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Determination of melamine and cyanuric acid in human urine by a liquid chromatography tandem mass spectrometry

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ABSTRACT

Melamine was found to be the etiological factor for the urinary stones epidemic in infants and young children in China in 2008. Urine level of melamine and its analog cyanuric acid may be useful markers for the evaluation of toxic effects. Liquid chromatography tandem mass spectrometry methods for the individual determination of melamine and cyanuric acid in human urine are described. Using isotope labeled internal standards during liquid–liquid extraction, the method was fully validated by verifying specificity, linearity, LLOQ, intra- and inter-assay precision and accuracy, matrix effect, recovery and stability. Calibration curves with good linearity (r = 0.9999) over the concentration range from 10 to 5000 ng/ml, intra-assay precision <10% and inter-assay precision <15%, accuracy between 93.0 and 111.6% were obtained with multiple reaction monitoring mode for melamine and cyanuric acid in human urine. The methods were successfully applied to the analysis of urine samples collected from 86 infants and 110 adults.

1. Introduction

Melamine (Fig. 1A) is an organic base and a trimer of cyanamide, with a 1,3,5-triazine skeleton. It is generally used as the chemical material for the manufacture of plastics, resin, fabrics and even illegally added to food products in order to increase the apparent protein content [1]. In 2007, it was reported that pet foods contaminated with melamine and several analogues were probably responsible for renal disease or death in dogs and cats [2,3]. Melamine combines with cyanuric acid (Fig. 1B) to form melamine cyanurate crystal sediment. The histopathological investigation of affected animals indicated that kidneys contained large numbers of melamine cyanurate crystals. These crystals obstructed and damaged renal tubules, leading to renal failure [4,5]. It was also recently reported that melamine, along with several analogues, may be responsible for a similar development of kidney stones resulting to infant deaths as a result of drinking tainted formula [6].

Previous toxicological studies have demonstrated that melamine itself is of low toxicity, but may lead to crystal formation and subsequent kidney toxicity after it combined with cyanuric acid [5,7,8]. Melamine remains unchanged in the body and is mainly excreted within 24 h by the kidney following its administration [8]. Urine is the main route of melamine excretion and the concentrations in urine can be directly linked to the observed toxic effects. Hence, the assay of melamine and cyanuric acid in human urine may be used for clinical toxicity monitoring and diagnosis.

Recently, Cheng et al. reported an UPLC–MS/MS method to determine melamine in childrens urine in Taiwan region [9]. Patel and Jones described an analytical method for the quantitative determination of cyanuric acid in human urine [10]. Furthermore, various analytical approaches based on LC–MS/MS, HILIC-MS/MS, GC–MS/MS, MALDI-MS technique for the determination of melamine or cyanuric acid in other materials including cows milk, infant formula, pet foods have been described [11–16]. However, although these methods have good sensitivity and selectivity, they do not give evidence for clinical monitoring and diagnosis for human. For this reason, we developed sensitive and reliable liquid chromatography isotope diluted tandem mass spectrometry (LC-IDMS/MS) methods for both melamine and cyanuric acid in human urine with liquid–liquid extraction procedure. The validated methods were later applied to screening melamine and cyanuric acid in urine collected from 86 children (0–8 years) and 110 adults (25–57 years) in Shanghai Xuhui Central Hospital.

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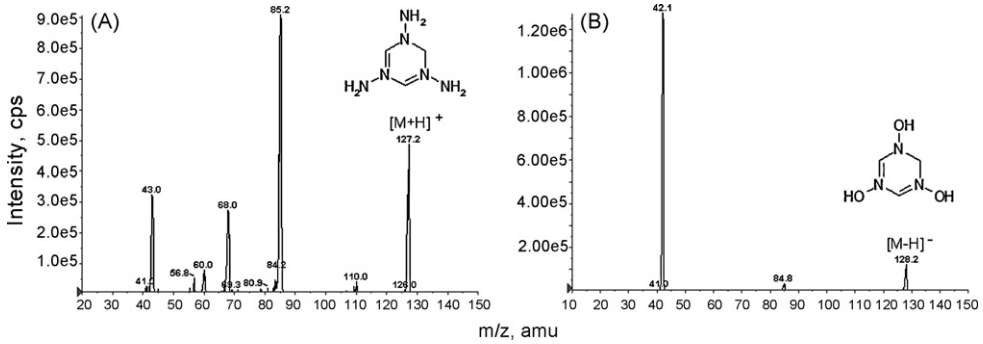

Fig. 1. Product ion mass spectra of (A) melamine, obtained with positive ionization mode and (B) cyanuric acid, obtained with negative ionization mode, by using single standard solution (10 μg/ml) with flow injection analysis.

