METHODOLOGY OF *IN VITRO* CHARACTERIZATION OF HUMAN UROLITHS

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Scopul lucrării este caracterizarea calculilor renali din punct de vedere al morfologiei şi compoziţiei. Din punct de vedere compoziţional, calculii renali au fost caracterizaţi prin XRD, FTIR în timp ce caracterizarea morfologică a fost efectuată prin SEM. Este important de menţionat compoziţia specială a calculilor renali extraşi de la pacient, foarte similară cu osul uman. Compoziţia calculilor renali, rezultat din ATD-TG este: apă–6,5%, componenta proteică (pe bază de colagen)–4,2%, $CaCO_3$–3,5%, oxalat de calciu dihidrat 1.8% şi hidroxiapatită–84%. Morfologia acestor calculi este foarte asemănătoare diferitelor tipuri de ţesuturi osoase şi a diverselor grefe osoase sintetice. Compoziţia şi morfologia calculilor renali este puternic influenţată de condiţiile de precipitare, condiţii care pot varia în domenii largi de concentraţii pe parcursul formării acestora.

The purpose of this study is the compositional and morphological characterization of human uroliths. The uroliths were characterized from the compositional point of view by XRD, FTIR while the morphological characterization was made by SEM. It is important to mention that the uroliths extracted from the subject have a special composition, very similar to natural human bone. The uroliths' composition, as result by DTA-TG is: water–6.5%; proteic matter (mainly based on collagen)–4.2%; $CaCO_3$–3.5%, calcium oxalate dihydrate 1.8% and hydroxyapatite–84%. The morphology of these uroliths is very similar to different kind of bone tissues and synthetic bone grafts. The composition and morphology of uroliths is highly influenced by the precipitation conditions which may vary in a large range of concentration during uroliths' formation.

Keywords: human uroliths; advanced characterization; instrumental methods

1. Introduction

Lithogenesis: Hyperconcentration of urine in crystalloids (crystalluria) is the central element of lithogenesis [1]. However, clinical considerations have shown that unstable supersaturation levels are unreachable unless other factors are

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involved (unbalanced promoter-inhibitor factors, mechanical fixation). Hyperconcetration usually occurs by oliguria and high rate of urinary crystalloids. Ions which compose the crystals form nuclei that become stable when their number is roughly over 100–thus forming *condensation nuclei*. These nuclei can be *homogenous* or, more often in humans, *heterogeneous*, which means that there is some kind of pre-existent solid phase (protein matrix, other types of nuclei). Once the nuclei formed, their growth no longer requires very high concentration of ionic components. Beyond that, uroliths growth is heavily influenced by the promoters [2]–inhibitors balance and requires immobilization of forming urolith inside the urinary tract. *Inhibitor mechanisms* are either chelating or direct growth impeding. Involving both mechanisms, *citrate* seems to be the most important agent. Other inhibitors are magnesium, oligoelements, unpolymerized Tamm-Horsfall protein, GAGs, acid glycoproteins, etc. *Promotor mechanisms* are: acidic or slight basic pH (4.5; 8.2), stasis, urothelium lesions, infection (infection modifies the characteristics of present proteins, some of them changing from inhibitors into promoters (eg. Polymerized Tamm-Horsfall protein, sulphated GAGs). Types of crystalline component include: calcium oxalate 63–69% (even up to 96); calcium phosphates 0.5–8%**;** $MgNH_4PO_4$10–20%; uric acid 15%; cistin 1%; xantin <1%; other 10% (mixed, iatrogen., etc.). The crystalline component forms the core of the urolith. Proteic matrix (mainly collagen) and water are also present [3, 4].

Establishing the type of lithiasis–the analysis of uroliths is the only investigation that provides the certain diagnostic of lithiasis type. It involves qualitative and quantitative determinations (such as chemical methods and instrumental methods: polarized–light microscopy [5], X–ray diffraction [6], IR spectroscopy [7], scanning electron microscopy [8] coupled or not with energy dispersive spectroscopy and differential thermal analysis coupled with thermogravimetry [9]). Many times, due to the overlapped peaks (for example, brushite and gypsum) more difractograms have to be recorded, at different temperatures. Due to the occurred thermal processes, some components are decomposed and the overlapped peaks are resolved [6]. Dual energy computed tomography–DECT may also be used. With DECT characterization, in vitro differentiation of different uroliths constituents may be done such as uric acid, cystine, struvite stones, etc. [10] but, the rational use of the above presented methods can identify all the components and also quantify them. For an advanced compositional analysis of the uroliths, implying trace elements analysis, the use of other instrumental methods are recommended: atomic absorption spectroscopy (AAS) [11], inductively coupled plasma mass spectrometry (ICPMS) [12] and/or electrochemical techniques.

