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ARTICLE

Oral Bioavailability, Urinary Excretion and Organ Distribution of Melamine in Sprague−Dawley Rats by High-Performance Liquid Chromatography with Tandem Mass Spectrometry

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High-performance liquid chromatography with tandem mass spectrometry (HPLC/MS/MS) was used to determine melamine oral bioavailability (BA) and urinary excretion. Organ distribution after a 14-day consecutive oral melamine administration (100 mg/kg/day, once a day) was also evaluated. A noncompartmental model was utilized to obtain pharmacokinetic parameters. According to the results, the BA of melamine was estimated to be 98.1%. Approximately 63% of administered melamine was recovered in urine within 96 h after a single oral administration (100 mg/kg). The bladder had the highest melamine concentration of all the organs after a 14-day consecutive oral administration of melamine, and almost no melamine was found in the rat brain. This result indicated that the oral absorption of melamine was almost complete and urinary excretion was the major route for its elimination. Repeated exposure to high-dose melamine may result in only slight accumulation in organs.

KEYWORDS: Melamine; pharmacokinetics; oral bioavailability; urinary excretion; distribution

INTRODUCTION

There was an extensive pet food recall in the United States due to pet foods contaminated with melamine and its analogues, which led to renal failure in cats and dogs who had eaten contaminated pet food in the spring of 2007 (*1*, *2*). In addition, melamine was deliberately added to raw milk to increase the apparent protein content, resulting in an increased incidence of renal failure in infants in China in 2008 following the consumption of infant foods contaminated with melamine (*3*). Several quantitative methods, including enzyme immunoassay (*4*), capillary zone electrophoresis (CZE) with mass spectrometry (*5*), gas chromatography with mass spectrometry (GC/MS) (*6*), and high-performance liquid chromatography with ultraviolet detection (HPLC/UV) (*7*), have been developed to measure this chemical. Recently, several methods using high-performance liquid chromatography with tandem mass spectrometry (HPLC/MS/MS) have been reported for the quantification of melamine in pork (*8*), fish (*9*), and kidney tissue (*10*, *11*).

Melamine (1,3,5-triazine-2,4,6-triamine) is a chemical used extensively by industry in the preparation of polymers for the manufacture of countertops, fabrics, glues, houseware items, and flame retardants (*12*). Toxicology studies have determined that melamine toxicity in rodents is low, with LD50 values in the rat and rabbit of around 3 and 5 g/kg, respectively (*13*). The etiology of melamine-induced renal failure has been discovered (*14*, *15*), but pharmacokinetic studies are few and limited to the determination of melamine in specific organs and tissues. A previous study reported the plasma and tissue kinetics of melamine in Fischer 344 rats after a single oral dose of 0.025 mCi (0.38 mg) [^{14}C]melamine (*16*). Another recent work described the plasma pharmacokinetics of melamine in pigs following intravenous administration (*17*). However, the exact extent of absorbed melamine via the oral route has not been reported. In addition, it has not been clarified if melamine accumulates in organs after acute and repeated exposure of melamine. Therefore, to determine its oral bioavailability (BA) and the possibility of accumulation after acute exposure to melamine, we set up a sensitive HPLC/MS/MS to determine urinary elimination, BA and organ

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distribution after 14-days consecutive oral administration of melamine.

MATERIALS AND METHODS

Chemicals. Melamine (purity: 99%) was obtained from Alfa Aesar (Ward Hill, MA). Carboxymethylcellulose sodium and heparin sodium were purchased from Sigma-Aldrich (St. Louis, MO). Pure water for all preparations was prepared by the Milli-Q system (Millipore, Milford, MA). Ammonium hydroxide (aq), formic acid, acetonitrile, and sodium chloride were of HPLC grade or GR for analysis grade from E. Merck (Darmstadt, Germany).

Animal Preparation. Male Sprague−Dawley rats weighing 200 ± 20 g (National Yang-Ming University Animal Center, Taipei, Taiwan) were housed in a 12 h light and 12 h dark cycle room. Free access to food (Laboratory rodent diet 5001, PMI Feeds, Richmond, IN) and water was allowed at all times. Animal experimental protocols were approved by the Institutional Animal Experimentation Committee of National Yang-Ming University.

To evaluate the BA of melamine, a jugular vein catheterization model was applied to perform repeated blood sampling in the unrestrained conscious rat (*18*). Briefly, rats were anesthetized with pentobarbital sodium (50 mg/kg, ip), and polyethylene tubes were implanted in the right jugular and right femoral veins. The catheter was exteriorized, fixed in the dorsal neck region and capped with a stopper. For the oral administration group, only the right jugular was catheterized for blood sampling. The patency of the tubing was maintained by flushing with heparinized normal saline (0.9% NaCl, w/v, solution containing 15 IU/mL heparin sodium salt). Rats were allowed a minimum of 24 h to recover prior to drug administration.

