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Analysis of renal stones by capillary isotachophoresis

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ABSTRACT

An analytical method for the determination of the composition of renal stones by capillary isotachophoresis with conductometric detection was developed. Using different leading/terminating electrolyte systems, the qualitative and quantitative analysis of organic compounds (urate, xanthate, oxalate) and inorganic ions (phosphate, Ca^{2+}, Mg^{2+}, Na^{+}, NH_4^{+}) species commonly present in mixed renal stones in three separate steps can be carried out with limits of detection about 10 μmol/L. The developed method was validated by the analysis of real samples and can be used for urinary calculi classification. In addition, it was verified that this method can also be employed for the determination of the above mentioned analytes in some other samples (bones, teeth) concerning apatite biominerals (fluoro-, carbonate-, chloro-apatite).

1. Introduction

In recent years many studies have been devoted to the determination of individual elements in human tissues. Lack or excess of certain elements causes many health problems. One of these diseases is urolithiasis–crystalline particles (kidney or urinary stones) are formed in a renal parenchyma or urinary tract [1–4] while about 15 crystalline substances have been determined in uroliths [1–4]. The main components of kidney stones are: hydrates of calcium oxalate ($Ca(COO)_2 \cdot nH_2O$, $n=1$ for whewellite, $n=2$ for weddelite), calcium phosphates ($Ca_x(PO_4)_y$) and some of its forms – hydroxylapatite (HA), brushite, dahlite, *etc.*; magnesium phosphates ($Mg_x(PO_4)_y$) mostly in struvite, newberyite and a mixture of Mg(II) and Ca(II) salts as whitlockite; uric acid and their sodium and ammonium salts [2,4] and cystine and xanthine in small amounts [1,4]. The uroliths can then be divided according to their chemical composition [1–4]. Urolithiasis occurs in patients regardless of gender, age or location. The highest incidence of urolithiasis is recorded in the USA, Great Britain, Scandinavia and the Middle East while a low occurrence is observed in Africa, Central and South America [1,2].

Classical methods of chemical analysis used in the past for the classification and analysis of kidney stones are not only time-consuming, but also yield results that are not sufficiently accurate [4–6]. At present the uroliths are usually analyzed by means of instrumental techniques, which obtain the information on major, minor and trace level elemental/compound composition but the techniques can also enable spatial analysis with structure determination [4,6]. Thus, the frequently used analytical techniques are IR and Raman spectrometry [4,7], polarization microscopy [4], scanning electron microscopy [8] and techniques using X-rays, *e.g.* diffraction analysis (XRD) [4] or X-ray fluorescence [9]. Some nuclear techniques, *e.g.* neutron activation (NAA) or nuclear reaction (NRA) analysis were also applied for the determination of the total elemental content [6]. In addition, atomic absorption and inductively coupled plasma (ICP) spectrometry with both optical and mass spectrometry (MS) detection have been employed for bulk analysis [6,9].

Since the urinary stones usually represent multiphase systems containing several minerals, more detailed information on their composition and structure can be obtained by imaging techniques. In addition to the above mentioned established techniques providing spatial resolution, such as microscopy techniques, IR spectrometry or XRD, the applicability of some relatively new or less common techniques has been examined. Synchrotron radiation X-ray microtomography has been used for the study of microstructure and mineralogical composition of urinary stones [10]. Laser induced breakdown spectroscopy (LIBS) has been utilized recently for the cross-sectional study of kidney stones and quantitative determination of elemental contents [9]. Spatial distribution of elements in uroliths has been investigated by means of laser ablation ICP-MS [9,11,12].

Bulk analysis of dissolved uroliths by atomic absorption/emission spectrometry or ICP-MS yields mean elemental contents which may be exploitable *e.g.* for studies of environmental

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influence on human health (contamination of food, water, air) but have little significance in identifying the causes of kidney stone formation. The techniques enabling solid state spatial speciation including mineral composition are more useful; however, they are time-consuming and expensive both in terms of investment and operating costs.

Another drawback of the above mentioned methods lies in their limited applicability for the identification and determination of organic compounds in renal stones. Although their contents can be estimated by recalculation to molecular species based on elemental analysis in some special cases (*e.g.* nitrogen to uric acid/urate), they may fail when the urinary calculi contain more organic compounds. In these cases, separation techniques are recommended for their analysis. Most of the applications are related to electromigration techniques due to the ionic character of the analytes present in urine [3,11–16].