2. Experimental

2.1. Standards and reagents

Melamine was purchased from Shanghai Anpel Scientific Instrument Co. Ltd. (Shanghai, China). Cyanuric acid was purchased from Sigma–Aldrich (Shanghai) Trading Co., Ltd. (Shanghai, China). Isotope labeled melamine-$^{13}C_3$, $^{15}N_3$ and cyanuric acid-$^{13}C_3$, $^{15}N_3$ used as internal standards (IS) were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). Acetonitrile, formic acid, methanol and ethyl acetate were purchased from Tedia Company Inc. (Fairfield, OH, USA). All the other reagents were analytical grade and obtained from Sinopharm Medicine Holding Co., Ltd. (Shanghai, China). Double distilled water was prepared in our laboratory and used throughout this study.

2.2. Preparation of calibration standards and quality controls

Stock solutions of melamine and cyanuric acid were prepared in 10% and 50% methanol from two separate weighing, one for calibrators and another for quality controls, respectively. Working solutions were obtained by serially dilution stock solutions. Stock solutions were stored at 4 °C and kept stable for 4 weeks. Calibration standards and quality controls were obtained by spiking 50 μl working solutions of melamine and cyanuric acid into 950 μl blank urine to give 10, 50, 100, 500, 1000, 2500, 5000 ng/ml for calibration standards and 10, 20, 2000, 4000 ng/ml for quality controls. Before preparation of calibration standards and quality controls in urine, blank urine was screened for the absence of melamine and cyanuric acid.

2.3. Sample preparation

Because the polarity of melamine and cyanuric acid is quite different, two separate sample preparation procedures were designed.

For melamine, 100 μl urine was transferred to the polypropylene tube, followed by adding10 μl IS working solution (2.5 μg/ml melamine-$^{13}C_3$, $^{15}N_3$ in 10% methanol) and 20 μl 1% (w/v) NaOH solution. This mixture was extracted with 1 ml ethyl acetate by vortex-mixing for 10 min, followed by centrifugation for 3 min at 36,220 × *g*. The upper organic layer was transferred to another clean tube and back-extracted with 100 μl 0.1% (v/v) formic acid in water solution by vortex-mixing for 10 min and centrifugation for 3 min at 36,220 × *g*. The water layer was used for LC-MS/MS analysis.

For cyanuric acid, 100 μl urine was transferred to a polypropylene tube, followed by adding 10 μl IS working solution (5 μg/ml cyanuric acid-$^{13}C_3$, $^{15}N_3$ in 50% methanol) and 20 μl 1% (v/v) formic acid solution. The mixture was extracted with 1 ml ethyl acetate: isopropanol (95:5, v/v) by vortex-mixing for 10 min and centrifugation for 3 min at 36,220 × *g*. The upper organic layer was transferred to another clean tube and blown to dryness under a stream of nitrogen under 37 °C. The residue was reconstituted with 100 μl 70% methanol solution and 5 μl was used for LC-MS/MS analysis.

2.4. LC–MS/MS conditions

The LC-MS/MS conditions for melamine and cyanuric acid were also different.

