CIENCIA 20(4), 264 - 271, 2012
Maracaibo, Venezuela

Attenuated total reflectance infrared spectroscopy as a screening tool for the urinary calculi characterization

A. Capella-Torres[1], D. Pérez-Guaita[1], J. Ventura-Gayete[2], M. Sancho-Andreu[2], S. Garrigues[1] and M. de la Guardia[1]*

[1] *Department of Analytical Chemistry, University of Valencia, 50 Dr. Moliner Street. 46100 Burjassot, Valencia, Spain.* [2] *University Hospital Doctor Peset Aleixandre, Av. Gaspar Aguilar, 90. 46017 Valencia, Spain*

Recibido: 13-06-2012 Aceptado: 03-09-2012

Abstract

The aim of this study has been the development of a fast and reduced cost screening tool for the kidney stones characterization based on attenuated total reflectance infrared measurements (ATR-FT-IR) and hierarchical cluster analysis (HCA). The method was compared with the traditional chemical methods used in hospitals based on reagents kits. 111 renal calculi samples were analyzed by a reference chemical method and their infrared ATR spectra obtained. The cluster analysis was made using the regions from 3662 to 2247 cm^{-1} and from 1956 to 565 cm^{-1}after vector normalization of spectra data and using Ward's linkage method and the Euclidean distance for the discrimination of the different sample groups. Dendrographic classification provides four groups of samples, according to the presence of uric acid, calcium oxalate or a combination of this compound with other phosphate salts. The IR spectra and dendrographic classification evidences the bad discrimination of some stone samples by the chemical method. As a conclusion, hierarchical cluster analysis of ATR-FT-IR spectra of kidney stones provides a fast, low-cost and environmental friendly method to classify samples in order to establish the clinical origin of urinary calculi, being evidenced that this methodology is free from the mistakes found in some cases using chemical kits.

Keywords: FT-IR, ATR, hierarchical cluster analysis (HCA), renal stones.

Espectroscopia infrarroja de reflectancia total atenuada como herramienta de criba para la caracterización de cálculos renales

Resumen

El objeto de este estudio ha sido el desarrollo de una herramienta de criba, rápida y de bajo coste, para la caracterización de piedras de riñón mediante medidas de reflectancia total atenuada (RTA) en el infrarrojo medio (MIR) y el análisis jerárquico de grupos. El método propuesto se ha comparado con los métodos químicos que habitualmente se emplean en los hospitales basados en la utilización de kits de reactivos. Un total de 111 muestras de cálculos renales fue-

* Autor para la correspondencia: salvador.garrigues@uv.e

ron analizadas por los métodos químicos de referencia, registrándose además los correspondientes espectros RTA. En el análisis de grupos se consideraron las regiones comprendidas entre 3662-2247 cm^{-1} y 1956-565 cm^{-1}, de los espectros previamente normalizados, y el método Ward's linkage y la distancia Euclidea para la discriminación de los diferentes grupos de muestras. La clasificación dendrográfica proporciona cuatro grupos de muestras, que corresponden con la presencia de ácido úrico, oxalato cálcico o una combinación de oxalato y diferentes fosfatos. El espectro IR y la clasificación dendrográfica ponen de manifiesto una inadecuada diferenciación de algunas de las muestras cuando se emplea el método químico. En conclusión, el análisis jerárquico de grupos a partir de los espectros RTA-IR de los cálculos renales proporciona un método rápido, de bajo coste y respetuoso con el medioambiente para la clasificación de las muestras con el objeto de establecer clínicamente el origen de los cálculos renales, evidenciando que mediante este procedimiento se evitan algunos de los errores de identificación observados para las muestras analizadas empleando kits de reactivos.

Palabras clave: FT-IR, ATR, análisis jerárquico de grupos, cálculos renales.

