This study characterized the concentration-time profile of melamine in the heart, liver, spleen, lungs, kidneys, bladder, feces, urine, and plasma after melamine (MM) administration. Female Sprague-Dawley rats received a single oral dose of 1.0 g/kg body weight. Samples (n = 4 per time point) were collected at 12, 24, 48, 72, 96, 120, 144, and 168 h. Based on calculations of the area under the concentration-time curves after dosing, ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS–MS) was used to detect MM concentration in tissues. Peak concentrations of MM are reached in the livers and lungs at 12 h after dosing and in hearts, spleens, kidneys, bladders, feces, urine, and plasma at 24 h after dosing. More than 90% of the ingested MM is excreted in feces and urine within 24 h. These results provided initial understanding of the tissue disposition of MM. Moreover, this study demonstrates that UPLC–MS–MS can be used to detect MM in biological samples.

Introduction

Reports of melamine (2,4,6-triamino-1,3,5-triazine, MM) being found in raw milk and pet food (1), in combination with increasing evidence of the toxicity of melamine, have recently increased the amount of concern surrounding this class of chemicals. MM is a nitrogen-containing compound used in the manufacture of plastics; in the production of melamine-formaldehyde resins for surface coatings, laminates, and adhesives; and in the production of flame-retardants. It is also used as a fertilizer (2,3). There are no established regulatory limits for melamine and related triazines in any type of food. Melamine and triazines are toxic at high doses, and therefore should never be present in food. Additionally, MM may cause urolithiasis and bladder cancer (4). However, MM can be found as a contaminant in a variety of foods and food-contacting materials because several manufacturers have added MM to pet food and milk to raise their apparent protein content, as the protein concentration is measured by analyzing the total nitrogen content (5–7).

Information on the toxicity of MM is extremely limited. The mechanisms of disposition and metabolism of MM in the body are unclear. There are no data or reports showing these procedures to be non-toxic, and there are no data concerning the carcinogenicity of melamine in humans. The carcinogenicity of melamine in animals was determined from studies in rats and mice. Uncertainty exists as to whether melamine causes direct renal toxicity over long-term exposure. However, studying the disposition of a chemical in relation to its effects is an important step in interpreting and understanding a chemical’s toxic actions. The utility of toxicokinetic data for the interpretation of toxicity findings and for cross-species toxicity assessment has long been recognized. Kinetics are widely used in support of toxicity studies, including in the consideration of species to be used (in terms of their relevance to humans and the availability of kinetic data), the doses selected (determining a high dose and a dose spread), and the route and frequency of administration (8–10).

As MM leads to outbreaks of renal problems in children, rapid screening or diagnosis using qualitative methods to detect MM is urgently needed. Gas chromatography (GC), liquid chromatography (LC), and capillary electrophoresis (CE) have been developed for the analysis of MM (11–14). GC and LC have been used to determine the presence and concentration of MM in varying matrices, such as soil, animal feeds and grains, plant matter, and raw milk and dairy products (15–18). Instrumental methods for the analysis of MM in food include high-performance liquid chromatography (HPLC) coupled with selective detectors such as ultraviolet absorption (UV) and diode-array detection (DAD), GC, or HPLC coupled with single-stage mass spectrometry (MS) and tandem MS (2,19,20). Based on previously reported methods in literature, this paper presents an approach using ultra-performance liquid chro-
matography (UPLC–MS–MS) to determine MM disposition in animal tissues.

Materials and Methods

Chemicals

The MM used was of analytical grade (≥ 99.0%, Sigma, St. Louis, MO). Sodium 1-heptanesulfonate monohydrate was of puriss grade (≥ 99.0%, Sigma). Methanol, formic acid, and acetonitrile were of LC grade (Merck, Darmstadt, Germany). Ammonium hydroxide and ammonium acetate were of reagent grade (Merck).