Although, since the early nineties, capillary zone electrophoresis (CZE) has started to be used in preference to capillary isotachophoresis (CITP) [17], CITP still remains a useful micro-analytical technique due to its unique capability both to separate and concentrate analytes simultaneously in one run [18–20]. This is a great advantage over CZE when the determination of some analytes at trace levels in a complex matrix is carried out. In such cases, the ITP procedure is simply applied as a so called transient-ITP preconcentration step prior to CZE analysis, thus giving rise to the ITP-CZE hyphenated technique [21–26]. This technique was also miniaturized in the form of a microchip [27]. Various applications have been already described in the literature, *e.g.* in bioanalysis and in the pharmaceutical and food industries [21–26], in the analysis of nanoparticles [28] or ionic liquids [29].

In this work, the analytical capillary isotachophoresis (CTIP) was used for the study of the composition of renal stones. The aim of this work was to develop an analytical method for the simultaneous determination of anionic/cationic species of both an inorganic and organic nature in real renal stones. So far, only one preliminary study describing anionic ITP analysis of model binary mixtures of calcium oxalate/calcium phosphate or calcium oxalate/uric acid has been described [30] and no other paper dealing with the complete ITP analysis of renal stones by this technique has been published in literature yet as was searched for in literature [20,21–26].

2. Materials and methods

2.1. Instrumentation and operating conditions

An isotachophoretic analyzer EA 102 (Villa Labeco, Spišská Nová Ves, Slovakia) with a contact conductomectric detector was used for the separation and determination of analytes. This analyzer is provided with a PTFE (polytetrafluorethylene) analytical column: diameter 0.3 mm, length 200 mm. The optimization described in Section 3.2 *Optimization of ITP operating condition* resulted in the following adjustment to the instrument.

The separation was carried out at a constant driving current of 100 μA for cationic system, 80 μA for the determination of oxalate and phosphate, and 70 μA for determination of uric acid. In order to improve sensitivity and limits of detection, the current was reduced for all systems to 50 μA prior to detection. Different driving currents were used because of system stability. Data acquisition and processing were performed by means of the ITP-Pro 32 software (KasComp, Bratislava, Slovakia). The analysis was repeated at least 2-times to get the representative results.

The analysis of all samples for most of the elemental content was carried on an ICP-MS Agilent 7500ce (Agilent, USA). The carbon content was determined on a LiquiTOC II (Elementar Analyser GmbH, Germany) while the nitrogen content was recalculated from the total analysis of major constituents.

Table 1
Operational systems employed in ITP analysis.

Parameters	Cationic analysis	Anionic analysis	
System no.	1	2	3
Leading electrolyte	7.5 mmol/L H_2SO_4	10 mmol/L Cl^-	
Terminating electrolyte	10 mmol/L BTP	10 mmol/L HEX	5 mmol/L TB
Counter ion	SO_4^{2-}	L-histidine	Imidazole
pH	2.1	5.5	7.2
Additive		0.1% HEC	

Where: BTP: 1,3-bis[tris(hydroxymethyl)methylamino]propane, HEX: hexanoic acid, TB: disodium tetraborate, HEC: hydroxyethylcellulose.

2.2. Chemicals

The following chemicals were used: hydrochloric acid (J.T. Baker), disodium tetraborate (TB) by Lachema, Czech Republic, sulphuric acid (Merck, Germany), while L-histidine, hexanoic acid (HEX), hydroxylapatite, uric acid, ammonium chloride, 1,3-bis-[tris(hydroxymethyl)methylamino]propane (BTP) were purchased from Sigma-Aldrich, (USA) and calcium oxalate monohydrate, magnesium and calcium chloride, hydroxyethylcellulose and imidazole were obtained from Fluka (Switzerland). The chemical compounds used in this study were of the highest purity and were used as received. Deionized water was used from Milli-Q RG (Millipore Corp., USA).

2.3. Characteristics of analyzed samples, decomposition procedures and sample pretreatment

Uroliths were collected and provided by Prof. Petr Martinec (Institute of Geonics, Czech Academy of Sciences, v. v. i, Ostrava, Czech Republic). The following sample dissolution procedure is based on experiments described in Section 3.1 *Sample preparation.* The samples were characterized by elemental analysis (see footnotes under Table 3) and some of them by IR spectroscopy for the determination of mineral composition.