1. Introduction

Urolithiasis is a common disease in humans that reaches a prevalence of 10% in developed countries (1-3). Urinary calculi can be pure compounds, but they are commonly composed by a mixture of different salts and acids. In spite of the fact that previous studies show pathological and geographical differences in kidney stones composition (1-5), the most common constituent is calcium (present in the 75-80% of all stones). Calcium is usually combined with oxalate to form calcium oxalate monohydrate, which is present in almost 50-78% of all analyzed stones, calcium oxalate dihydrate (30%) and in lesser extent with phosphate (15%). Uric acid or uric acid dihydrate (10-20%), magnesium ammonium phosphate (6-14%) and cysteine (0.8-6%) are also frequent compounds of kidney stones. In the context of this study, the determination of the above mentioned compounds is very useful for the correct evaluation of the etiology of the stone formation and consequently to orientate the diagnostic of diseases and to establish the appropriate therapies. In this sense it has been reported that calcium oxalate stones are commonly associated with dietary factors and intestine disorders, uric acid are also due to metabolic disorders and, on the other hand, cysteine stones evidence a hereditary tendency to suffer from cystinuria (6).

The characterization of the nature of urinary calculus can be made through the use of colorimetric measurements and classical drop analysis or based on modern spectrometry techniques. In the last years a great number of instrumental techniques have been reported for the characterization of kidney stone components (5, 7). X-ray power diffraction is a powerful tool providing information about quantitative phase abundances of composite materials and micro-structural features intrinsic to the crystalline environment (7). Scanning electron microscopy with elemental distribution analysis (SEM-EDAX) has been proposed to understanding the crystal formation through spatial distribution of major and trace elements (8). In spite of the great potential of these methods, it is their high cost and complexity in operation that these techniques are not implemented as screening methods in ordinary hospitals. In the last 20 years the importance of vibrational spectroscopy, coupled with chemometrics data treatment, for the analysis of kidney stones has been emphasized while the use of the chemicals methods developed has been decreased (9). Using KBr pellets, infrared spectroscopy characterizes successfully the

main constituents of kidney stones (10-17). This technique does not use any reactive and provide a low cost, easy and environmentally-friendly method. However, the transmission based KBr-pellet technique involves time-consuming sample preparation. This handicap can be solved with the use of attenuated total reflectance measurements (ATR-FT-IR) which offers excellent tools for the urinary calculi characterization based on single reflection direct measurements (18-20) or measurements acquired through microscopic measurements (21).

The aim of this study has been the development of a fast and reduced cost screening tool for the kidney stones characterization based on ATR-FT-IR measurements and hierarchical cluster analysis. The method was compared with the traditional chemical methods used in hospitals and some mistakes in the characterization of samples were detected.

2 Experimental part

2.1. Apparatus and reagents

A Bruker Tensor 27 (Bremen, Germany) FTIR spectrometer equipped with a temperature-stabilised deuterated lanthanum tryglycine sulphate (DLATGS) detector and an in-compartment DuraSampleIR accessory from Smiths Detection Inc. (Warrigton, UK) using a three reflection diamond ZnSe DuraDisk plate. During measurements the instrument was purged with nitrogen.

Reference standards for the characterization of renal calculi with ATR-FTIR were, uric acid (99.0%), calcium carbonate (99.0%), ammonium phosphate (98.0%), calcium phosphate (96.0%), calcium oxalate (99.9%), all obtained from Sigma-Aldrich.

2.2. Reference procedure

Samples were characterized at the hospital by a chemical method based on use of the kit Ecoline from Diasys (Wokingham, UK). After milling the stones and mixing of the resulting powder, samples were acidified with 5 drops of sulfuric acid and diluted to 50 mL volumes. These solutions were transferred inside small reaction vessels for each determination envisaged. The semi-quantitative determinations, based on colorimetric reactions, used a great number of reagents (table 1) and were carried out in a slow way under constant supervision.

2.3. ATR-FTIR procedure

A small amount of standard or kidney stones were milled, homogenized and deposited on the ATR accessory surface. Spectra were recorded from 4000-600 cm^{-1} wavenumber interval by accumulating 100 scans at a resolution of 4 cm^{-1}. For background collection the clean ATR crystal was used, measured in the same instrumental conditions as during data collection. Triplicate measurements of the sample were carried out and the final spectrum was obtained averaging the replicates. Clusters were established from the spectra using the software OPUS version 6.5 from Bruker (Bremen, Germany).

