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Increased detection rate of melamine-containing calcium urolithiasis by using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry technique in clinical practice

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ABSTRACT

Background: Studies have shown that melamine may be associated with urolithiasis. A more sensitive method is needed to analyze melamine in urinary stones to identify potential causes of urolithiasis.
Methods: Here we compare the analytical methods of detecting melamine in urinary stones by Fourier transform infrared (FTIR) spectrophotometer and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) in the laboratory and clinic. First, we established the melamine detection limit in melamine-cyanurate standard by the methods of FTIR spectrophotometer and MALDI-TOF MS. Subsequently, we applied these methods to 54 adult patients with upper urinary tract calcium urolithiasis.
Results: The detection limit of melamine in melamine cyanurate standard by MALDI-TOF MS was ~10,000-fold more sensitive than FTIR. We applied both instruments to 54 stone specimens from 54 calcium urolithiasis patients. In those without distinctive melamine pattern in the FTIR spectra, melamine could be detected by MALDI-TOF MS in an additional 12 out of 42 subjects' stone specimens (28.6%). Compared to MALDI-TOF MS-negative subjects ($n = 30$), those positive subjects ($n = 12$) excreted significantly higher urinary melamine levels ($P < 0.05$).
Conclusions: Compared to FTIR, MALDI-TOF MS is a more sensitive method to detect the content of melamine in melamine-containing kidney stones.

1. Introduction

Since 2008, exposure to melamine has emerged as a public health concern due to an outbreak of kidney stones and renal failure in infants caused by the melamine-adulterated formula in China [1]. Until now, melamine has been ubiquitously present in food and feed products [2–4]. There is increasing evidence that low melamine exposure may be a culprit of urolithiasis formation, even in adults [5–7]. Since understanding of the stone composition can determine the subsequent strategies of its prevention, management, and treatment in conventional clinical practice, Fourier transform infrared (FTIR) spectrophotometer is the most commonly used method to analyze and diagnose stone composition such as calcium, uric acid, or cystine calculi [8]. However, whether its sensitivity and specificity of analytical ability is enough to detect melamine in the stone specimens in the coming era of newly-emerging environmental diseases such as melamine-containing calculi is doubtful [9]. Recently, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been suggested to be a sensitive tool to screen and determine the presence of melamine in urinary stones in clinical practice [10]. Nevertheless, this state-of-the-art technique has not been routinely applied to adult calcium calculi, although this illness is the most prevalent one of urolithiasis and its recurrent rate is relatively high [11,12]. Our previous preliminary study found that melamine content could be qualitatively detected in 9 out of 9 (100.0%) calcium stones [5]; however, that study did not address any quantitative issue of detection of melamine in human stone specimens.

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2. Materials and methods

First, we established the melamine detection limit in melamine-cyanurate standard by the methods of FTIR spectrophotometer and MALDI-TOF MS. The detailed analytical methods were described previously [13]. Subsequently, we applied these 2 methods to 54 adult patients with upper urinary tract calcium urolithiasis and whose stone specimens were still available from our previous cohort study [5]. The study was approved by the Institutional Review Board of Kaohsiung Medical University Hospital and written informed consent was obtained from each participant.

2.1. Preparation standard of melamine-cyanurate complex

Melamine and cyanuric acid were purchased from Sigma-Aldrich. Preparation standard of melamine-cyanurate complex has been described in detail elsewhere [13]. It is believed that melamine encounters cyanuric acid to form a stable compound with poor aqueous solubility, precipitate in renal tubules, and result in the formation of kidney stones [14]. Thus, we chose this complex as the standard.

2.2. Analytical method of FTIR spectrophotometer

Melamine content in the melamine-cyanurate complex was analyzed by FTIR (Spectrum RX I Fourier Transform-Infrared System) using the technique of potassium bromide (KBr) disc and infrared spectroscopy [12,15] and has been described in detail previously [13].

To define the detection limit of melamine in the melamine-cyanurate complex by FTIR, we used 0.35 mg of complex mixed with 1% KBr as the original reference standard, and then made different standards with different amounts of complexes (0.117 mg, 0.039 mg, 0.013 mg, 4.333 μg, and 1.444 μg) based on the same ratio of reduction to a third amount of complex in order with KBr. The measurements of melamine were done in three independent experiments. The typical absorbance peaks of melamine structure were near 3500–3000/cm due to the overtone NH2 stretching.

After the establishment of FTIR analytical method and the typical pattern of melamine, the FTIR spectra of stone specimens from urolithiasis patients were evaluated by two independent scientists (CC Wu and CC Liu) who were blinded to subject urinary melamine levels to detect any typical melamine absorbance peaks. If the readings were different, further discussion was followed to reach a consensus.