Animals

Specific pathogen-free (SPF) young adult female Sprague-Dawley (SD) rats (8–9 weeks of age, 161–220 g) were obtained from the Experimental Animal Center of Guangdong Province (Guangzhou, China) and maintained under SPF conditions for toxicity studies. The animals were acclimatized to laboratory conditions for at least 4 days prior to initiating the studies in a room with a 12-h light/dark cycle designed to control relative humidity at 50 ± 5% and temperature at 22 ± 1°C. Commercial food pellets and tap water were available ad libitum. For the single oral and intravenous dose studies, animals were fasted for 12 h before administration. Food was returned 2 h after dosing. Animals were not fasted for the dermal and repeated oral dose studies. The study was conducted in compliance with all regulations, including the Institutional Animal Care and the NIH Guide for the Care and Use of Laboratory Animals. Animals were treated humanely, and there were no potentially painful procedures.

Animal dosing and sample collection

The oral dosage given was 1.0 g MM/kg body weight. Groups of four rats were used. Researchers determined it was not advisable to exceed 1.0 g/kg body weight due to the limitations of a group 3 carcinogenic risk set by the World Health Organization. Rats were provided water ad libitum but were fasted for 12 h prior to treatment to avoid intersubject variability in GI absorption due to varying food intake. Access to food was provided 2 h after dosing. The dosed animals were maintained in metabolism cages for urine and feces collection before weighing, etherization, and sampling of blood and tissues. Groups of rats were euthanized with CO2 after 12, 24, 48, 72, 96, 120, 144, and 168 h, as well as 2 and 3 weeks post-dosing. Blood samples were drawn from the inferior vena cava and collected in heparinized tubes. The heart, liver, spleen, lungs, kidneys, and bladder were collected and weighed at necropsy, then stored at −80°C until analysis.

Sample preparation

The frozen tissue samples (< 1.0 g) were pulverized into a fine powder under liquid nitrogen using a medium tissue pulverizer (Spectrum Laboratories, Rancho Dominguez, CA). The tissue powder was then collected into a 15-mL polypropylene centrifuge tube, and 10 mL 2.5% formic acid was added. The samples were vortex mixed for 1 min and then were agitated using an ultrasonic agitator for 30 min to elute melamine from tissue samples. The tissue samples were then centrifuged at 9000 g for 5 min. A 1-mL supernatant aliquot was transferred into another new 15-mL polypropylene centrifuge tube, and 4 mL acetonitrile was added. The sample was vortex mixed for 1 min and then centrifuged at 5000 × g for 3 min at room temperature. The supernatant was then filtered through a 0.22-mm nylon syringe filter into a glass LC vial for UPLC–MS–MS analysis.

Plasma and urine (1 mL) were poured into a 15-mL polypropylene centrifuge tube, and 10 mL 2.5% formic acid was added. The sample was vortex mixed for 1 min and then agitated using an ultrasonic agitator for 30 min to extract melamine from the samples. The samples were then centrifuged at 9000 × g for 5 min. A 1-mL supernatant aliquot was transferred into another new 15-mL polypropylene centrifuge tube, and 4 mL acetonitrile was added. The sample was vortex mixed for 1 min and then centrifuged at 5000 × g for 3 min at room temperature. The supernatant was then filtered through a 0.22-mm nylon syringe filter into a glass LC vial for UPLC–MS–MS analysis.

Standard solution preparation

MM (10 mg; Dr. Ehrenstorfer GmbH, Germany) was dissolved in acetonitrile and diluted to a stock solution of 10 µg/mL.

Analytical conditions

UPLC–MS–MS analysis was carried out using a Waters (Milford, MA) Acquity™ UPLC system coupled to a Micromass1 Quattro Premier XE triple-quadrupole MS (Waters) equipped with an electrospray ionization (ESI) source and Masslynx software for separation, detection, and quantification. The analytical column was an Acquity UPLC1BEH HILIC column (3 µm, 2.1 × 150 mm, Waters) maintained at 30°C. The mobile phase was acetonitrile/20 mM ammonium acetate (95:5, v/v), and the flow rate was maintained at 0.3 mL/min. The injection volume was 10 µL.