2.4. Sample description

111 renal calculi samples were collected from patients of the hospital *Dr. Peset Aleixandre* in Valencia (Spain) and characterized by the reported method (see section 2.2). This method could identify the presence of oxalate, calcium, phosphate, uric acid, magnesium and cysteine. Using this technique, 15.31% of calculi were considered as pure calcium oxalate and 27.92% as calcium oxalate with traces of phosphates. The rest were composed by uric acid-calcium oxalate mixture (15.31%), calcium oxalate-ammonium phosphate mixture (6.31%), calcium oxalate-ammonium magnesium phosphate mixture (9.09%) and calcium oxalate-calcium phosphate mixture (25.22%).

Table 1
Principle of the semi-quantitative chemical methods used in the hospital.

Analyte	Principle
Calcium	Titrimetric determination with ethylenediamine tetraacetic acid disodium salt using calconcarboxylic acid as an indicator.
Oxalate	Decoloration of the colour complex formed by iron (III) and sulfosalicylic acid.
Ammonium	Yellow to brown solution obtained with Nessler's reagent (dipotassium tetraiodomercurate and sodium hydroxide).
Phosphate	Formation of molybdenum blue by reaction of molybdatophosphoric acid formed upon addition of ammonium molybdate which is reduced by means 4-methyl-aminophenol sulphate and sodium disulfite.
Magnesium	Red complex formed in a buffered solution by magnesium reacts with a 1-azo-2-hydroxy-3-(2,4-dimethyl-carboxoanilido)-naphtalene-1'-(2-hydroxy benzene-5-sodium sulfonate) solution.
Uric Acid	Formation of molybdenum blue in a buffered solution of molybdatophosphoric acid.
Cystine	Red colour with sodium nitropusside produced by Cystine in an alkaline environment after reduction to cysteine by sodium sulphite.

3. Results

3.1. ATR infrared spectra of samples

Figure 1 shows the spectra of some samples evaluated in this study. The presence of different bands in the 3500 to 2500 cm^{-1} range as well as in the fingerprinting region (1800-600 cm^{-1}) can be used for fast classification of the nature of kidney stones composition.

3.2. Spectra of major components of kidney stones

Figure 2 shows the spectra of three different standards and real samples with close similarities with the considered compounds. It can be seen that calcium oxalate can be identified by bands at 1605, 1314 and 778 cm^{-1} and uric acid by several overlapping bands between 3400 to 2500 cm^{-1} and characteristics bands at 1120 cm^{-1} and 990 cm^{-1}. Calcium phosphate standard presents the main band at 1015 cm^{-1} and a second one near 560 cm^{-1}. In stones of mixed composition, identification of all compounds is possible because of the ease in which the characteristic bands of each compound can be identified, even in the presence of other ones.

3.3. Dendrogram classification of spectra

Differences between spectra permit us to do a cluster classification of all considered samples and standards. The cluster analysis was made using the regions from 3662 to 2247 cm^{-1} and from 1956 to 565 cm^{-1} after vector normalization of spectra data and using Ward's linkage method and the Euclidean distance for the discrimination of the different sample groups (figure 3).

4. Discussion

Dendrographic classification provides four groups (table 2), class A which corresponds to pure oxalate samples, class B which includes oxalates combined with calcium phosphate and traces of other com-

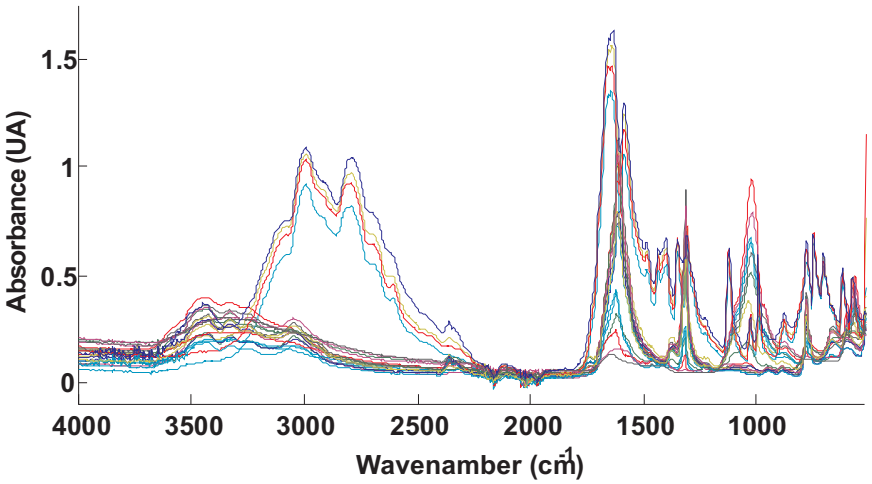

Figure 1. ATR-FT-IR spectra of different samples considered in this study.