For melamine, the chromatographic separation of melamine was carried out on Shimadzu LC-20AD HPLC system (Kyoto, Japan) equipped with two LC-20AD pumps, a HIL-HTc auto-sampler and an online DGU-20A3 vacuum degasser. The analytical column was Allure PFP Propyl (100 mm × 2.1 mm I.D., 5 μm, Restek, USA) coupled with a C_{18} guard column (4.0 mm × 3.0 mm I.D., 5 μm, Phenomenex, USA). The mobile phase was composed of phase A (50% methanol/50% acetonitrile, containing 0.02% formic acid) and phase B (water, containing 0.02%formic acid). Melamine was eluted at a flow rate of 0.4 ml/min with an isocratic program of A/B at 70:30 (v/v). An aliquot of 5 μl was injected for HPLC analysis, melamine showed retention time about 1.9 min and a total run time of 3.5 min was achieved. An API 3000 triple quadrupole tandem mass spectrometer (Applied Biosystems/MDS Sciex, Toronto, Canada) equipped with a Turbo Ionspray source was operated in positive ionization mode. Ion transitions of *m*/*z* 127/68 and *m*/*z* 127/85 were used for quantitative and qualitative detection of melamine, respectively and *m*/*z* 133/72 for IS detection. High pure nitrogen was used for mass spectrometry analysis. The source/gas parameters were set as: nebulizer gas 10, curtain gas 10, collision gas 7, ion spray voltage 5500 V and the source temperature 450 °C.

For cyanuric acid, the HPLC mode used was same as melamine. The analytical column was an XBridge Phenyl (150 mm × 2.1 mm I.D., 5 μm, Waters, USA) coupled with a C_{18} guard column (4.0 mm × 3.0 mm I.D., 5 μm, Phenomenex, USA). The mobile phase was methanol containing 0.01% formic acid at a flow rate of 0.35 ml/min. A sample volume of 5 μl was injected and a total run time of 3 min was achieved. The typical retention time was about 1.3 min for cyanuric acid and internal standard. The 3200 QTrap mass spectrometer (Applied Biosystems/MDS Sciex, Toronto, Canada) was operated in negative ionization mode. Ion transitions of *m*/*z* 128/42 and *m*/*z* 128/85 were used for quantitative and qualitative detection of cyanuric acid, respectively and *m*/*z* 134/44 for IS detection. The source/gas parameters were set as: nebulizer gas 50, curtain gas 20, collision gas High, ion spray voltage −4500 V and the source temperature 500 °C. Analyst 1.4.0 Software (Applied Biosystems/MDS Sciex, Toronto, Canada) was used for instrument control and data acquisition.

2.5. Method validation

The methods were validated by verifying linearity, lower limit of quantification (LLOQ), intra- and inter-assay precision and accu-

racy, matrix effect, extraction recovery and stability for melamine and cyanuric acid in human urine. It was carried out according to the Guidance for Industry Bioanalytical Method Validation recommended by Food and Drug Administration (FDA) [17].

3. Results and discussion

3.1. Blank residue of melamine and cyanuric acid

In this study, an interesting finding was the significant cyanuric acid peak (Fig. 2) observed in the blank urine. A similar finding has been reported in our previous study in monkeys after oral administration of melamine [18]. And we also found that melamine generally presented in human urine. To determine if it is the source of the contamination, the distilled water and methanol were assayed for melamine and cyanuric acid residue. No such residue or contamination was found in water or methanol used in our experiment. After different sources of blank urine were investigated, although melamine and cyanuric acid generally present in a low level (ranged 10–100 ng/ml in most case) in urine, some "pure" blank urine (no residue found) were screened and later used as the blank matrix for method development and validation. Melamine is commonly used as industrial material in the production of melamine resins, laminates, glues, adhesives, moulding compounds, coatings and flame retardants. Cyanuric acid may be found as an impurity of melamine. And cyanuric acid was also used as FDA-accepted component of feed-grade biuret or water disinfectant for swimming pool [7]. Due to widespread use of melamine and cyanuric acid, not necessarily due to adulteration or contamination, as material in contact with food, low levels may be detected in food, so we presume that the source of melamine and cyanuric acid detected in human urine may be the result of migration from food contact material into foods.

3.2. Method validation

3.2.1. Specificity

Melamine and cyanuric acid at three concentration levels of 20, 2000, 4000 ng/ml were added to six different sources of urine. The accuracy of melamine and cyanuric acid was in the range of 95.5–113.7% and 77.3–118.8%. The RSD was respectively lower than 7.2% and 15.1%, which indicated that the specificity of these methods was sufficient. No significant batch-to-batch variation was observed.