2. Experimental

The analyzed uroliths were obtained from human subjects. Once obtained, they were lyophilized and investigated by X-ray diffraction, IR spectroscopy, scanning electron microscopy and complex thermogravimetrical methods.

X-ray diffraction analysis was performed using a Shimadzu XRD 6000 diffractometer at room temperature. The samples were scanned in the Bragg angle, 2θ range of 10–80°, with a scan step of 0.02°.

SEM analyses were performed on a HITACHI S2600N electron microscope on samples covered with silver layer.

For IR spectroscopy (Shimadzu 8400 FTIR Spectrometer) measurements, the spectra were recorded in the wave number range of 500-4000 cm^{-1}, with a resolution of 2 cm^{-1}.

The differential thermal analysis (DTA) coupled with thermo gravimetric analysis (TGA) was performed with a Shimadzu DTG-TA-50H, at a scan rate of 10 ^{0}C/min, in static air.

3. Results and discussion

Extensive calcification (mineralization) of the arterial wall and soft tissues is a relative frequent feature for the patients with end-stage chronic kidney disease. As a result of the renal failure, cardiovascular and extraskeletal tissues mineralization but also bone alteration occurs. The arterial calcification is induced by a "real ossification" process. The most susceptible tissues to mineralization are: cardiac myocytes, aorta, various arteries, renal tubules, gastric mucosa, muscularis, lung, etc. [13-17].

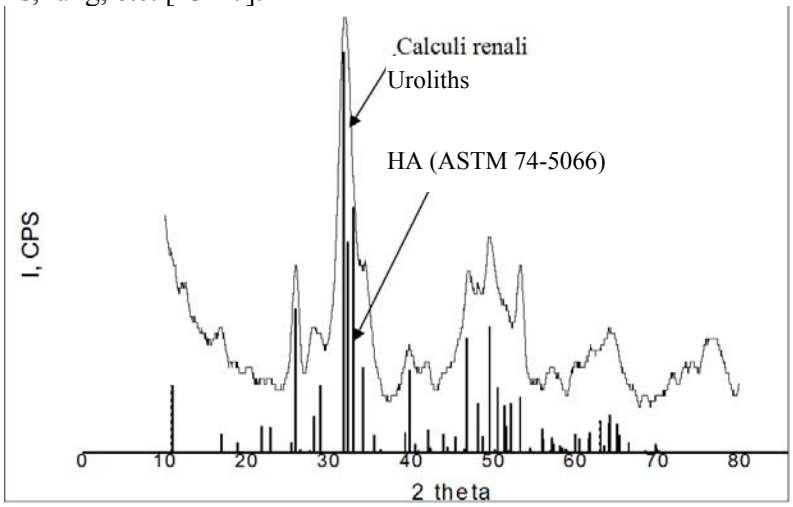

Fig. 1. XRD pattern of uroliths

Uroliths were characterized from the composition and morphology point of view. The qualitative assignments were based especially on the XRD and FTIR analysis while the quantitative ones based on the thermogravimetric measurements.

Based on the literature data on hydroxyapatite based composite materials, the X-ray diffraction pattern is similar to the difractograms recorded for collagen/ hydroxyapatite (COLL/HA) composite materials [18, 19]. The difference between this difractograms (Fig. 1) and that of the COLL/HA consists in the crystallinity of the mineral, the uroliths exhibiting a lower crystallinity degree.

Besides, uroliths contain small amount of other minerals, such as calcium oxalate dihydrate, $CaCO_3$ and even sodium chloride.