MM was analyzed using ESI in positive ion (ESI+) mode. Multiple reaction monitoring (MRM) mode was used with the characteristic fragmentation transitions m/z 127 > 85 for quantitative analysis and 127 > 68 for confirmatory analysis. The MS–MS conditions were as follows: capillary voltage, 5.5 kV; cone voltage, 40 V; source temperature, 130°C; desolvation gas temperature, 450°C; desolvation gas flow, 900 L/h; and cone gas flow, 50 L/h nitrogen. The argon collision gas pressure was adjusted to 3.5 × 10⁻² mbar for MS–MS. The collision energy was varied and optimized for each MRM transition. The transitions monitored for MM were m/z 127 > 85 at 17 V and 127 > 68 at 22 V. The ion ratio of transition ions m/z 127 > 68 and 127 > 85 was 0.14, calculated by the average of a series of standard solutions. The dwell time for each transition was 0.05 s.

Quantification

MM was quantified using a linear calibration curve with standard solutions of MM dissolved in acetonitrile at concentrations ranging from 0.01 to 0.5 µg/mL. MM disposition in tissue samples, plasma, and urine was determined from a calibration curve constructed by plotting the peak-area ratios.
(m/z 85 and 89) against the concentration of MM. The linear regression analysis was carried out with the known melamine concentrations against corresponding peak areas, and the determination coefficient, slope, and intercept of the resulting calibration curve were calculated. The calibration curve with a coefficient of a correlation ($r^2$) greater than 0.995 was recognized to be linear. The limits of detection (LOD) and quantification (LOQ) were calculated using the data from the linearity test with the following equations: LOD = 3.3 SD/m and LOQ = 10 SD/m, where SD is the standard deviation of the intercept, and m is the slope of the calibration curve. The recovery of this method was determined the standard addition method. Three different concentrations (0.01, 0.25, and 0.5 µg/mL) of the melamine standards were added into the plasma from control rats in triplicate. The solutions were extracted and quantified as previously described.

Statistical analysis
Data are presented as mean ± standard deviation unless otherwise stated. Statistical comparisons were performed by ANOVA with post hoc Dunn or Tukey tests for multiple comparisons and two-tailed paired or unpaired Student t-tests, where appropriate. Correlation analyses used the Pearson product moment or the Spearman rank order test. A value of $p < 0.05$ was considered significant.

Results
Changes in the concentration of melamine in tissues of female SD rats after dosing
After oral administration of MM (1.0 g/kg) to rats, the compound was absorbed and had already reached an apparent peak concentration of $5.9 \pm 0.6$ µg/g in female SD rat hearts at the sampling time point of 24 h after the dose (Figure 1A). The concentration of MM fell rapidly up to 96 h period after dosing and was below the limit of detection within 96 h.

Changes in the concentration of melamine in female SD rat livers after dosing
MM was detected in livers at 12 h after dosing. The concentration of MM reached an apparent peak concentration of $6.2 \pm 0.3$ µg/g in female SD rat livers at 12 h after the dose (Figure 1B). After 12 h, the concentration of MM fell rapidly up to 72 h after dosing and was below the LOD within 96 h.

![Figure 1. Tissue disposition of MM in SD rats that received an oral dose of MM (1.0 g/kg): time course of melamine in the hearts of female SD rats (A); time course of melamine in the livers of female SD rats (B); time course of melamine in the spleens of female SD rats (C); time course of melamine in the lungs of female SD rats (D); time course of melamine in the kidneys of female SD rats (E); time course of melamine in the bladders of female SD rats (F); time course of melamine in feces of female SD rats (G); time course of melamine in urine of female SD rats (H); and time course of melamine in plasma of female SD rats (I).]
Changes in the concentration of melamine in female SD rat spleens after dosing

MM was detected in spleens at 12 h after dosing. The concentration of MM increased rapidly after that and reached an apparent peak concentration of 22 ± 1.3 µg/g in female SD rat spleens at 24 h after the dose (Figure 1C). After 24 h, the concentration of MM fell rapidly up to 72 h after dosing and was below the LOD within 96 h.

Changes in the concentration of melamine in female SD rat lungs after dosing

MM was detected in the lungs at 12 h after dosing. The concentration of MM reached an apparent peak concentration of 4.0 ± 0.3 µg/g in female SD rat lungs at 12 h after the dose (Figure 1D). The concentration of MM fell rapidly up to 72 h after dosing and was below the LOD within 96 h.