A known amount (~15 mg) of material was taken from the homogenized kidney stone powder for analysis. This subsample was transferred into a boiling flask and treated in 1 ml of 1-M NaOH for uric-acid determination or in 1 ml of 1-M HCl for the determination of other compounds. The resulting solution containing usually a small undissolved residue was diluted with 15–20 ml of distilled water and subjected to boiling under reflux to dissolve the remaining solids. Then the clear solution was transferred into a 25 ml volumetric flask and filled up to the mark with distilled water. In the case of cationic analysis, the acidic samples were neutralized to pH 6–8 by the addition of a BTP solution. Samples were analyzed by ITP using leading and terminating electrolyte (LE/TE) systems (see Table 1). The aqueous solutions used for uric acid analysis were prepared from carbon dioxide-free water in order to eliminate the bicarbonate zone during ITP analysis. Carbon dioxide-free water was prepared from redistilled water saturated by argon.

3. Results and discussion

3.1. Sample preparation

Artificial model samples prepared as binary/ternary mixtures of the most abundant compounds in the examined urolith specimens, *i.e.* hydroxylapatite, calcium oxalate, and uric acid, were

subjected to dissolution experiments carried out with selected reagents (HCl, $HClO_4$, H_2SO_4, citric acid, EDTA, NaOH, KOH, tetrabutylammonium hydroxide) to design the optimum dissolution procedure. Moderately elevated temperatures (~50–60 °C) proved to be insufficient for the complete sample dissolution and therefore the reaction mixture was kept at boiling point under reflux.

Selection of a suitable dissolving agent was based on the optimization of LE/TE composition, which is described in Section 3.2 *Optimization of ITP operating conditions*. In addition, the urolith composition was taken into account. HCl and NaOH are considered suitable reagents since Na^+ ions of low concentration are present in samples (see Table 3) and Cl^- ions are present in the leading electrolyte (see section 3.2 *Optimization of ITP operating conditions*). Consequently, those ions do not interfere in course of the ITP analysis by a mixed zone establishment. Their concentration was also optimized since their addition increases the time of analysis as a consequence of prolongation of LE zones *(e.g.* H^+ or Cl^- ions).

3.2. Optimization of ITP operating conditions

The determination of urolith constituents cannot be carried out simultaneously due to different ionic nature of the analytes. Therefore, the ITP analysis was divided into three steps: (i) determination of anionic species ($C_2O_4^{2-}$, HPO_4^{2-}); (ii) determination of organic compounds (urate); (iii) determination of cationic species (Ca^{2+}, Mg^{2+}, Na^+, NH_4^+).

We have focused on anionic analysis since information about the presence and content of oxalate, phosphate or uric acid makes it possible to classify particular renal stones [3,4]. The procedure originally developed for the determination of phosphate and their species during their condensation reaction [31] was adopted (see System No. 2 in Table 2) for analysis of the mixture of phosphate and oxalate (see Fig. 1A). The pH was adjusted to 5.5 to increase the mobility of species and to shorten the time of analysis. This can also be optimized by the application of a higher current. The electric current in the ITP procedure was therefore adjusted to 80 μA and then decreased to 50 μA when a change of conductivity signal decreased by 10 mV.

The ITP determination of uric acid (pK_a~5.2 [1]) is more complicated since the molecule of acids starts to dissociate at pH > 5 and therefore the LE/TE system for ITP analysis should be operated at pH > 7 where bicarbonate can interfere with the ITP analysis by the presence of new zone. There are two methods describing the ITP analysis of uric acid in serum [32] or in a test mixture [30]. Both systems, namely (i) (LE: 10 mmol/L HCl/ε-aminocapronic acid/0.25% HEC, pH=5.5; TE: 5 mmol/L morpholinoethanesulfonic acid-MES/tris(hydrosymethyl)aminomethane-TRIS, pH=6.5 [32]); and (ii) (LE: 5 mmol/L HCl/ tris(hydroxymethyl)aminomethane/0.1% HEC, pH=7.7; TE: 10 mmol/L phenol/$Ba(OH)_2$, pH=9.4 [30],) were tested by analysis of binary uric acid-xanthine sample.

