Identification of Melamine/Cyanuric Acid-Containing Nephrolithiasis by Infrared Spectroscopy

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Melamine-contaminated milk formula caused infant nephrolithiasis in some areas of China. Its combination with cyanuric acid causes crystallization in renal tubules. Following this renal damage and even renal failure that require long-term hemodialysis has been reported. Therefore, correct and timely diagnosis of these complex diseases is critical. Melamine containing stone is a combination of equal molar ratios of common stone compositions that has been reported from previous animal studies. We have previously identified the compositions of urinary tract stones with infrared (IR) spectroscopy. We hypothesized that the absorbance of wavelength of IR can identify melamine/cyanuric acid in the presence of mixing human stone compositions. In this study, we made an artificial stone

composition and examine under IR absorbance by mixing equal molar ratios of melamine/cyanuric acid with different types of human urinary stones, and established a reference of IR analysis for the identification of melamine/cyanuric acid-containing human urinary tract stones. Knowledge of the precise stone composition allowed institution of appropriate prophylactic dietary and medical therapy and this may help in the prevention of urinary stone recurrence. The results are promising that melamine and cyanuric acid can be identified clearly in a low percentile (~1%) of stone mixture pellet. Therefore, IR seems to be an ideal tool for the identification of melamine/cyanuric acid-containing stones. *J. Clin. Lab. Anal.* 24:92–99, 2010.

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Key words: cyanuric acid; infrared spectroscopy; melamine; nephrolithiasis; urinary stone analysis

Grant sponsor: National Science Council of Taiwan; Grant numbers: NSC 97-2815-C-039-028-B; NSC 97-2320-B-039-022-MY3; Grant sponsor: China Medical University; Grant numbers: CMU97-031; CMU97-267; CMU97-279; Grant sponsor: Taichung Medical Association, Taiwan Medical Association.

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Received 7 July 2009; Accepted 11 November 2009

DOI 10.1002/jcla.20362

Published online in Wiley InterScience (www.interscience.wiley.com).

INTRODUCTION

In 2008, melamine [2,4,6-triamino-1,3,5-triazine, a chemical intermediate used to manufacture amino resins and plastics (1) and has also been marketed as a fertilizer because of its high nitrogen content (2)]-contaminated milk formula caused infant nephrolithiasis in some areas of China. Its combination with cyanuric acid or the analogues, including ammeline and ammelide, appear to cause crystallization in renal tubules (3). Most of the infants were asymptomatic, and a few suffered from stone-induced urinary obstruction resulting in acute renal failure (4). The department of health in Taiwan also initiated services to screen exposed children to address public concerns about melamine and cyanuric acid. Wang et al. showed that consumption of melamine-contaminated formula appeared to be a major factor in the nephrolithiasis development. Because of the lack of symptoms and signs, they recommend that ultrasonography be performed in children exposed to melamine-contaminated dairy products (5). Therefore, correct and timely diagnosis as well as active treatment of this complex disease are critical factors that help the infants to recover the renal function and prevent multiple organ system failure (6).

There have been many methods suggested for the analysis of the composition of urinary stones (7). Infrared (IR) absorption spectrometry, a tool for the identification of organic chemicals, is used to get information about the compound's structure and purity, as well as sometimes to compare with each other (8). This technique is specific, rapid, and versatile, and can be used with crystalline and amorphous substances. IR spectroscopy is based on the interaction between the infrared light and the molecules in the compounds. Different types of bonds and different functional groups of compounds absorb IR radiation of different wavelengths. Therefore, identification from bonds or functional groups can be made (9). Since 1972, Fourier transform IR (FT-IR) (10) has been widely used in the analysis of human stone compositions because different stone compositions have different characteristic absorbance wavelengths (7). However, little information is available yet concerning human urinary stone, which contains melamine and/or cyanuric acid. This issue maybe because there are no ideal and rapid methods for the identification of urinary stone containing melamine.

Melamine causing stone is a combination of equal molar ratios of common stone compositions (e.g., uric acid) that has been reported from previous animal studies (11) and we have previously identified the compositions of urinary tract stones with IR spectroscopy (12). We hypothesized that the absorbance of

wavelength of IR can identify melamine/cyanuric acid in the presence of mixing human stone compositions. In this study, we made an artificial stone composition and examine under IR absorbance by mixing equal molar ratios of melamine/cyanuric acid with different types of human urinary stones to establish a reference of IR analysis for the identification of melamine/cyanuric acid-containing stones.

