JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2010, 58, 943–948 943
DOI:10.1021/jf9026316

Tissue Deposition and Residue Depletion in Lambs Exposed to Melamine and Cyanuric Acid-Contaminated Diets

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Many countries have introduced maximum residue limits for melamine in foods since its adulteration in infant formula in China. However, more animal feeding studies are needed to understand the fate of melamine-contaminated animal feed. In this study, the melamine contents in tissues and serum were tested using LC-MS/MS for up to 60 days for lambs fed with diets containing 2−100 mg of melamine per kg of diet. A higher dose of melamine in the diet resulted in a higher concentration in tissues and serum, with the maximum melamine content in the kidney. When cyanuric acid was coadministered at an equal concentration to that of melamine, the deposition of melamine in lambs was similar to the treatment with melamine only and was much higher than the deposition of cyanuric acid. When melamine was withdrawn from the diet, melamine concentrations decreased below 20 μg/kg in all tissues after 4 days. The present study may provide available information for future work about the risk assessment of melamine to human health.

KEYWORDS: Melamine; cyanuric acid; deposition; elimination

INTRODUCTION

Melamine (MEL) is a compound used for syntheses of a large variety of materials for applications such as cleaning supplies, dry erase boards, and plastics (*1*). The global production of melamine in 2007 was approximately 1.2 million tons, with the predominant producers located in China and Western Europe (*2*). The commercial MEL product is synthesized from urea and varies in purity dependent on synthesis conditions and subsequent purification steps (*3*). Low-purity MEL products are conventionally recovered from the processing wastewater stream and contain a mixture of MEL and oxytriazines (*4*). Major symptoms observed from animals fed with MEL are urolithiasis or development of bladder stone, while some studies reported correlations to cancers and malfunctions of kidney (*5*−*8*).

The adulteration of powdered infant formula by MEL in China was in the spotlight (*9*, *10*) due to its significant harm to human health (*11*, *12*). Several studies reported the accumulation of MEL in various animal tissues after administration of MEL. When catfish was administered with diets containing 400 mg/kg of MEL and 400 mg/kg of cyanuric acid (CYA), 33 mg/kg of MEL and 0.14 mg/kg of CYA were detected in fish muscles (*13*). A recent report from South Africa (*14*) suggested that the presence of MEL in raw cow's milk may have originated from MEL-contaminated raw materials used to make animal feed. A separate study reported the resultant MEL contents in tissues of swine fed with diets containing 69−74 mg/kg of MEL, and the concentrations of MEL in loin and ham were 9−12 μg/kg in three hogs slaughtered after feeding for 1 day (*15*).

MEL contains a substantial amount of nitrogen—66% by mass—and MEL has previously been investigated for use as a possible alternative nitrogen source for ruminant animals. In 1966, Clark (*16*) reported crystalluria and mortality of sheep exposed to high doses of MEL—100 g (approximately 50 000 mg/kg in the diet) daily for 11 days. MacKenzie (*17*) reported weight losses and mortalities of sheep fed with diets containing either 9.8 or 19.6 g of MEL (approximately 6250 or 12 500 mg/kg in the diet). In addition, a couple of studies demonstrated that the toxicity of MEL and cyanurate complexes was qualitatively different from that of MEL or CYA administered alone (*18*, *19*). An early study by Aleksandrian (*20*) reported that orally administered MEL and cyanurate complexes induced some renal toxicity in rats. Recent data from Reimschuessel et al. (*13*) showed that catfish did not form crystals after being dosed with 400 mg/kg bw of the complex for 3 days. However, these toxicity studies were not correlated with the levels of MEL or CYA in animal tissues.

Although many countries have set maximum residue limits (MRLs) for MEL in infant formula and other foods (*21*) and the above sporadic studies were reported, a quantitative assessment of the carry-over of MEL from animal feed into foods is still needed for a better assessment of animals exposed to MEL

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Published on Web 12/28/2009

Table 1. Levels of MEL and CYA in Different Animal Treatments

group	level of MEL in diet	level of CYA in diet
control	30 μg/kg[a]	0
Mel-2	2 mg/kg	0
Mel-10	10 mg/kg	0
Mel-30	30 mg/kg	0
Mel-100	100 mg/kg	0
Mel-Cya	100 mg/kg	100 mg/kg

[a] As detected in the control basal diet.

existing in the diet. In this study, we focused on determining the levels of MEL in edible tissues of lambs fed with diets with low-doses of MEL. We also investigated the accumulation of MEL and CYA in tissues of lambs because of the above-mentioned need of studying animals simultaneously administered with the two compounds.

MATERIALS AND METHODS

Chemicals. MEL, with a purity of 99.4%, was obtained from Sigma Aldrich (Shanghai, China). The stable-isotope-labeled internal standard was used in the LC-MS/MS experiments; [$^{15}N_3$]-MEL (99% isotopic purity) and [$^{13}C_3$]-CYA (99% isotopic purity) were products from Cambridge Isotope Laboratories, Inc. (Beijing, China). All other chemicals and solvents used in the analyses were reagent grade.