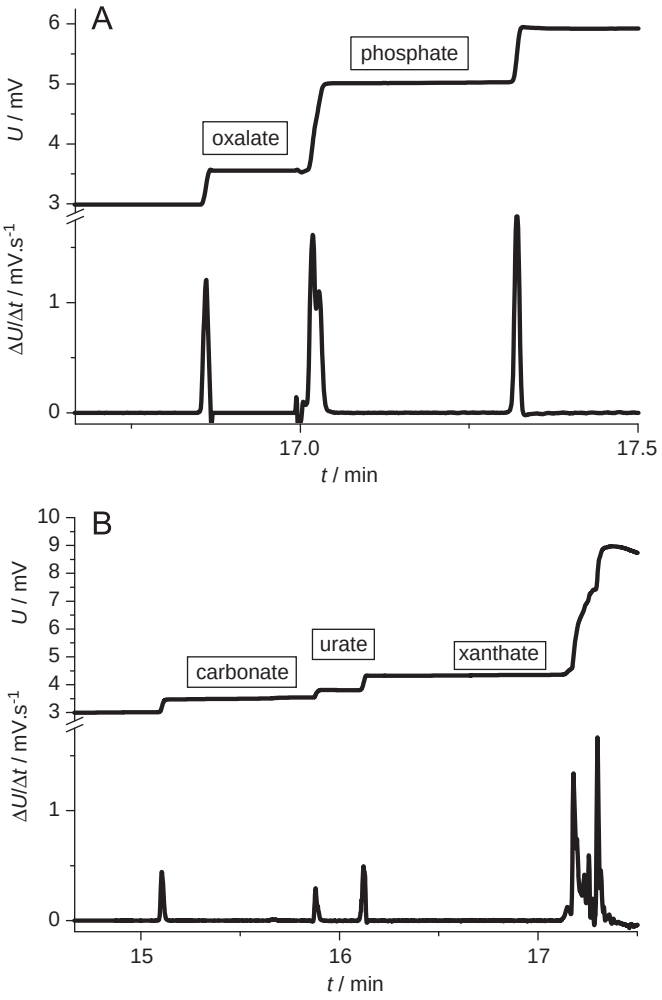

Fig. 1. Anionic analysis of model mixture in (A) system 2, (B) system 3 (c_{Oxal}= 0.19 mmol/L, c_{PO_4} =0.13 mmol/L, $c_{uric\ acid}$=0.46 mmol/L, $c_{Xanthine}$=0.33 mmol/L).

Table 2
Analytical properties of applied ITP analytical method.

Analyte	RSH[d]	Sensitivity (Zone length/c) s/mmol/L	LOD/LOQ[e] (μmol/L)	Dynamic linear range (mmol/L)	Intra-assay[f] RSD (%)
NH_4^+ [a]	0.304 (3)	428 (19)	40/132	0.04–0.200	2.9
Na^+ [a]	0.481 (3)	441 (24)	25/75	0.025–0.150	3.1
Ca^{2+} [a]	0.594 (3)	164 (4)	16/54	0.016–0.27	4.4
Mg^{2+} [a]	0.625 (3)	245 (3)	13/41	0.013–0.200	3.7
$Oxalate^{2-}$ [b]	0.125 (2)	51.3 (2)	13/43	0.013–0.500	2.1
$Phosphate^{3-}$ [b]	0.691 (4)	150 (1)	25/82	0.025–0.500	2.5
Urate [c]	0.271 (5)	124.0 (8)	17/62	0.017–0.350	1.5
Xanthine [c]	0.354 (7)	153 (3)	14/45	0.014–0.270	1.0

[a] System 1.
[b] System 2.
[c] System 3.
[d] "Relative Step Height" (RSH) is a qualitative parameter of the substance in the system defined as $RSH_X=(h_X-h_L/h_T-h_L)$, where h_X is the height of analyte, h_L is the height of leading electrolyte and h_T is the height of terminating electrolyte.
[e] The limit of detection (LOD) was calculated as $3 \times s_{y,x}$/slope of calibration plot, the limit of quantification (LOQ) was calculated as $10 \times s_{y,x}$/slope of calibration plot.
[f] Repeated measurement, $n=15$.