FTIR spectrum (Fig.2) was recorded in order to analyse, from the qualitative point of view, the composition of uroliths. The advantage of FTIR over the XRD is the possibility to analyze also noncrystalline phases such as organic matter. In the FTIR spectrum, HA but also collagen were detected [20, 21]. The main peaks of collagen can be identified only after deconvolution because of the overlapping of these peaks with the peaks of HA. They are attributed to: amide I (C=O) at 1654cm^{-1}; amide II (N-H) at 1550cm^{-1}; pyrrolidine ring vibrations at ~1420cm^{-1}. The main vibration bands of hydroxyapatite, corresponding to phosphate groups are: 1030, 601 and 563 cm^{-1}. The large band from 3000-3600 cm^{-1} is due to the associated hydroxyl groups from collagen, hydroxyapatite and water. Between the components which usually occur in uroliths, uric acid and urates are not present (based on the lack of the specific absorption bands of urates) [22] and calcium oxalate and carbonate is present as minor component (main peaks intensity is very low) [23].

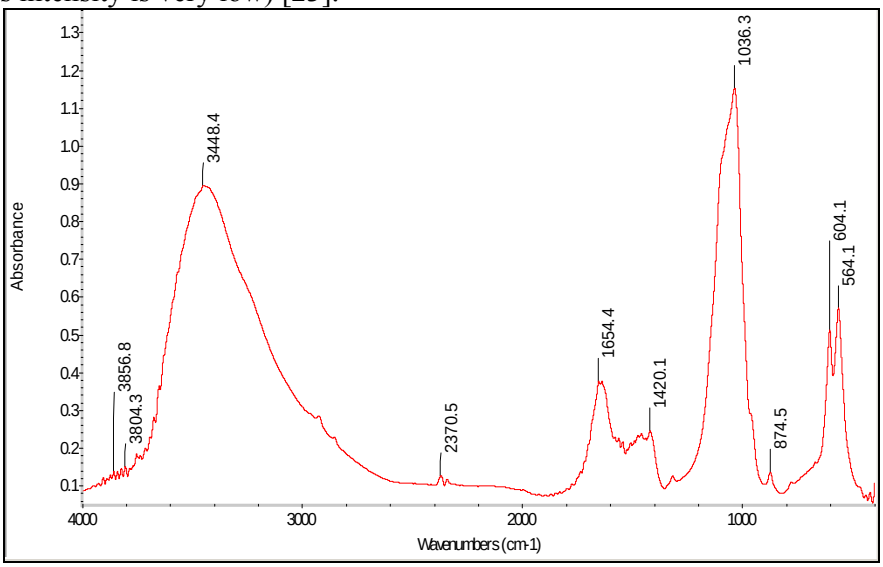

Fig. 2. Infrared spectrum of uroliths

SEM images were recorded at different magnifications in order to study the micro and also the nanoscale morphology of uroliths. At relatively low magnifications (X 1000 or X 1.500) one can observe the mineralized organic network (especially Fig. 3b) but also spherical-shaped grains which probably resulted due to the activity of different kidney cells. Fig. 3b presents the microstructure of a fracture. One can observe the rectangular shapes which probably are sodium chloride crystals.