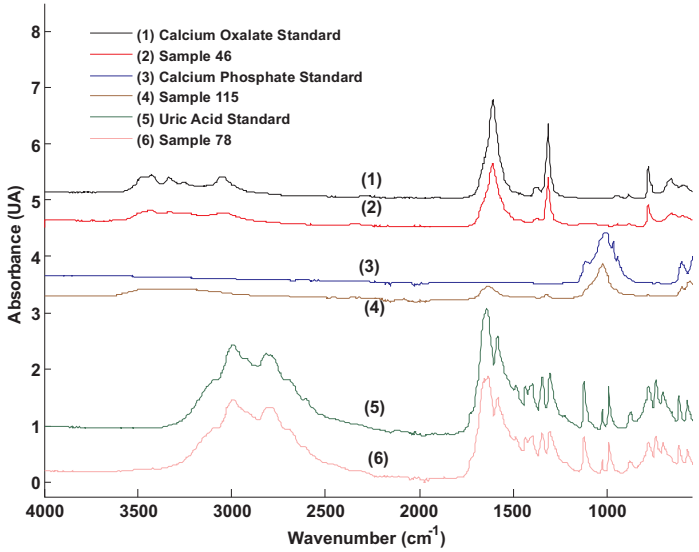

Figure 2. ATR-FT-IR spectra of pure standards and real samples. Note: Spectra were shift on the y-axis to clearly show their bands and avoid overlapping.

pounds, class C corresponding to oxalate and uric acid mixtures and class D including oxalate mixed with other salts as calcium phosphate or ammonium and magnesium phosphate. If we compare the average spectra of each class with their respective pure standards spectra (figure 4), class A average spectra fits perfectly with calcium oxalate spectrum and the class C average spectrum fits perfectly with uric acid spectrum but there is no coincidence between calcium oxalate spectrum. For class B or D, average spectra of both classes fit well with a mixture of calcium oxalate and calcium phosphate being the contribution of oxalate stronger in class B than in class D and the contribution of phosphate stronger in class D than in class B. This facts evidence, once again, the capability of this tool for sample discrimination.

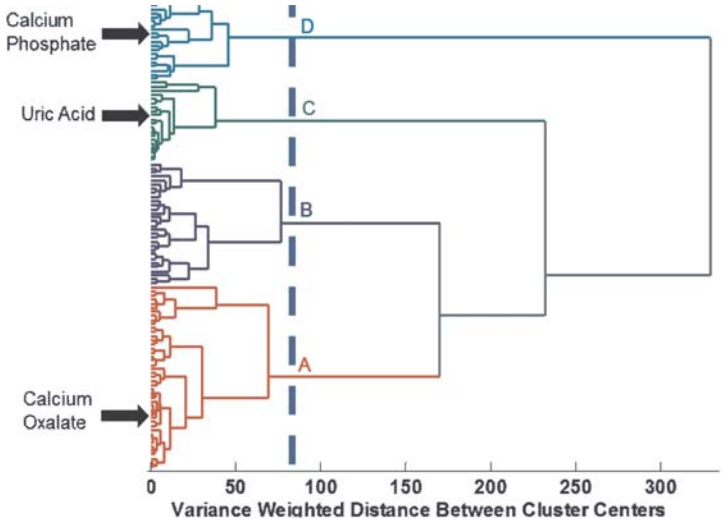

Figure 3. Hierarchical cluster analysis of kidney stone samples and standards using the Ward linkage method and the Euclidean distance applied to ATR-FT-IR spectra. Arrows indicate the location of the standards spectra inside the dendrogram.

Table 2
Classification of samples based of hierarchical cluster analysis of their ATR-FT-IR spectra.