2.3. Analytical method of MALDI-TOF MS

First, we used our established analytical method of melamine-cyanurate complex by MALDI-TOF MS [13] with slight modifications. The melamine-cyanurate complex (20 mg) was extracted with 1 ml 50% acetonitrile/water (v/v), sonicated for 1 h, and centrifuged at 9000 rpm for 10 min. An aliquot (1 μl) of the clear extract was mixed with saturated 2,5-dihydroxybenzoic acid (DHB) matrix solution instead of α-cyano-4-hydroxycinnamic acid (CHCA) in equal volume on the MALDI target. After the sample/matrix solution had been air-dried, the target was transferred into the ion source of a MALDI-TOF mass spectrometer (MALDI-TOF MS, Autoflex III, Bruker Daltonics GmbH). The following procedure was the same as previously published [13]. The typical melamine signal of [M + H]+ was at *m/z* 127 in MALDI-TOF MS spectra [5,10,13].

To determine the limit of detection of MALDI-TOF MS analysis for melamine, we spiked different amounts of melamine cyanurate complexes (41.3, 4.13, 0.413, 0.041, and 0.004 μg/ml) in 50% acetonitrile/water. The solution was then ultrasonicated, centrifuged, and the resultant supernatant was used for MALDI-TOF MS analysis. The sample/matrix solutions (1:1, v/v) were also prepared by on-spot mixing of the analytes with the saturated DHB solution on the MALDI plate and examined using MALDI-TOF MS.

2.4. Application of human stone specimens

Two-hundred eleven adult patients underwent surgical procedures to remove their upper urinary stones, and the stone specimens were confirmed to be calcium urolithiasis by the analyses of FTIR routinely in our urological clinic [5]. The rest of the stone specimens were kept in sterile containers for long-term storage. We retrospectively retrieved their FTIR spectra to evaluate whether they had the presence of the typical pattern of melamine. Since only 54 stone specimens from 54 case subjects were still available for further analyses by MALDI-TOF MS (Fig. 1), we described the analytical results in those 54 subjects.

2.5. Statistical analyses

Data were expressed as medians with interquartile range (IQR) unless otherwise indicated. Quantitative variables between different groups were compared by Kruskal–Wallis test, whereas qualitative variables were compared by Fisher's exact test. All tests were performed by SAS 9.2 statistical software.

3. Results

3.1. The detection limit of melamine by MALDI-TOF MS and FTIR spectrophotometer

The detection limit of melamine in melamine cyanurate standard by MALDI-TOF MS was 0.413 ng (injected 1 μl of 0.413 μg/ml melamine into the instrument), and mean (SD) signal-to-noise (S/N) ratio in 5 repeated experiments was 12.6 (1.5). In contrast, the negative control and saturated 2,3-dihydroxybenzoic acid (DHB) blank had no detectable melamine signal. For FTIR spectrophotometer, the detection limit of melamine was 4.333 μg; the differences of detection limit in these two instruments showed a ~10,000-fold difference (Fig. 2).

3.2. Detection of melamine by FTIR spectrophotometer and MALDI-TOF MS in calcium stone specimens

In our previous 211 calcium urolithiasis patients [5], 54 stone specimens from 54 case subjects were still available for further analyses by MALDI-TOF MS. There were no significant differences regarding demographic characteristics between case patients with (n = 54) and without (n = 157) stone specimens for further analyses (Table A.1).

Twelve (22.2%) out of 54 subjects' stone specimens had a distinctive melamine pattern in the FTIR spectra and the rest of 42 FTIR diagrams from 42 subjects' stone specimens did not show the distinct melamine pattern as compared to the typical melamine spectra in melamine concentration of 4.333 μg of melamine cyanurate standard (Fig. 2, A1).

In those without distinctive melamine pattern in the FTIR spectra, melamine could be detected via MALDI-TOF/MS in 12 out of 42 subjects' stone specimens (28.6%) (Figs. 1 and 3). Compared to MALDI-TOF MS-negative subjects (n = 30), MALDI-TOF MS-positive subjects (n = 12) excreted significantly higher urinary melamine levels without and with correction for urinary creatinine (P = 0.019 and 0.048 respectively) (Table 1). Other stone-related indexes, including stone episodes, stone number, stone size, severity score and family history of urinary stones were not significantly different between these subjects (data not shown). In contrast, urinary melamine levels corrected by urinary creatinine (Cr) were not significantly different between FTIR-positive subjects (median, 0.32 μg/mmol Cr, n = 12) and FTIR-negative subjects (0.29 μg/mmol Cr, n = 42, P = 0.693) (data not shown).

Among the 12 positive melamine spectra in 12 subjects, we analyzed their stored stone specimens by MALDI-TOF MS and found that in two out of 12, melamine could be detected (data not shown).