MATERIALS AND METHODS

Sample Preparation and IR Spectroscopy

Samples of stones were obtained from previous analyzed specimens, where the compositions were verified by IR spectroscopy and scanning electron microscopy with energy-dispersive analysis by X-ray (EDAX, JEOL Ltd., Tokyo, Japan) (7,12). Four stones representative of calcium oxalate (CaOx), uric acid, cystine, and calcium phosphate (CaP) were used. Melamine and cyanuric acid were purchased from ChemService (West Chester, PA) and Sigma-Aldrich (St. Louis, MO), respectively. Each stone was fragmented into powdered format, compressed into a nearly transparent wafer with potassium bromide (KBr) (ACROS, Morris Plains, NJ) as original stone group reference examined by IR spectroscopy. The experimental group used half the amount of stone powder with half the amount of melamine (with or without cyanuric acid) and KBr to compress a wafer. Different ratios (50, 40, 30, 20, 10, 5, and 1%) of melamine/cyanuric acid were added to the stone powder and analyzed to identify the detection limit of melamine-cyanuric acid in stones.

Spectra were obtained by KBr disc technique and KBr itself had no absorption. Samples to be analyzed were thoroughly ground with an agate pestle and mortar and intimately mixed with KBr powder at a sample concentration of approximately 1% and their spectra were obtained in the KBr matrix. Suitable discs were obtained for the analysis, prepared by ensuring adequate grinding of the sample, dryness of the KBr powder, and application of sufficient pressure during pressing of the disc.

The FT-IR instrument is an automatic recording double beam infrared spectrophotometer that measures and records percent transmittance of a sample at any frequency in the infrared zone. Components of the stone are evaluated according to the wave that is absorbed. All samples were examined and IR data was recorded between 4,000 and 400 cm^{-1} by the SHIMADZU IRPrestige-21 (SHIMADZU, Kyoto, Japan) infrared spectroscopy. The standard procedure of the interpretation of the data from stone powder was based on the previous reports (12–14). This spectrum has absorption peaks corresponding to the

vibration frequency of the bonds of atoms comprising the stone. The peak size directly correlates with the quantity of the specific chemical. The FT-IRS spectrum is then computer matched against a library of spectra (of over 1,000 various bio and inorganic materials) so as to generate a precise report on the various stone components. Data from the mixture of stones with melamine/cyanuric acid were compared to the pure stone group by analyzing the difference of absorbance (3,470, 3,420, 3,335, 3,215, 1,653, 1,555, 1,470, 1,440, 1,030, and 815 cm^{-1} for melamine and 3,210, 3,060, 1,710, 1,425, 1,050, and 535 cm^{-1} for cyanuric acid) wave.

RESULTS

The presence of melamine is shown by absorptions at 3,470, 3,420, 3,335, 3,215, 1,653, 1,555, 1,470, 1,440, 1,030, and 815 cm^{-1} in its IR spectrum (Fig. 1A), which is most typical of melamine IR (Table 1) (15,16). In addition, the presence of cyanuric acid is shown by absorptions at 3,210, 3,060, 1,710, 1,425, 1,050, and 535 cm^{-1} in its IR spectrum (Fig. 1B), which is most typical of cyanuric acid IR (Table 2) (17,18). The wavenumber positions of transmission peaks, peak intensities, and peak widths are useful for functional

Fig. 1. Concordance of FT-IR transmission spectra of (A) melamine, (B) cyanuric acid, (C) CaOx, (D) uric acid, (E) cysteine, and (F) CaP.

TABLE 1. Infrared Absorptions of Melamine

Wave numbers (cm ⁻¹)	Assignment
3,470–3,420	NH ₂ stretch, typical of melamine, absent in salts of strong acids
3,335	Asymmetric NH ₂ stretch
3,125	Symmetric NH ₂ stretch
1,653–1,630	NH ₂ deformation
1,580–1,555	1,3,5- <i>s</i> -triazine ring “quadrant stretch”
1,470–1,440	1,3,5- <i>s</i> -triazine ring “semicircle stretch”
1,030	C–N stretch, primary amines, tertiary C
815	1,3,5- <i>s</i> -triazine, out of plane ring bending by “sextants”

TABLE 2. Infrared Absorptions of Cyanuric Acid

Wave numbers (cm ⁻¹)	Assignment
3,210–3,060	Degenerate N–H stretch
1,800	Symmetric C–O stretch
1,710	Degenerate C–O stretch
1,425–1,410	Degenerate N–H in-plane bend
1,065–1,050	Degenerate in-plane ring stretch
545–535	Degenerate C–O in-plane bend

group and sample identification. Wavenumber positions of transmission bands are specific to the functional groups in a sample; thus, each sample has a unique “fingerprint” absorbance spectrum. Different stones (CaOx, uric acid, cystine, and CaP) that do not contain melamine/cyanuric acid are also shown (Fig. 1C–F) to contrast the differences in spectra caused by addition of the melamine or cyanuric acid.