Using both LE/TE systems, the measurements for analysis of uric acid were not reproducible because of a high voltage. For the second LE/TE system, this may probably be due to the presence of the uncharged $BaCO_3$ species formed from bicarbonate originating from dissolved carbon dioxide. To avoid this undesirable phenomenon, the TE in the latter system was substituted with 10 mmol/L TB solution. Because this adverse effect still persisted, TE concentration was decreased to mmol/L a value of 5 mmol/L. As a result, this considerably improved the behavior of the system. The final composition of the LE/TE system (System No. 3) is given in Table 2. The example of analysis of the artificial sample concerning uric acid and xanthine using the optimized LE/TE composition is shown in Fig. 1B. It can also be seen that carbonate (RSH ~ 0.123) is present in the solution, although all aqueous solutions were prepared without dissolved CO_2. This is probably due to carbon dioxide bound to imidazole as a free base. Nevertheless, uric acid, xanthine and bicarbonate can be determined simultaneously in the mixture.

The ITP procedure for the determination of alkaline and alkaline earth metal ions (see Fig. 2) was developed for the analysis of agricultural and environmental samples [33,34]. The first system consisting of LE (20 mmol/L KOH/hydroxyisobutyric acid (HIBA), pH = 5.0/acetic acid, pH = 4.1) and TE (5 mmol/L acetic acid) [33] was tested for a model mixture of Ca^{2+} and Mg^{2+} ions with successful results. However, this method could not be employed for the separation of K^+ and NH_4^+ ions since they are not capable of complex formations with HIBA and they have almost the same mobility [18–20]. This is an important aspect in the case of uric acid when their salts can be present in renal stones and therefore another system (LE: 7.5 mmol/L H_2SO_4/0.1% HEC, pH = 2.05; TE: 10 mmol/L BTP [34]) was utilized where the separation of K^+ and NH_4^+ ions was ensured by adding 18-crown-6 ligand (see Fig. 2). This system is unbuffered, because it assured by controlled migration of H_3O^+ ions of the terminating ion using a weak acid since as the leading ion is used H_3O^+, counterion is a base, terminating ion is a cation with a low mobility [34].

The analytical properties of developed method for all analytes of interest are given in Table 2. The achieved values of limit of detection can be compared with those values given in literature for the CE method [13,14,16]. The ITP procedure can also be used for qualitative analysis since RSH values for important species (oxalate, phosphate, urate) are significantly differing and therefore they can be used for possible stone classification.

The representative real samples were chosen for analysis in order to cover all possible composition of renal stones (oxalate or urate and their mixture). Then these samples concerning macrospecies (oxalate, phosphate, urate, Ca^{2+}, Mg^{2+}) were analyzed by ITP using optimized LE/TE systems (see Table 1) while the content of microspecies was below the limit of detection of this method and therefore they could not be determined (see Table 3). Examples of analysis of samples are given in Fig. 3 and the results of the analysis are presented in Table 3. The content of uric acid in samples is loaded by a higher error since they were determined indirectly from elemental analysis (C, P, Ca, Mg) after recalculation of their results. Anyway, all the results of analysis were correlated with values obtained by ICP-MS and elemental analysis taken from [35] (see Fig. 4). The linear plot $Y = 1.01(4) \times X$, where X and Y are values found by the proposed ITP and used ICP-MS

Table 3
Results of determination of ions in real urinary stone samples. The standard deviations are given in the brackets for the sake of clarity and they are related to the last digit.

Analyte (%)	Sample[c]				
	1	2[d]	3[d]	4	5[d]
Ca^{2+}	2.3 (1)[a]	26.8 (2)[a]	21.6 (2)[a]	9.0 (1)[a]	24.3 (8)[a]
	5.4 (1)[b]	24.3 (2)[b]	21.1 (2)[b]	7.1 (1)[b]	29.3 (2)[b]
Mg^{2+}	< LOD[a]	1.2 (3)[a]	2.18 (6)[a]	< LOD[a]	< LOD[a]
	< LOD[b]	0.49 (1)[b]	1.98 (2)[b]	0.102 (1)[b]	< LOD[b]
$Oxalate^{2-}$	13.7 (4)[a]	18.5 (7)[a]	11.8 (7)[a]	0.62 (8)[a]	47.0 (1)[a]
	11.9[b]	16.5[b]	11.9[b]	< LOD[b]	50.5[b]
$Phosphate^{3-}$	< LOD[a]	19.4 (1)[a]	19.6 (3)[a]	10.3 (1)[a]	6.9 (3)[a]
	< LOD[b]	16.1 (1)[b]	31.5 (2)[b]	11.1 (1)[b]	9.5 (1)[b]
$Urate^-$	69.4 (6)[a]	36.3 (3)[a]	12.4 (1)[a]	90.2 (7)[a]	< LOD[a]
	78.5[b]	41.8[b]	16.9[b]	79.5[b]	< LOD[b]