3.2.2. Linearity and LLOQ

After the sample preparation and LC-MS/MS conditions were defined, a full validation was performed to assess the performance of the methods. A seven-point calibration standard curve ranging from 10 to 5000 ng/ml was used in duplicate for each analytical run (three runs in total) with $1/x$ weighting. The mean correlation coefficients (r) of all of the curves (both melamine and cyanuric acid) were all above 0.999. The RSDs at each level of melamine were less than 10%, with accuracy ranging from 91.5 to 105.0%. The corresponding values for cyanuric acid were RSDs less than 7%, with accuracy ranging from 89.3 to 106.0%. Thus, the calibration curves exhibited good linearity within the chosen range. The lower limit of quantification (LLOQ), which reflects the signal-to-noise ratio (S/N), was more than 6 at 10 ng/ml for both melamine and cyanuric acid (Fig. 3). As can be seen from Table 1, acceptable precision and accuracy for the LLOQ were obtained.

3.2.3. Accuracy and precision

Inter- and intra-assay accuracy and precision for assays were characterized by the four levels of QCs run on three sequential batches in six replicates. All QC samples were randomized daily, processed and analyzed, together with calibration samples. The precision was expressed as a relative standard deviation (RSD) by calculating the standard deviation as the percentage of the mean calculated concentration, while the accuracy of the assay was determined as the percentage of the mean with reference to the nominal concentration. Table 1 showed the accuracy and precision of melamine and cyanuric acid. Based on the accuracy and precision data presented here, it was concluded that the proposed method was precise and sufficiently accurate for the determination of melamine and cyanuric acid in human urine.

Table 1
The intra- and inter-precision and accuracy of melamine and cyanuric acid in quality controls of the analytical method.

Nominal conc. (ng/ml)	Intra-assay (n = 6)		Inter-assay (n = 18)	
	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)
Melamine				
10	5.8	111.6	10.1	100.9
20	4.0	104.5	7.3	103.0
2000	4.0	94.7	5.9	101.1
4000	0.9	93.0	4.0	97.7
Cyanuric acid				
10	7.4	100.2	14.4	97.8
20	5.4	98.0	10.0	98.6
2000	3.5	99.0	3.6	99.2
4000	2.4	97.7	2.7	99.1

3.2.4. Recovery and matrix effect

To compare the detection response of melamine and cyanuric acid in urine from different subjects or sources, the recovery and matrix effects were evaluated by calculating the peak area ratio using six lots of blank urine. The recovery was assessed by comparing the percentage of peak areas of melamine and cyanuric acid spiked to blank urine with those in neat QC chemical standards and matrix effect was assessed by comparing the percentage of peak areas of melamine and cyanuric acid in neat QC chemical standards with those of standards spiked to blank urine after extraction. The experiment was performed in six different lots of urine at three QC levels. The recoveries at 20, 2000, 4000 ng/ml were 17.7%, 20.2%, 20.9% for melamine and were 70.9%, 71.5%, 70.1% for cyanuric acid, respectively. The recoveries of IS for melamine and cyanuric acid were 19.7% and 66.7%, respectively (Table 2). The methods demonstrated almost no matrix effect from biological material for melamine and cyanuric acid at 20, 2000, or 4000 ng/ml.

3.2.5. Stability

The melamine and cyanuric acid stabilities were investigated in terms of auto-sampler stability, bench top stability and freeze-thaw stability. The bench top stability was assessed by analyzing QC samples spiked in urine kept at room temperature for 0, 2, 6 and 24 h. The auto-sampler stability was assessed by analyzing extracted quality control samples in auto-sampler at room temperature for 0, 2, 4 and 6 h. Freeze-thaw stability was evaluated after three cycles of QC samples in urine from −20 °C to room temperature. The stability of stock solution in 10% methanol (melamine) and 50% methanol (cyanuric acid) was also evaluated at 4 °C for 0, 2, 4 weeks. The relative standard deviation (RSD) was expressed by calculating the standard deviation as the percentage of the mean calculated concentration at time intervals investigated, while the accuracy of the assay was determined as the percentage of the mean at time intervals with reference to the nominal concentration. These results are outlined in Table 3. The analytes remained considerably stable under the above conditions at all time intervals investigated. The good stability of melamine and cyanuric acid simplified the precautions needed for laboratory manipulations during the assay procedures.