Previous studies have shown that several types of stones including CaOx, uric acid, cystine, and CaP were the most common forms. The comparisons of FT-IR spectrum of the commonest stone types encountered melamine–cyanuric acid are shown in Figure 2: melamine, melamine+CaOx, melamine+uric acid, melamine+cystine, and melamine+CaP. The IR transmission peaks at 3,500–3,000 and 1,700–1,300 cm⁻¹ in the melamine spectra are attributed to the stretching and bending vibrations of amino groups present in the melamine. The absorption band present at 1,653 cm⁻¹ results from the stretching vibrations of >C=N bonds present in the triazine ring of melamine (15).

The combinations of melamine with cyanuric acid appear to cause crystallization in renal tubules. The IR spectrums of mixture of melamine with cyanuric acid (7:3 w/w) and the different types of urolithes (CaOx, uric acid, cystine, and CaP) with melamine–cyanuric acid are shown in Figure 3. Melamine and cyanuric acid can be easily recognized and distinguished from IR spectra of other compounds-containing urolithes. In addition,

Fig. 2. Comparison of FT-IR transmission spectra of melamine, melamine+CaOx, melamine+uric acid, melamine+cystine, and melamine+CaP. Blue arrows indicate the infrared absorptions of melamine at 3,470, 3,420, 3,335, 3,125, 1,653, 1,555, 1,470, 1,440, 1,030, and 815 cm⁻¹.

these two concern agents can be differentiated from each other in a compound spectrum by different infrared absorptions and assignments.

Since previous urinary calculi specimen analysis showed that calculus was composed mainly of melamine and uric acid (19), we next tested the detection limit of FT-IR with uric acid stone sample. Representative absorbance spectra are shown in Figure 4. Absorbance peaks related to the NH₂ group can be clearly identified in the melamine spectrum. The peaks near 3,470–3,420 cm⁻¹ are due to the overtone of NH₂ stretching, which also showed marked absorption in the spectra of pure melamine (Fig. 1A). Decreases in the absorption in this region were notable as the melamine concentration was decreased (Fig. 4). The results of detection limit for melamine–cyanuric acid in stones showed that melamine and cyanuric acid can be

Fig. 3. Comparison of FT-IR transmission spectra of melamine-cyanuric acid, melamine-cyanuric acid+CaOx, melamine-cyanuric acid+uric acid, melamine-cyanuric acid+cystine, and melamine-cyanuric acid+CaP. Blue arrows indicate the infrared absorptions of melamine at 3,470, 3,420, 3,335, 3,125, 1,653, 1,555, 1,470, 1,440, 1,030, and 815 cm^{-1} ; Red arrowheads indicate the infrared absorptions of cyanuric acid at 1,050 and 535 cm^{-1} .

identified clearly in a low percentile ($\sim 1\%$) of stone mixture pellet. Therefore, IR seems to be an ideal tool for the identification of melamine/cyanuric acid-containing stones.

DISCUSSION

The exact knowledge of renal stone composition may be able to predict the underlying medical disorder (20). The possibility of melamine-containing stone compositions

was examined by IR spectroscopy. In this study, we provide the first evidence that the absorbance of wavelength of IR can identify melamine (and its derivative cyanuric acid), with a computerized reference library match of the closest IR spectrum, in the presence of different types of mixing human stone compositions including CaOx, uric acid, cystine, and CaP.

To understand the chemical composition of urinary calculi is important for the etiology/management and for the prevention of recurrences; however, the

Fig. 4. IR absorption spectra, after automatic baseline correction and smoothing, of uric acid with melamine/cyanuric acid at 0.5, 1, 5, 10, 20, 30, 40, and 50% w/w: (a) entire IR spectral region ($4,000\text{--}400\text{ cm}^{-1}$) with a peak appearing at $3,470\text{--}3,420\text{ cm}^{-1}$ (due to the overtone of NH_2 stretching), which also showed marked absorption in the spectra of pure melamine, attributed to NH_2 , identified as the smallest visual spectral difference between uric acid and uric acid with melamine/cyanuric acid. Magnified image of the peak at $3,470\text{--}3,420\text{ cm}^{-1}$ region, where spectra with 1–50% w/w melamine can be clearly differentiated.

appropriate method for this has still not been completely defined (14,21). Currently, the methods are available for stone analysis including chemical analysis, emission spectroscopy, polarizing spectroscopy, X-ray diffraction (XRD) and coherent scatter/crystallography, thermogravimetry, scanning electron microscopy, and IR spectroscopy (7,14).