Changes in the concentration of melamine in female SD rat kidneys after dosing

MM was detected in kidneys at 12 h after dosing. The concentration of MM reached an apparent peak of 23 ± 1.1 µg/g in female SD rat kidneys at 24 h after the dose (Figure 1E). The concentration of MM decreased rapidly up to 72 h after dosing and was below the LOD within 120 h.

Changes in the concentration of melamine in female SD rat bladders after dosing

MM was detected in bladders at 12 h after dosing, and the levels were still increasing within 24 h. The concentration of MM increased rapidly and reached an apparent peak concentration of 208 ± 10 µg/g in female SD rat bladders at 24 h after the dose (Figure 1F). The concentration of MM fell rapidly up to 72 h after dosing and was below the LOD at 168 h.

Changes in the concentration of melamine in female SD rat feces after dosing

MM was detected in feces at 12 h after dosing, and the levels were still increasing rapidly within 24 h. The concentration of MM reached an apparent peak of 10,000 ± 130 µg/g in female SD rat feces at 24 h after the dose (Figure 1G). The concentration of MM fell rapidly up to 48 h after dosing, and the levels were still detected at 168 h.

Changes in the concentration of melamine in female SD rat urine after dosing

MM was detected in urine at 12 h after dosing, and the levels were still increasing rapidly within 24 h. The concentration of MM reached an apparent peak of 4100 ± 100 µg/mL in female SD rat urine at 24 h after the dose (Figure 1H). The concentration of MM fell rapidly up to 48 h after dosing, and the levels were still detected at 168 h.

Changes in the concentration of melamine in female SD rat plasma after dosing

MM was detected in plasma at 12 h after dosing, and the levels were still increasing rapidly within 24 h. The concentration of MM reached an apparent peak of 2270 ± 99 µg/mL in female SD rat plasma at 24 h after the dose (Figure 1I). The concentration of MM fell rapidly up to 48 h after dosing, and the levels were still detected at 168 h.

Tissue-to-plasma ratios of MM in female SD rats at different times after dosing

The tissue-to-plasma ratios of MM (g tissue/mL plasma) in all the tissues examined were found not to be dependent on the times of sampling (Table I), thus suggesting that the distribution of MM into these tissues had attained equilibrium with the peripheral plasma shortly after MM administration. The tissue-to-plasma MM ratios of heart, liver, spleen, lungs, kidneys, and bladder were significantly (*p<0.05) higher at 48 h than at 12 h after dosing. The tissue-to-plasma MM ratios of feces and urine were significantly (*p<0.05) higher at 96 h than at 12 h after dosing. Delays in attaining a distributional equilibrium of MM between these tissues and the peripheral plasma might have been responsible for the time-dependent differences in tissue-to-plasma ratios.

Clearance of MM in female SD rats at different times after dosing

Excretion of MM was detected in the urine and feces of female SD rats after administration of a single dose. The clearance of MM was 7.5% and 4.0% in urine and feces at 12 h after dosing, respectively, and it was 25% and 61% at 24 h in urine and feces, respectively. Excretion of MM reached 97% at 24 h in female SD rats after dosing. The excretion of MM decreased

Table I. Tissue-to-Plasma Ratios of MM in Female SD Rat at Different Times after Dosing