Oral Bioavailability. In the intravenous group (n = 5), melamine dissolved in normal saline was given via the femoral vein (10 mg/kg, iv), and in the oral group (n = 5) melamine suspended in 2% (w/v) carboxymethylcellulose sodium (CMC) solution was administered by gastric gavage (50 mg/kg, po). A 250 μL blood sample was withdrawn manually via the jugular vein catheter and placed into a vial rinsed with heparin. Blood was taken at 10, 20, 30, 60, 120, 180, 240, 300, 360, 420, and 480 min after drug administration, and the removed blood after each sampling was replaced with an equal volume of heparinized normal saline. Plasma was separated by centrifugation at 6000*g* for 10 min at 4 °C. The resulting plasma sample was stored at −20 °C before analysis.

Urinary Excretion after Once Oral Administration. Rats (n = 5) were fasted for 24 h before dosing. Melamine suspension (50 mg/mL) was prepared in 2% (w/v) CMC solution and given to each rat by gastric gavage (100 mg/kg, po). Urine samples were collected by metabolic cages (Mini Mitter, Bend, OR) and then weighed before being stored in a −20 °C refrigerator.

Organ Distribution after a 14 Day Consecutive Oral Administration. Six rats were used in this experiment. Melamine suspension (50 mg/mL) was prepared in 2% (w/v) CMC solution and given to each rat once a day by gastric gavage (100 mg/kg, po) for 14 consecutive days. On day 15, the rat was anesthetized, a 3 mL volume of blood was taken by cardiac puncture, and the rat was sacrificed by decapitation. Liver, kidney, spleen, urinary bladder, and brain were removed and weighed immediately, and the brain cortex, striatum, hippocampus, cerebellum, stem, and the rest of the brain were further dissected and weighed for the determination of regional distribution of melamine. Each tissue sample was stored at −20 °C before sample preparation.

Sample Preparation. Plasma samples were extracted by the solid-phase extraction (SPE) cartridge (Oasis MCX 60 μm, 30 mg) washed with 1% (v/v) formic acid in water, acetonitrile, and eluted with acetonitrile alkalized by ammonium hydroxide (5%, v/v) and evaporated to dryness at 50 °C. Organ homogenate samples, including liver, kidney, spleen, urinary bladder, and brain regions, were treated by 1% (w/v) trichloroacetic acid solution for homogenization and extracted by the solid-phase extraction. The dried residue was reconstituted in 90% (v/v) acetonitrile before HPLC/MS/MS analysis.

HPLC/MS/MS and Method Validation. An Xbridge hydrophilic interaction chromatography (HILIC) column (100 × 2.1 mm, 3.5 μm, Waters, Milford, MA) was used as the stationary phase. The mobile phase contained 900 mL of acetonitrile and 100 mL of ammonium acetate (10 mM) containing 0.1% acetic acid (pH 4.5) delivered at 200 μL/min. A 5 μL volume of sample was introduced into the analytical system. A Quattro Ultima MS/MS system (Micromass, Manchester, U.K.) equipped with an electrospray ionization (ESI) probe was used, and multiple reaction monitoring (MRM) transition m/z 127 → 85 was applied to quantify melamine. Other optimized instrument settings were as follows: capillary voltage 3.0 kV, cone voltage 40 V, collision energy 17 eV, source temperature 100 °C, desolvation temperature 300 °C, cone gas 100 L/h, desolvation gas 500 L/h. The MassLynx 3.5 (Micromass) software was used for data processing. Samples that were higher than the calibration range were diluted with the extracted blank matrices to accurately quantitate melamine concentrations. Method validation was performed according to the guidance proposed by USFDA (*19*). Calibration curves ranging from 20 to 500 ng/mL were prepared by mixing blank biological matrices (e.g., plasma and tissues) with melamine working standard solutions and then processed as described in Sample Preparation. The calibration curve with a coefficient of correlation (r^2) greater than 0.995 was recognized to be linear, and precision and accuracy tests indicated the method was reproducible. The matrix effect ranged from 66.2 to 95.5%, and recovery ranged from 79.8 to 113.0% (*20*).