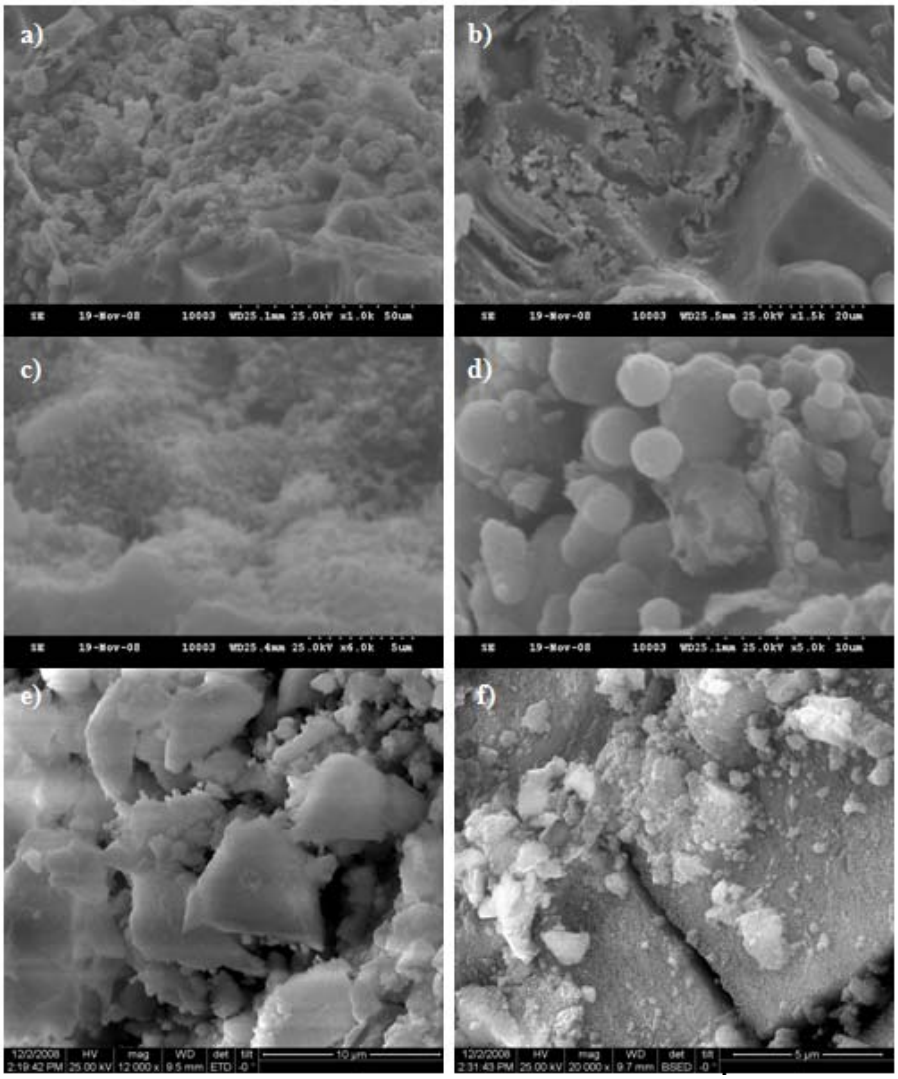

Fig. 3. Scanning electron micrographs of uroliths at different magnifications:
a) x1k, b) x1.5k, c) x5k, d) x8k, e) x12k and f) x20k

Because uroliths are formed during long periods of time (years), their microstructure is very heterogeneous. This heterogeneous architecture of uroliths becomes obvious at higher magnification. The particle size can vary from dozen of nanometers up to micrometers.

At much higher magnification (Fig. 3e, f) the nanometric structuring of hydroxyapatite can be analysed.

Thermal analysis (Fig. 4) was used in order to study the thermal behaviour of human uroliths and to quantify the content of components. Based on the thermal behaviour and mass loss, the content of water, organic matter, calcium oxalate dihydrate, calcium carbonate and hydroxyapatite was determined.