<table>
<thead>
<tr>
<th></th>
<th>Heart</th>
<th>Liver</th>
<th>Spleen</th>
<th>Lung</th>
<th>Kidney</th>
<th>Bladder</th>
<th>Feces</th>
<th>Urine</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 h</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>12 h</td>
<td>0.02 ± 0.00*</td>
<td>0.40 ± 0.02</td>
<td>0.07 ± 0.01</td>
<td>0.03 ± 0.00</td>
<td>0.12 ± 0.01</td>
<td>0.98 ± 0.1</td>
<td>4.3 ± 0.6</td>
<td>7.9 ± 0.6</td>
</tr>
<tr>
<td>24 h</td>
<td>0.01 ± 0.00</td>
<td>0.01 ± 0.00*</td>
<td>0.01 ± 0.00</td>
<td>0.01 ± 0.00</td>
<td>0.01 ± 0.00*</td>
<td>0.09 ± 0.01</td>
<td>4.4 ± 0.5</td>
<td>1.8 ± 0.02</td>
</tr>
<tr>
<td>48 h</td>
<td>0.14 ± 0.01*</td>
<td>0.10 ± 0.04*</td>
<td>0.47 ± 0.03*</td>
<td>0.04 ± 0.00*</td>
<td>0.31 ± 0.1*</td>
<td>2.8 ± 0.4*</td>
<td>4.5 ± 0.5</td>
<td>4.3 ± 0.6</td>
</tr>
<tr>
<td>72 h</td>
<td>0.08 ± 0.01</td>
<td>0.04 ± 0.00</td>
<td>0.24 ± 0.02*</td>
<td>0.06 ± 0.00</td>
<td>0.04 ± 0.00</td>
<td>0.55 ± 0.03</td>
<td>4.3 ± 0.5</td>
<td>6.4 ± 0.9</td>
</tr>
<tr>
<td>96 h</td>
<td>0.08 ± 0.01</td>
<td>0.04 ± 0.00</td>
<td>0.24 ± 0.02*</td>
<td>0.06 ± 0.00</td>
<td>0.04 ± 0.00</td>
<td>0.55 ± 0.03</td>
<td>4.3 ± 0.5</td>
<td>6.4 ± 0.9</td>
</tr>
<tr>
<td>120 h</td>
<td>0.08 ± 0.01</td>
<td>0.04 ± 0.00</td>
<td>0.24 ± 0.02*</td>
<td>0.06 ± 0.00</td>
<td>0.04 ± 0.00</td>
<td>0.55 ± 0.03</td>
<td>4.3 ± 0.5</td>
<td>6.4 ± 0.9</td>
</tr>
<tr>
<td>144 h</td>
<td>0.08 ± 0.01</td>
<td>0.04 ± 0.00</td>
<td>0.24 ± 0.02*</td>
<td>0.06 ± 0.00</td>
<td>0.04 ± 0.00</td>
<td>0.55 ± 0.03</td>
<td>4.3 ± 0.5</td>
<td>6.4 ± 0.9</td>
</tr>
<tr>
<td>168 h</td>
<td>0.08 ± 0.01</td>
<td>0.04 ± 0.00</td>
<td>0.24 ± 0.02*</td>
<td>0.06 ± 0.00</td>
<td>0.04 ± 0.00</td>
<td>0.55 ± 0.03</td>
<td>4.3 ± 0.5</td>
<td>6.4 ± 0.9</td>
</tr>
</tbody>
</table>

* p < 0.01 compared with tissue-to-plasma ratios at other time points.
† p < 0.05 compared with tissue-to-plasma ratios at other time points.
rapidly in urine and feces within 48 h. However, excretion of MM was still detected at 168 h after dosing (Figure 2).

**Method linearity, LOD, LOQ, and recovery**

A calibration curve was built with melamine solutions in the concentrations of 0.01 to 0.5 µg/mL. The method was linear in the range of concentrations used. It was found that the behavior of the method is described by the equation $y = 1.47x + 1.5$, where $y$ was the area of the peak and $x$ is the concentration of the melamine solution; a determination coefficient ($r^2$) of 0.9998 was found. They showed good linear relationships between $y$ and $x$. LOQ for melamine was 0.01 µg/mL, and the LOD was 0.0025 µg/mL. According to the data obtained from the recovery results, recovery ranged from 83% to 110%.

**Discussion**

MM was an unknown substance to nephrologists until recently. It became headline news after the occurrence of an outbreak of urinary stones in infants and children consuming melamine-tainted milk in 2008 in China (21–23). Studies concerning the toxicity of melamine taken orally in humans are nonexistent. Toxicity data mainly come from studies in sheep, cats, dogs, mice, and rats (24–28). The most commonly reported chronic renal toxicity is stone formation (6,7). Uncertainty exists as to whether melamine results in any chronic toxic effects other than aggressive stone formation. There are no data showing the deposition of MM. This lack of toxicology data is one of the main problems preventing the progress of diagnosis and therapy. The aim of this study was to delineate tissue uptake and elimination time-profiles for melamine.