[a] Results of ITP analysis – the LOD's are given in Table 2.
[b] Results of ICP-MS analysis (Ca, Mg, P – phosphate) and elemental analysis C – oxalate, N—uric acid). The LOD's (in mg/kg) are for Ca (0.04), Mg (0.001), P (0.004) for ICP-MS and 0.1% for C while LOD for nitrogen was not determined.
[c] Elemental analysis for all samples: Na (2.25–7.53 mg/g); K (0.47–16.61 mg/g); other ions in μg/g units - Rb (0.304–18.670); Sr (123.30–376.80); Ba (4.18–29.38); Zn (376–990); Cu (0.22–0.28); Al (8.4–15.6); Fe (22.6–102.2); Ni (0.22–0.28); Cd (0.153–0.420); Pb (15.09–115.90); Cr (0.069–0.092); Mn (0.139–0.207); Se (0.1–0.8); Zr (0.035–0.155); Mo (0.07–0.45); Sn (0.446–6.524); Sb (0.014–0.026); Co (≈ 0.015); Pt (≈ 0.01); V (0.01–0.02); As (0.01–0.04); Hg (0.0213–0.0483).
[d] Mineral analysis from IR spectroscopy: sample 2: uric acid (35%); whewelit (30%); dahlit (carbonate-HA, 20%); struvite (15%) sample 3: uric acid (20%); weddelit (30%); HA (30%); struvite (20%) sample 5: weddelit (70%); HA (30%).

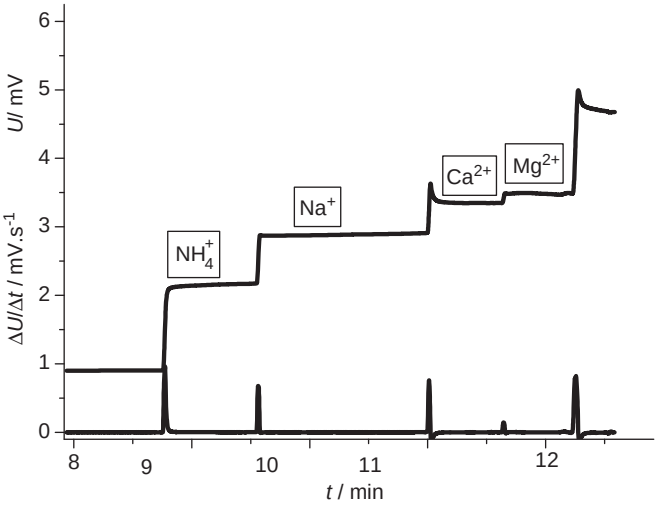

Fig. 2. Cationic analysis of model mixture ($c_{NH_4^+}$ = 0.13 mmol/L, c_{Na} = 0.14 mmol/L, c_{Ca} = 0.18 mmol/L, c_{Mg} = 0.12 mmol/L).

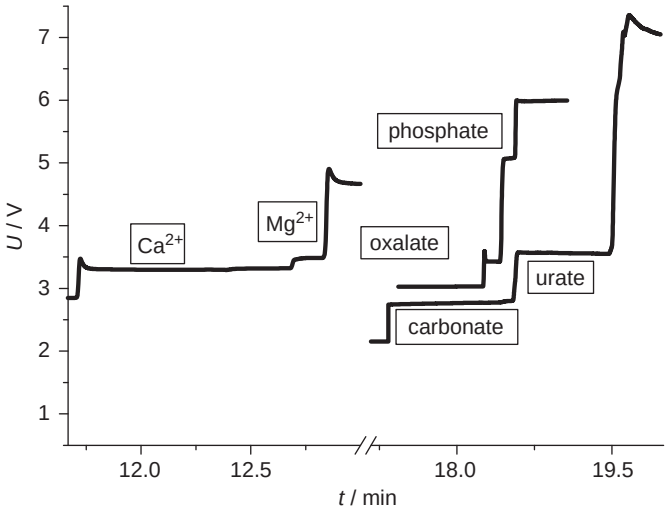

Fig. 3. Example of analysis of renal stone (Ca^{2+}/Mg^{2+} No. 1, phosphate/oxalate No. 2, urate No. 3—see Table 3).