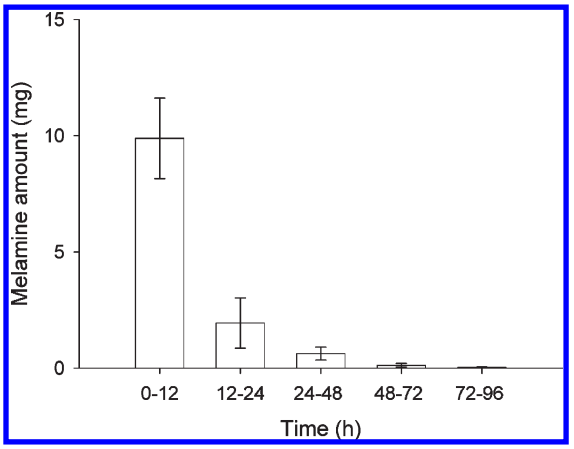

Figure 1. Urinary excretion profile of melamine following a single oral administration of melamine (100 mg/kg); results are expressed as mean ± SD (n = 5).

Pharmacokinetic Data Analysis. The drug concentration data were processed by WinNonlin Standard Edition Version 1.0 (Scientific Consulting, Apex, NC) to generate pharmacokinetic parameters of each rat with a noncompartmental model. The results are represented as mean ± standard deviation.

RESULTS AND DISCUSSION

The present work first reports the exact value of melamine absorption estimated by BA in the unrestrained conscious rat model, because previous studies performed either in rats (*16*) or in swine (*17*) did not clearly indicate the absorption extent of melamine after oral ingestion. Our study design using the unrestrained conscious animal model avoided the altered physiological conditions due to restraint stress (*21*) and influences of anesthetics (*22*) on drug absorption of experimental animals and provided more accurate pharmacokinetic data. **Figure 1** shows melamine levels in rat urine after a single oral ingestion of melamine (100 mg/kg, n = 5). About 63.2% of the administered dose was recovered from the urine within 96 h. The previous study indicated a value of 90% elimination within the first 24 h (*16*), and this discrepancy might result from a much higher dose (100 mg/kg, po) in our study, possibly leading to the saturation of urinary elimination and delayed elimination time. This result suggests that most of the administered melamine was absorbed and eliminated via the urine in unchanged form.

The concentration versus time profile of melamine after a single intravenous dose and a single oral dose administered to five individual rats is shown in **Figure 2**. Melamine was absorbed rapidly

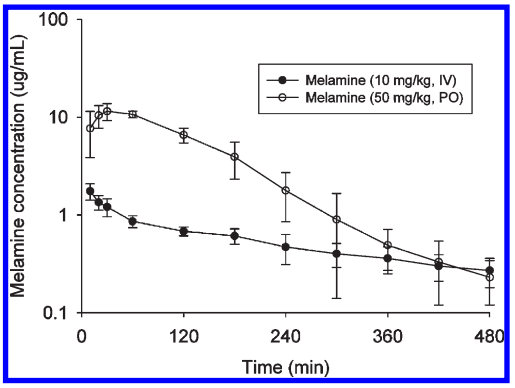

Figure 2. Concentration versus time curves of melamine after drug administration (10 mg/kg, iv and 50 mg/kg, po) in rats; data is expressed as mean ± SD (n = 5 for each group).

Table 1. Pharmacokinetic Data of Melamine in Rats[a]

	iv, 10 mg/kg	po, 50 mg/kg
C_{max} (μg/mL)	2.29 ± 0.64	12.20 ± 1.29
$t_{1/2}$ (min)	194 ± 38	79 ± 12
AUC (min μg/mL)	360 ± 84	1766 ± 357
MRT (min)	293 ± 59	116 ± 17
bioavailability (%)		98.1

[a] Values are expressed as mean ± SD. C_{max}: peak plasma melamine concentration. $t_{1/2}$: half-life. AUC: the area under the concentration−time curve. MRT: mean residence time. Bioavailability was obtained by dividing the mean AUC of the po group with that of the iv group corrected by doses according to the equation $(AUC_{po} \times dose_{iv})/(AUC_{iv} \times dose_{po})$.

from the gastrointestinal tract in rats, and a maximum concentration of 12.20 ± 1.29 μg/mL was observed at around 30 min following oral administration. The areas under the concentration−time curve (AUC) values were 360 ± 84 min μg/mL for the intravenous (10 mg/kg, iv) group and 1766 ± 357 min μg/mL for the oral (50 mg/kg, po) group (**Table 1**). According to the equation $(AUC_{po} \times dose_{iv})/(AUC_{iv} \times dose_{po})$, BA obtained by dividing the mean AUC of the po group with that of the iv group corrected by doses was approximately 98.1%. We found the half-life ($t_{1/2}$) of melamine was 194 ± 38 min after iv dosing, which is comparable to the reported values of 2.71 h in the rat (*16*) and 4.07 h in the pig (*17*). The mean residence time (MRT) values, which are the average time that molecules of a drug reside in the body, were 293 ± 59 and 116 ± 17 min, in the iv and po groups, respectively.