In this study, we characterized the fate of MM in tissues of SD rats that received MM orally. Oral administration of MM to SD rats resulted in the detection of MM in plasma and tissues. These results provided initial understanding of the tissue disposition of MM. These data showed that MM distributes rapidly into all of the tissues sampled in this study after absorption into systemic circulation. Indeed, high peak concentrations of MM were observed in tissues during the 24 h after absorption, and tissue concentration profiles of MM followed the same pattern as those for plasma, indicating equilibrium MM distribution between tissues and plasma.

An important finding of our study was that the concentration-time profile of MM in the tissues studied was not readily predictable from the concentration-time profile of MM in the plasma. Peak concentrations of MM were reached in the livers and lungs at 12 h after dosing and in hearts, spleens, kidneys, bladders, feces, urine, and plasma at 24 h after dosing. We found that the concentration-time courses did not increase or decrease in parallel in the tissues after MM administration. This finding was primarily because MM concentrations in different tissues did not increase or decrease as rapidly as plasma concentrations after MM administration. To the best of our knowledge, when a chemical is distributed rapidly into the tissue after absorption, its disposition and steady-state concentration in the body should depend on its metabolism and rate of biotransformation by the liver. Its elimination from the body should depend on its excretion by the kidneys (29). In this study, the concentration of MM in the liver increased constantly within 12 h after dosing. However, the concentration of MM in the kidneys reached a peak concentration at 24 h after dosing; the concentration of MM then decreased constantly from 12 h to 96 h. All of these data demonstrate that the distribution of MM after dosing was different among the tissues sampled in our study.

However, these data also demonstrate an apparent rise and fall in tissue-to-plasma concentration ratios after MM administration. The tissue-to-plasma concentration ratios in the heart, liver, spleen, lungs, kidneys, and bladder at 48 h were significantly different from those at other time points. Additionally, the tissue-to-plasma concentration ratios at 96 h were significantly different from those at other time points in feces and urine. These data showed that high levels of MM could be detected in tissues within 48 h and in feces and urine within 96 h. We suggest that accumulation of MM would occur at 96 h if the female SD rats were administered a repeat dose.

MM was distributed into all sampled tissues, with the highest concentrations found in the feces. Peak levels in the feces were significantly higher than levels in the heart, liver, spleen, lungs, kidneys, bladder, urine, and plasma. MM is not metabolized by female SD rats and is rapidly eliminated in the urine. More than 90% of the ingested MM is excreted in feces and urine within 24 h. However, MM could still be detected at a low level by the end of our experiment. It was demonstrated that MM still existed in the bodies of female SD rats after 168 h of dosing.

Observational data reported by several authors reveal an association between the prevalence of kidney stones as determined on ultrasonography of the kidney at one point in time and a history of melamine exposure based on the amounts in infant formulas as reported by the Chinese authorities. Kidney stones were seen in nearly 10% of the children studied in Beijing who received formula with a high melamine content (> 500 ppm) or a moderate melamine content (< 150 ppm) (22–24). Thus, according to our data in this study, kidney stones must be associated with MM accumulation in the body.
In LC–MS–MS, good chromatography is critical to overall performance. Packed with very small particles, the short UPLC column used in this study provided sufficient retention and allowed for a relatively high flow rate of 0.3 mL/min of a mobile phase with substantial organic content. The LOD for melamine was 0.0025 µg/mL, which was much lower than those reported in the literature. Venkatassami et al. (30) found the LOD was 0.1 µg/mL by using HPLC method for determination of melamine in infant formula. Wu et al. (31) observed the LOD was 0.02 µg/mL by using HPLC–MS–MS for determination of melamine in SD rats. With further validation, this simple, accurate, and high-throughput LC–MS–MS may replace existing traditional methods for detection of MM, especially in busy and high-volume laboratories.

Conclusions

In conclusion, MM was distributed rapidly into the heart, liver, spleen, kidneys, bladder, feces, urine, and plasma after a single dose administration of 1.0 g/kg body weight. More than 90% of the ingested MM was excreted in feces and urine within 24 h. But high levels of MM were detected in all of the tissues sampled within 48 h and were detected constantly in feces, urine, and plasma at 168 h after dosing. UPLC–MS–MS is able to detect MM efficiently in female SD rat tissues. Application of this approach to the study of other disease markers is under investigation. Further studies are needed to relate MM accumulation to kidney stones found in children who received formula contaminated by MM.

Acknowledgments

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