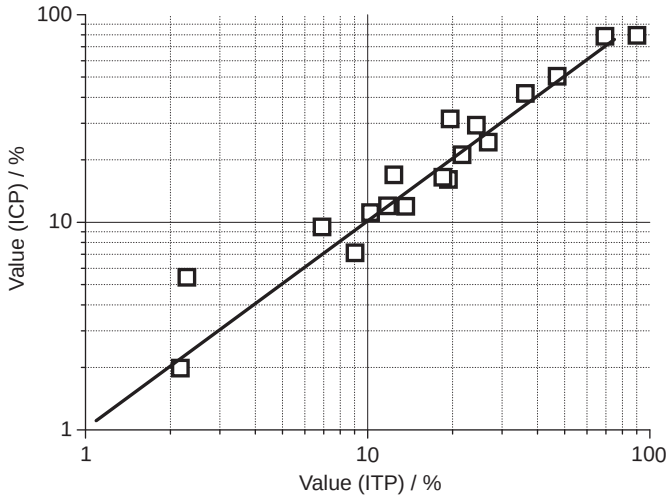

Fig. 4. Comparison of all results obtained by ITP method and elemental analysis for the determination of ions in urinary stones.

analytical method, demonstrates no systematic error in determination of all analytes within a broad concentration range. The RSD for repeated measurements does not exceed 5% for quantitative analysis. In addition, for some samples, the values from ITP analysis can also be compared with a mineral analysis obtained by IR spectroscopy. As it can be seen, the uric-acid and oxalate content is also in rough agreement with the results obtained from IR spectroscopy (see footnote d under Table 3).

The developed ITP procedure was then applied for the determination of concentration of analytes in urine which was only diluted 20-times prior to analysis. In the sample provided by a volunteer, the required analytes and also citrate (RSH$\sim$0.388) were found in the physiological range [36]. In addition, this procedure can be utilized for routine control in clinical analysis. When some component concentrations are higher than their physiological concentrations as a consequence of some pathobiochemical changes (*hypercalciurie*–Ca^{2+}, *hyperphosphateurie*–phosphate, *hyperoxalurie*–oxalate [36], there is a risk of formation of renal stones [1,2]. Thus this method can be used for the well-timed prediction of the possible formation of renal stones and the patients can be treated in advance with citrate to eliminate their health problems [1].

The developed procedure was tested for accuracy of method. Since it is difficult to find the suitable certified reference material, the procedure was utilized for analysis of bone NIST 1400 sample. The results of analysis are (the declared values in % are in brackets): P 17.29 (17.91 $\pm$ 0.19), Ca 36.98 (38.18 $\pm$ 0.13), Mg < LOD (0.684 $\pm$ 0.013). The comparison for declared and found values is satisfactory. The analysis of human tooth shows the composition 30.41% Ca and 20.47% similar to bone sample.

4. Conclusions

The article describes the development and validation of the ITP procedure suitable for the analysis of urinary calculi. This method was optimized for sample treatment as well as for ITP analysis. The procedure can be used for qualitative analysis (uroliths classification) and quantitative determination of Ca^{2+}, Mg^{2+}, Na^{+}, NH_4^{+}, $C_2O_4^{2-}$, PO_4^{3-}, urate and xanthate ions in renal stones. In addition, this procedure can be applied to the analysis of mixtures of inorganic ions and/or organic compounds of a similar chemical nature in other biominerals (*e.g.* teeth, bones) and biofluids (urine) concerning K^{+}, CO_3^{2-}, F^{-} and citrate ions. The robust method is fast (total time of ITP analysis is about 1 h) and cheaper when compared with other analytical techniques (ICP-MS, ion chromatography, CZE).

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