Exposure to melamine in our daily life can come from tableware products, pesticides and cleaning solutions. Migration of melamine from tableware products made of melamine resin has been studied under controlled conditions (*23*). Results found that heating and acidity affected the migration of melamine greatly (*24*, *25*). Though consecutive migration of melamine happens during the lifetime of these products, it is considered that the concentration of melamine in foods from migration is likely to be less than 1 mg/kg (*26*). In addition, melamine is a metabolite of cyromazine, which is used as a pesticide. Residues of cyromazine and melamine have been detected on vegetable crops after spray application (*27*). Melamine residues in bean plants have been measured after applying cyromazine in solution to the bean roots, though melamine residues in the vegetative part of the bean remained below 1 mg/kg (*28*). Furthermore, melamine can be produced from the decomposition of trichloromelamine, which is approved for use in the USA in cleaning solutions for food processing equipment and food contact articles. The USFDA estimated a melamine dietary concentration of approximately 0.14 mg/kg based on the assumption that all sanitizers contain

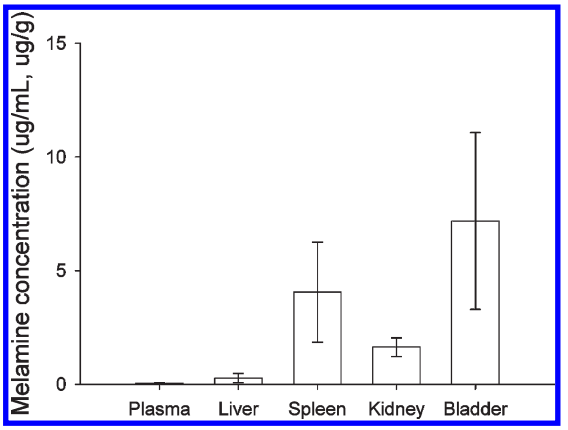

Figure 3. Rat plasma, liver, spleen, kidney, and bladder levels of melamine after a 14 day consecutive oral ingestion of melamine (100 mg/kg/day); data are expressed as mean ± SD (n = 6). The melamine concentration units of plasma and organs are μg/mL and μg/g, respectively.

trichloromelamine (*26*). Relatively high oral BA indicates that melamine is absorbed well in the gastrointestinal tract if melamine is ingested. However, the exposure amount and toxicity of melamine is very limited, and according to animal experiments melamine produces neither crystals in the kidney nor acute toxicity without the presence of cyanuric acid (*29*, *30*).

Only a trace amount of melamine could be determined in the rat brain, and the levels were all approaching the limit of detection, indicating that this highly polar compound cannot easily enter the brain. **Figure 3** presents melamine levels in the rat plasma, liver, kidney, spleen, and bladder after a 14 day oral ingestion of melamine (100 mg/kg/day). Melamine concentration in plasma after a 14 day oral ingestion of melamine was 44.7 ± 16.1 ng/mL. The bladder exhibited the highest concentration of melamine, while the liver exhibited the lowest among these organs. Buur et al. (*31*) observed little accumulation in porcine liver and kidney following a twice daily oral administration regimen (5.12 mg/kg) for 7 days. Melamine contamination was determined to be in the range of 30−120 ppm in swine feed (*32*), and therefore we designed a high dose with a 14 day continuous dosing to estimate the condition. Our results indicated that acute and repeated exposure to high-dose melamine may result in only limited accumulation (less than 12 ppm) in organs. In sum, the oral absorption of melamine was almost complete and urine was the major elimination route. Repeated exposure to high-dose melamine may result in only slight accumulation in organs.

ABBREVIATIONS USED

HPLC/MS/MS, high-performance liquid chromatography with tandem mass spectrometry; C_{max}, maximum concentration; AUC, area under the concentration−time curve; BA, oral bioavailability; CZE, capillary zone electrophoresis; GC/MS, gas chromatography with mass spectrometry; HPLC/UV, high-performance liquid chromatography with ultraviolet detection; ip, intraperitoneal administration; iv, intravenous administration; CMC, carboxymethylcellulose sodium; po, oral administration; SPE, solid-phase extraction; HILIC, hydrophilic interaction chromatography; MRM, multiple reaction monitoring; RSD, relative standard deviation; LOQ, limit of quantification; $t_{1/2}$, half-life; MRT, mean residence time.

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