Melamine is a chemical intermediate used to manufacture amino resins and plastics. However, an epidemic of melamine contamination of powdered-milk formula-induced urinary tract stones in young children occurred recently in China. The addition of melamine to food elevates the apparent protein content, since melamine contains 66% nitrogen; this covert practice appears to

have been the cause of the melamine contamination of milk formula. In 2007, there was a major pet food recall in the USA following complaints about pet foods (22,23). The epidemic of renal failure and some deaths had occurred in animals exposed to pet food also contaminated with melamine (24,25); however, the relationship between melamine ingestion and urinary stones in humans was initially unclear (26). Cyanuric acid, a by-product of melamine synthesis, was also identified in the pet food as a co-contaminant in the pet food recall investigation (27). Although melamine and cyanuric acid are relatively nontoxic individually, it has previously been demonstrated that the combination of melamine and cyanuric acid (a stable compound-

melamine cyanurate with poor aqueous solubility) (28–30) is responsible for acute renal failure in cats (31). This compound precipitates in renal tubules and results in the formation of kidney stones and eventually leads to renal toxicity (32). Therefore, our study established a reference of IR analysis for the identification of melamine/cyanuric acid-containing stones.

Increased analytical methods for the determination of melamine in food were developed. The techniques used for this purpose so far are gas chromatography, liquid chromatography, mass spectrometry, or surface-enhanced Raman spectroscopy, etc. (27,33,34). However, biochemical analysis of biological solid samples is still a challenging task. An accurate analysis of the composition is important for understanding the cause of urinary tract stone formation and determining the proper clinical treatment. In vivo ultrasound imaging is common for kidney stone screening but cannot provide chemical composition information. FT-IR and powder XRD are the two most common and relatively fast methods adopted for in vitro composition analysis of kidney stones (14,35), but a large sample amount is required. Optical and electronic microscopy has also been applied to examine the morphology of kidney stones (36,37). Tang et al. described a sensitive method for the chemical analysis of solid urine stones/residues containing melamine using matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) (32). Another group also presented a method for the determination of melamine in milk powder samples using capillary zone electrophoretic with either ultraviolet or MS detection (38). More recently, Lu et al. and Mauer et al. established methods for detecting pure melamine in infant formula powder using IR spectroscopy (39,40). With partial least-squares models, infrared spectroscopy methods could be used to evaluate for the detection and quantification of melamine in infant formula powder; their data showed that FT-IR methods could enable detection of 1 ppm melamine in infant formula powder. By contrast, the purpose of our study was to rapidly identify (not for the detection limit or quantification) melamine/cyanuric acid-containing nephrolithiasis (with different clinical stone samples) by infrared spectroscopy, since it will not be considered as a melamine/cyanuric acid-containing nephrolithiasis clinically if the concentrations of melamine/cyanuric acid are very rare in the stone samples.

With a computerized reference library match of the closest IR spectrum, FT-IR can characterize virtually most stone samples. FT-IR allows the detection of both crystalline and non-crystalline substances detectable. In addition, FT-IR is a sensitive, reliable, accurate, safe and quick, and moderate costs method of accurate stone analysis suitable for use in a clinical laboratory (41–43).

However, time-consuming preparation and difficulty in differentiation for some mixed stones are its limitations. X-ray differentiation and MALDI-MS have been proved to be the best methods for stone analysis, but the disadvantage of the highest cost make was that it was not popularly used in Taiwan. Instead, IR spectroscopy became the suitable choice for stone analysis in most laboratories. Nevertheless, FT-IR has some limitations: (1) the procedure is dependent on a proper homogenization of the sample with KBr, which may affect the IR spectrum quality; (2) resolution of the apparatus and reproducibility of the spectrum bands may affect its reliability; (3) certain mixtures may be problematic and difficult to interpret in cases of low proportions (44).

CONCLUSIONS

In this study, we made an artificial stone composition and examined under IR absorbance by mixing equal molar ratios of melamine with different types of human urinary stones and for the first time established a reference of IR analysis for the identification of melamine-containing human urinary tract stones. Knowledge of the precise stone composition allowed institution of appropriate prophylactic dietary and medical therapy and this may help in the prevention of urinary stone recurrence.

ACKNOWLEDGMENTS

We thank Miss Yun-Chen Hsu for manuscript preparation.

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