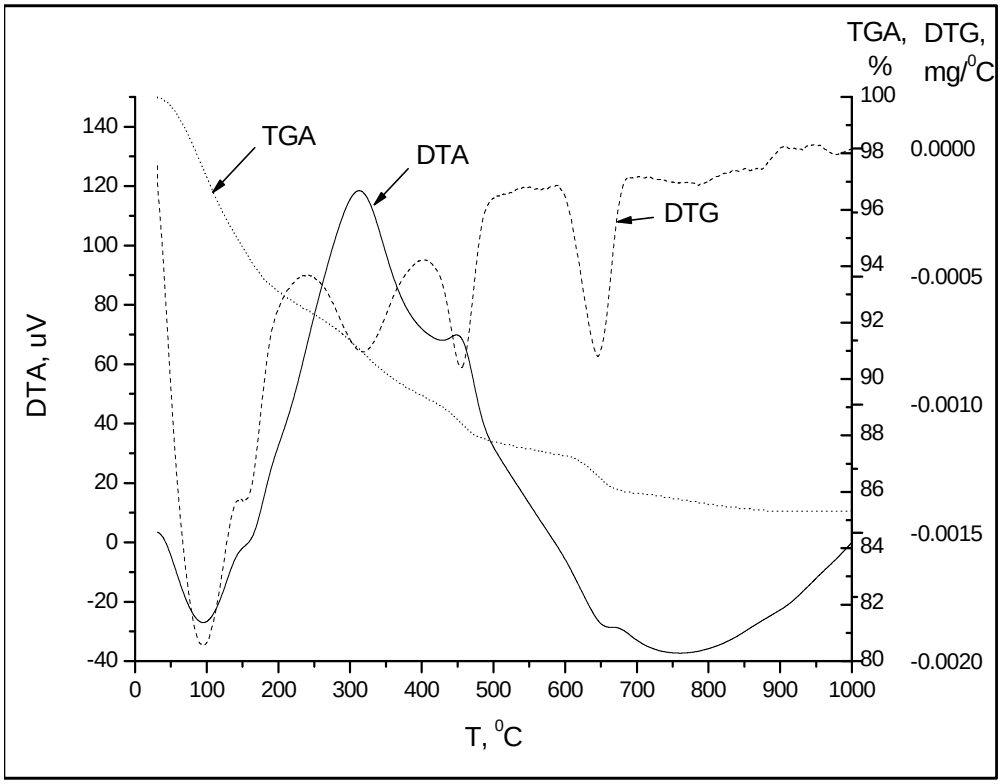

Fig. 4. DTA–TG analyses of human uroliths

The content of the uroliths' components was estimated based on the TGA and DTG curves. In order to be able to make these estimations it is very important to assign each DTG peaks and mass loss to the corresponding process [24, 25]. These processes are:

- water evaporation – endothermic effect which occurs with mass loss at temperature below 200°C (with a maximum rate at 96°C);

- deshidratation of calcium oxalate dihydrate (Eq.1)– endothermic effect, superposed over the water evaporation which occurs with mass loss at temperatures between 140 and 161 °C a (with maximum rate at 151°C)

$$CaC_2O_4 \cdot 2H_2O \rightarrow CaC_2O_4 + 2H_2O \qquad \text{Eq.1}$$

- organic matter total decomposition, mainly associated with collagen degradation by oxidation, occurs with two exothermic effects accompanied with mass loss between 200 and 500°C (with the maxima rates at 318 and 455°C);

- calcium oxalate decomposition (Eq.2) is an endothermic effect, with maximum rate at aprox. 480°C, which occurs with mass loss between 400 and 500°C; due to the overlapping of the secondary decomposition step of the organic matter with carbon monoxide release, in the DTG curve it appears the resulted effect (slight exothermic effect)

$$CaC_2O_4 \rightarrow CaCO_3 + CO \qquad \text{Eq.2}$$

- calcium carbonate decomposition (Eq.3) is an endothermic process accompanied with mass loss which occurs between 600 and 700°C – especially the decomposition of the calcium carbonate obtained by calcium oxalate decomposition and, at higher temperature (up to 900°C) the calcium carbonate from uroliths is decomposed;

$$CaCO_3 \rightarrow CaO + CO_2 \qquad \text{Eq.3}$$

Very important to note that calcium oxalate dihydrate is estimated based on the mass loss due to the deshidratation process while the content of water, organic matter and calcium carbonate from the uroliths are determined taking into account the mass loss contribution of the three step decomposition of the calcium oxalate dihydrate.

Base on TGA data, the uroliths composition was estimated. The values are reported comparatively with the healthy human bones (Table 1) [26]. The composition of the human bones varies in a large range, function of many factors (age, type, sex, etc.). In the case of patients with primary hyperoxaluria or oxalosis-unrelated renal failure the oxalate content can vary up to 13% [27].

It can be seen that the examined uroliths contain the same components, but the mixing ratio differs. Anyway, there are a lot of unhealthy bones, with similar composition with these uroliths, which contain lower quantity of organic phase (especially collagen) and higher quantity of hydroxyapatite [25-27].

Table 1.

Uroliths vs. human healthy bones composition

Component	Uroliths	Human healthy bone
Water	6.5%	7–10%
Organic matter	4.2%	10–20%*
Calcium carbonate	3.5%	3–5%
Calcium oxalate dihydrate	1.8%	1.5%
Hydroxyapatite	84%	65–80%

* mainly composed by collagen type I

4. Conclusions

The uroliths were surgically obtained from human subjects. The qualitative assignments were based on XRD and FTIR analyses, the morphological investigation are based on SEM, while the mass quantification was based on the complex thermogravimetric analysis.

Considering the obtained data we can conclude that the main components of uroliths are: hydroxyapatite (~84%), calcium carbonate (3.2%), calcium oxalate (~1%), organic matter, mainly composed by collagen (4.2%) and water (7.1%). These results, corroborated with literature data, suggest that, at the kidney level, the synthesized uroliths are similar to natural bone, these uroliths being formed similarly with the human bones.

Acknowledgements

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R E F E R E N C E S

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