Chemical Kit Classification	Cluster Classification			
	A	B	C	D
CO-UA	2	0	15	0
CO	13	1	3	0
CO-AP	0	4	2	1
CO-AMP	2	4	0	5
CO-CP	5	13	0	10
CO-PT	20	8	0	3

CO (Calcium Oxalate), UA (Uric Acid), AP (Ammonium Phosphate), AMP (Ammonium Magnesium Phosphate), CP (Calcium Phosphate), PT (Phosphate traces).

For some samples the cluster classification carried out do not fit well with the chemical method results (table 2) and, as for example, in class C we can find samples classified as pure calcium oxalate by the chemical method although their spectra totally fits with that of uric acid standard (figure 5a). In this regard, we report a couple of samples which were identified as calcium oxalate-uric acid mixture according by the Ecoline kit but, as can be seeing in their spectra (figure 5c), there was no evidence of the presence of uric acid. In addition, other samples classified by the chemical kit as ammonium phosphate showed spectra witch matches well with a calcium phosphate-calcium oxalate mixture (figure 5b). In summary, considering the high reliability of the renal calculi classification based on the big differences among the FTIR spectra of those compounds, it can be concluded that this classification error is caused by misidentification problems using chemical kits.

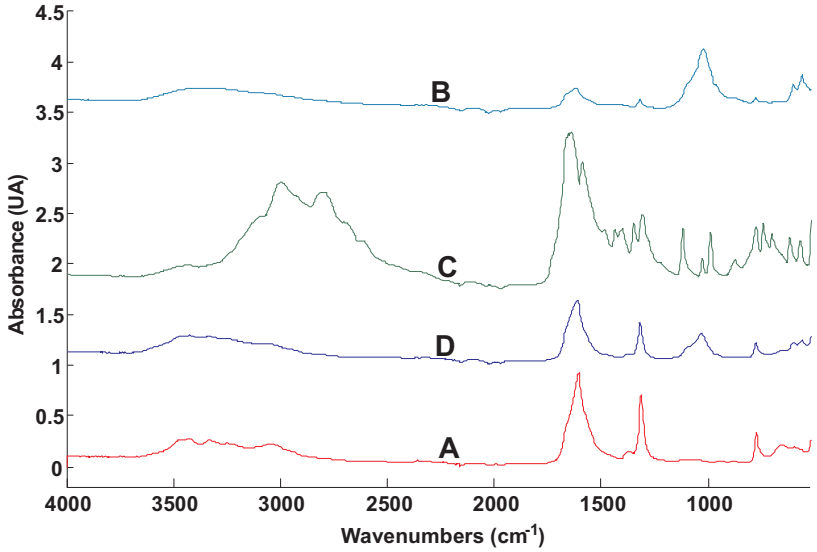

Figure 4. Average spectra of the four different sample classes established from hierarchical cluster analysis. A, B, C, D corresponding to the sample class indicated in Figure 3 and Table 2. Note: Spectra were shift on the y-axis to clearly show their bands.

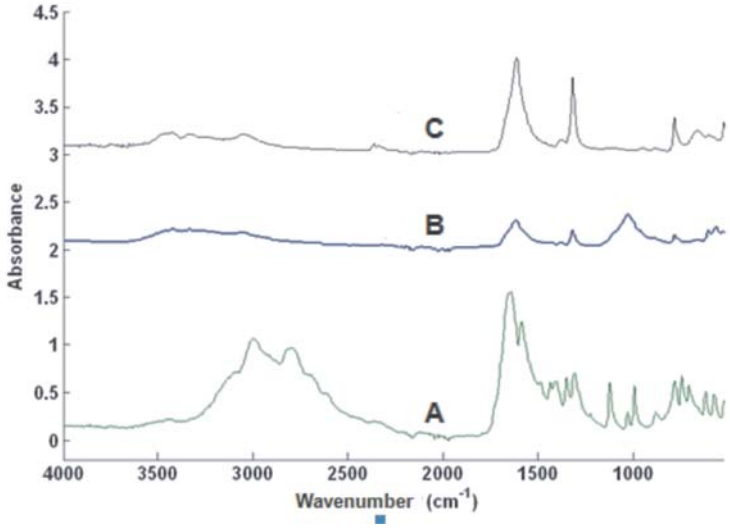

Figure 5. Spectra of misclassified samples according with the use of the chemical kit: A) sample 14, classified as calcium oxalate, B) sample 26, classified as ammonium phosphate and C) sample 16, classified as mixture of calcium oxalate-uric acid.