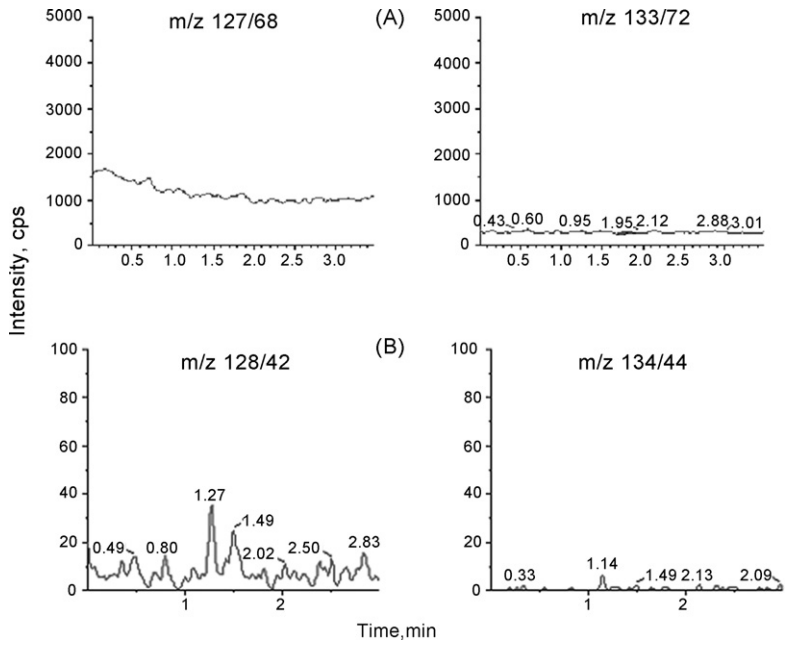

Fig. 2. MRM chromatograms resulting from the analysis of (A) melamine (*m*/*z* 127/68) and its internal standard (*m*/*z* 133/72), and (B) cyanuric acid (*m*/*z* 128/42) and its internal standard (*m*/*z* 134/44) acquired from blank human urine. No other interferences were detected except for a low level of cyanuric acid background.

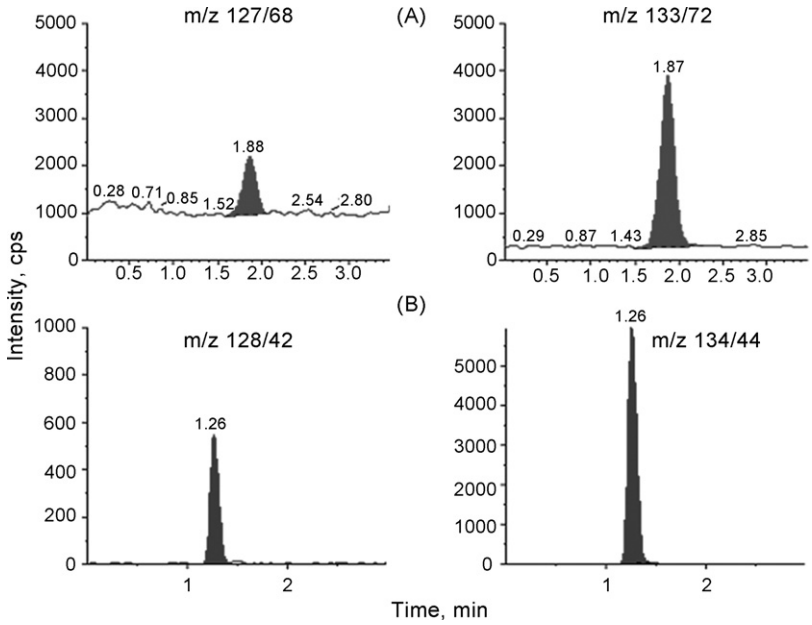

Fig. 3. MRM chromatograms resulting from the analysis of (A) melamine (*m*/*z* 127/68) and its internal standard (*m*/*z* 133/72), and (B) cyanuric acid (*m*/*z* 128/42) and its internal standard (*m*/*z* 134/44) acquired from lower limit of quantification (LLOQ, 10 ng/ml) spiked to blank human urine. The sensitivity was demonstrated by the signal-to-noise ratio (S/N) >6 at LLOQ level.

