) and weddellite (15%) in stone 5 are worthy of comment. Sutor [21] has shown that in a struvite-oxalate mixture such concentration ratios are only "just detectable" by XRD when a sophisticated X-ray camera system is used. Our X-ray analysis, with equipment inferior to that used by Sutor, failed to detect the presence of oxalate in this stone, thereby confirming Sutor's prediction and lending confidence to our XRD and XRF measurements.

While providing useful quantitative data, XRF cannot be applied independently of other procedures. Only *elements*, amongst them Ca, P and Mg (as well as H_2O content) are determined. Qualitative data from another source are thus necessary to determine the origin of each element and hence to complete the quantitative analysis. This is illustrated in stones 5 and 9 where IR identification of calcium oxalate and struvite respectively was required before the XRF data could be properly interpreted.

Besides the elements mentioned above, other major and trace elements such as Al, Fe, Mn, K, Cr, Ni and Ti can be detected by XRF. Since trace elements are considered by some workers to play a significant role in urolithiasis [7, 10], the technique could prove most useful in this regard as it offers a complete quantitative analysis on a routine basis while requiring only one sample preparation for the entire analysis.

The use of a density gradient column in the analysis of urinary calculi has recently been reported [14]. As in that study, none of the present samples separated into discrete bands lending support to the conclusion that sequential deposition of pure components within these stones was not the major means of crystal aggregation. For the 2-component stone systems (stones 1, 2, 7 and 8) the calculated compositional figures show good agreement with the XRF and AA data where these were available. Similarly, the composition of the 3- and 4-component systems show good agreement with those XRF values *not incorporated* into the density calculations, lending confidence to the determinations. The sample from stone 10 was treated as a two-component system since the presence of apatite (detected by IR) was not confirmed by any of the other procedures, including XRF, thus suggesting a very low concentration grain in the IR sample.

As with XRF studies, prior identification of constituents present in the sample is necessary before density calculations can be undertaken. This can be achieved by either XRD or IR analysis. Although density gradient analysis can provide information concerning the matrix content of stone samples [14], this aspect was ignored in the present study. In general, the technique is quick, easy to apply and, when combined with XRD (or IR) and XRF (say), yields accurate quantitative information.

AA has been reported in but few cases of urinary stone analysis and does not appear to have been widely used for this type of study [3, 24]. Since the technique yields information concerning metal content only, the elements of interest in urinary calculi are Ca, Mg and Na. No distinction between whewhellite, weddellite and/or apatite can be made when these are present in the same sample [stones 3, 4, 5, 6, 9 and 10]. On the other hand, in those samples where there is only one Ca containing component and hence no confusion as to its origin, quantitative compositional values can be obtained. Because AA is limited to the determination of metals, it does not appear to have as wide an application as the previously described techniques in routine stone analysis. However, it may prove extremely useful in the determination of trace metals in urinary calculi.

In this study a multiple technique approach has been applied and has yielded a very detailed picture of stone composition and structure. Perhaps just as important however is the *clinical* significance of such data. Effective treatment must be based on a thorough understanding of the chemical conditions prevailing at the time of stone nucleation. Identification of *all* the constituents present in a urinary calculus is thus essential. In stone 5 for example, the deposition of the minor constituent, calcium oxalate, might well have been the primary event in this stone's formation with the subsequent deposition of struvite and hydroxyapatite occurring in secondary processes. Treatment of this patient for a urinary infection, as indicated by the presence of struvite in the calculus, is obviously an inadequate procedure.

The quantitative data deserve comment. For any one stone the data obtained from the different procedures showed excellent agreement lending confidence to their accuracy and reliability. As far as stone composition itself is concerned the data is remarkable in that different samples from within a given calculus yielded closely agreeing concentration values. In stone 2 for example the apatite : struvite ratio was found to be 41 : 59 by *all three* quantitative procedures despite the fact that fresh samples were used in each case. Although as noted earlier, sequential deposition probably does not occur, the constant average composition throughout the stone indicates that the deposition mechanism of constituents in such calculi is uniform and continuous.

It is interesting to note that of the stones analysed, all consisted of at least two components. Indeed, multiple technique analyses might very well cast some doubt as to the existence of the so-called "pure" stone.

Approaches similar to that outlined in this paper have been reported in only a few cases. In some instances a multiple technique approach was adopted to characterise and identify new components in urinary calculi [3, 17], while in other instances routine analyses were being conducted [1, 16]. The present study has shown that a multiple technique approach can yield not only a detailed picture of stone composition with respect to major and minor constituents but can provide accurate quantitative data as well. Both "fingerprint" methods, XRD and IR, have their merits, each complementing the other with respect to their advantages and limitations. Coupled with SEM they provide a wealth of information concerning stone composition and structure. Where XRF facilities are available, investigators should seriously consider utilising this technique with density gradient procedures. AA, however, has been shown to be useful in the analysis of certain types of stone only and is not recommended as a routine analytical method.

It is by adoption of a multiple technique approach that true insight into stone initiation and growth may be obtained.

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