5. Conclusions

It has been evidenced that ATR-FT-IR direct spectra measurements of kidney stones coupled with hierarchical cluster analysis provides a fast, low-cost and environmental friendly method to classify samples in order to establish the clinical origin of urinary calculi. We have shown that ATR-FTIR methodology avoids mistakes found in

some cases when chemical kits have been used for calculi studies, being suitable for rapid characterization of this type of alterations. As compared with previous work on the use of infrared ATR for renal calculi determinations, we offer the possibility of an increasing of sensitivity by using a three internal reflections accessory, and do not require the use of microscopy or complex interpretation algorithms for spectra processing and interpretation.

Acknowledgments

Authors gratefully acknowledge the financial support of the Ministerio de Educación y Ciencia (Project CTQ2011-25743) and the Generalitat Valenciana Project PROMETEO 2010-055 and the kindly support from the Fundación para la Investigación from University Hospital Dr Peset. D.P.G. acknowledges the "V segles" grant from the University of Valencia.

References

1. DAUDON M. ***Annal D'Urol*** 39(6): 209-231. 2005.
2. SCHUBERT G. ***Urol Res*** 34(2): 146-150. 2006.
3. CHOU Y.H., LI C.C., WU W.J.; JUAN Y.S., HUANG S.P., LEE Y.C., LIU C.C., LI W.M., HUANG C.H., CHANG A.W. ***Kaohsiung J Med Sci*** 23(2): 63-66. 2007.
4. SOUCIE J.M.; THUN M.J.; COATES R.J.; MCCLELLAN W.; AUSTIN H. ***Kidney Int*** 46(3): 893-899. 1994.
5. ZHANG J., WANG G.Z., JIANG N., YANG J.W., GU Y., YANG F. ***Urol Res*** 38(2): 111-115. 2010.
6. ESTEPA L., DAUDON M. ***Biospectrosc*** 3(5): 347-369. 1997.
7. GHOSH S., BASU S., CHAKRABORTY S., MUKHERJEE A.K. ***J Appl Crystallogr*** 42: 629-635. 2009.
8. MARICKAR Y.M.F., LEKSHMI P., VARMA L., KOSHY P. ***Urol Res*** 37(5): 271-276. 2009.
9. CARMONA P., BELLANATO J., ESCOLAR E. ***Biospectrosc*** 3(5): 331-346. 1997.
10. HALL P.M. ***Clin J Med*** 76(10): 583-591. 2009.
11. CORNS C.M. ***Ann. Clin. Biochem.*** 20: 20-25. 1983.
12. KHALIL S.K.H., AZOOZ M.A. ***J Appl Sci Res*** 3(5): 387-391. 2007.
13. LEHMANN C.A., MCCLUREB G.L., SMOLENSA I. ***Clin Chim Acta*** 173(2): 107-116. 1988.
14. OLIVER L.K., SWEET R.V. ***Clin Chim Acta*** 72(1): 17-32. 1976.
15. SINGH I. ***Int Urol Nephrolog*** 40(3): 595-602. 2008.
16. VOLMER M., BOLCK A., WOLTHERS B.G., DERUITER A.J., DOORNBOS D.A., VANDERSLIK W. ***Clin Chem*** 39(6): 948-954. 1993.
17. VOLMER M., WOLTHERS B.G., METTING H.J., DEHAAN T.H.Y., COENEGRACHT P.M.J., VANDERSLIK W. ***Clin Chem*** 40(9): 1692-1697. 1994.
18. BENRAMDANE L., BOUATIA M., IDRISSI M.O.B., DRAOUI M. ***Spectrosc Lett*** 41(2): 72-80. 2008.
19. GULLEY-STAHL H.J., HAAS J.A., SCHMIDT K.A., EVAN A.P., SOMMER A.J. ***Appl Spectrosc*** 63(7): 759-766. 2009.
20. VOLMER M., DE VRIES J.C.M., GOLDSCHMIDT H.M.J. ***Clin Chem*** 47(7): 1287-1296. 2001.
21. GULLEY-STAHL H.J., BLEDSOE S.B., EVAN A.P., SOMMER A.J. ***Appl Spectrosc*** 64(1): 15-22. 2010.