Table 2
Extraction recovery and matrix effect of melamine and cyanuric acid in human urine.

Nominal Conc. (ng/mL)		20	2000	4000	250 (IS)
Melamine	ER (%)	17.7	20.2	20.9	19.7
	ME (%)	96.7	98.3	96.9	97.2
Cyanuric acid	ER (%)	70.9	71.5	70.1	66.7
	ME (%)	98.5	99.3	97.4	96.5

ER: extraction recovery; ME: matrix effect.

Table 3
Stability of melamine and cyanuric acid investigated in auto-sampler, on bench top, after freezing-thaw and in stock solution.

Nominal Conc. (ng/ml)	Melamine		Cynuic acid	
	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)
Auto-sampler stability (room temperature for 6 h after processing)				
20	3.8	106.2	7.4	105.2
2000	2.0	98.8	1.6	99.5
4000	2.6	97.9	3.0	101.5
Bench top stability (room temperature for 24 h)				
20	14.7	116.6	7.9	104.1
2000	4.7	106.0	2.9	101.3
4000	5.3	104.6	2.5	103.0
Freeze-thaw stability (three cycles from −20 °C to room temperature)				
20	11.9	110.8	7.5	105.5
2000	5.5	105.8	2.9	102.0
4000	2.6	104.3	2.2	101.5
Stock solution stability (4 °C for 4 weeks)				
500	4.2	99.0	3.4	98.7

Table 4
Cases and ratio in concentration range of melamine and cyanuric acid in baby and adult urine.

Conc. range (ng/ml)		<10	10–100	100–1000	1000–10,000	>10,000
Melamine	Baby	15(17.4%)	40(46.5%)	15(17.4%)	14(16.3%)	2(2.3%)
	Adult	14(12.7%)	76(69.1%)	15(13.6%)	5(4.5%)	0(0%)
Cyanuric acid	Baby	2(2.3%)	48(55.8%)	34(39.5%)	1(1.2%)	1(1.2%)
	Adult	1(0.9%)	85(72.3%)	22(20.0%)	2(1.8%)	0(0%)

Number of case (percentage of case).

3.3. Application

In this study, in order to investigate the correlation of melamine and cyanuric acid concentrations in human urine to the kidney disease involved, urine samples were collected from 86 infants and children (0–8 years) who were suspected of having ingested melamine-contaminated powdered formula and from 110 adults (25–57 years) who had presented for health examination in our hospital after the outbreak of incidence of milk products contaminated with melamine in China in September, 2008. The study protocol was approved by the Hospital Ethical Committee. The potential profit and risk of the study were fully evaluated. As can been seen from Table 4, most cases were found in the concentration range of 10–100 ng/ml for melamine and cyanuric acid in the investigated child and adult urine samples. Furthermore, a significantly higher melamine concentration ($p < 0.01$) in children's urine than in adult urine was noted. However, no such difference was noted in cyanuric acid concentration. No correlation between the urine concentration of melamine and cyanuric acid was observed in our study. Though no one of these investigated subjects was diagnosed to be kidney failure or kidney stone, these urine data are still helpful to explore the etiology and pidemiology related to melamine and evaluate its risks for specific population for the further clinical observation. Considering melamine and cyanuric acid may exist in food contact material in low level, it is important to set an acceptable threshold in food as well as in human urine.

4. Conclusions

In this study, we developed two LC–MS/MS methods that could determine melamine and cyanuric acid in human urine. The main advantages arising from these methods are: (1) two rapid liquid–liquid extraction procedures were verified in human urine (2) both melamine and cyanuric acid were quantitatively determined at the low level of ng/ml in human urine (3) using of isotope labeled internal standard to confirm the reliability and sensitivity of both detection methods (4) low levels of melamine and cyanuric acid were found in many urine samples collected from investigated subjects, which may be helpful to set an acceptable threshold in human urine.

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