Animal Experiments. The lambs used in this work were the breed of Xinjiang, China. The lambs were 5−6 month-old and had a body weight of 39.5−57.8 kg, averaging ca. 50 kg. The 180 lambs were randomly divided into 18 groups; each group of 10 lambs received the same diet treatment. The body weight of lamb was recorded, and there was no significant difference among the groups. The 1000 g basal diet contained approximately 600 g of silage maize, 100 g of *Leymus chinensis* (Trin.) Tzvel., 165 g of maize, 75 g of soybean meal, 45 g of wheat bran, and 15 g of premix containing vitamins and minerals. The 18 groups were randomly assigned to one of the six treatments (3 groups per treatment) detailed in **Table 1**. Lambs were fed *ad libitum* and presented with unlimited fresh water.

The lambs were fed with the same diet for up to 60 days. For each group, one lamb was sacrificed on days 7, 14, 21, 35, 49, and 60. Specimens of gluteal muscle, longissimus dorsi muscle, kidney, liver, and blood were collected for chemical analysis. The tissue samples were kept at −20 °C until analyses.

To evaluate the clearance of MEL from tissues and blood, the lambs after the 60-day Mel-100 treatment were switched to the control treatment. After switching the diet for 12, 36, 60, 84, and 108 h, lamb muscle, liver, and kidney samples were collected and kept at −20 °C until analyses. In addition, the lamb blood was sampled after switching the diet for 1, 3, 12, 36, 60, 84, and 108 h. The blood samples were centrifuged at 16 100 g for 10 min, and the supernatant (serum) was transferred and stored at −70 °C.

Preparation of Standard Solutions. The stock solution of 1000 μg/mL of MEL and CYA was prepared as follows: 100 mg of MEL and 100 mg of CYA were separately transferred into 100 mL volumetric flasks and diluted with 0.2% formic acid in water. The solution was stored at 4 °C (stable for 6 months). Intermediate standard mixtures combining MEL and CYA at 10, 2.5, and 0.5 μg/mL were prepared with stock solution and diluted with 2% formic acid in water. The intermediate standard mixture solutions were kept at 4 °C (stable for 2 weeks). Five-point calibration standards of MEL and CYA were prepared at 2, 10, 50, 250, and 400 ng/mL by diluting intermediate standard mixture solutions with acetonitrile, which contained 20 ng/mL [$^{15}N_3$]-MEL and 40 ng/mL [$^{13}C_3$]-CYA.

Sample Preparation. The lamb tissue samples were prepared for LC-MS/MS using the following steps. The sample was first homogenized with a tissue homogenizer and then 2 g of sample was weighed into a 15 mL screw-cap glass test tube and 40 μL of a [$^{15}N_3$]-MEL standard solution (10 mg/L in acetonitrile) and 80 μL of a [$^{13}C_3$]-CYA standard solution (10 mg/L in acetonitrile) were added. The sample was respectively vortexed for 1 min with a 10 mL mixture of acetonitrile and water (70:30, v/v) for MEL analysis and with 2 mL of acetonitrile and 8 mL of 2% acetic acid (v/v, in deionized water) for CYA analysis (*22*, *23*). After being sonicated in a sonic-bath at 50 °C for 30 min and centrifuged at 16 100 g for 10 min, 3 mL of the supernatant was transferred to 10 mL tubes and vortexed for 1 min with 2 mL of *n*-hexane. After centrifugation at 16 100 g for 5 min, the upper organic phase (*n*-hexane) was carefully discarded, and 0.5 mL of the bottom extract was vortexed for 1 min with 0.5 mL of acetonitrile. After centrifugation at 16 100 g for 10 min, the supernatant was filtered through a 0.22 μm filter into a 2 mL autosampler vial.

Table 2. Mass Spectrometry Parameters for Determination of MEL and CYA by LC-MS/MS

compound	retention time (min)	Q1 mass (amu)	Q3 mass (amu)	DP (V)	CE (V)
CYA	1.78	128.0	42.0[a]	−23	−23
			85.0	−20	−12
$^{13}C_3$-CYA	1.78	131.0	43.0[a]	−37	−26
MEL	6.77	127.0	85.0[a]	22	28
			68.0	24	43
$^{15}N_3$-MEL	6.77	130.0	87.0[a]	23	29

[a] MRM used for quantification.

To prepare the serum samples for LC-MS/MS, 0.5 mL of sample was mixed with 2 mL of acetonitrile, 7 mL of 2% formic acid (v/v, in deionized water), and 0.5 mL of water. After sonication at 50 °C for 30 min, vortexing for 1 min, and centrifugation at 16 100 g for 10 min, 0.5 mL of the supernatant was mixed with 40 μL of the [$^{15}N_3$]-MEL standard solution (10 mg/L in acetonitrile), 80 μL of the [$^{13}C_3$]-CYA standard solution (10 mg/L in acetonitrile), and 3.38 mL of acetonitrile. After vortexing for 10 min and centrifugation at 16 100 g for 10 min, the supernatant was filtered through a 0.22 μm filter into a 2 mL autosampler vial.

Quantification of MEL and CYA Concentrations. The above samples prepared from lamb tissues and serum were quantified for the concentrations of MEL and CYA using a LC-MS/MS method, described in detail elsewhere (*23*, *24*). A prepacked silica-bonded ZIC-HILIC column (2.1 mm × 150 mm, Merck SeQuant, Umea Sweden) was used. The chromatographic separation was carried out employing a 10ADVp pumping system (Shimadzu, Columbia, MD) interfaced to a Sciex API 4000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA), employing the multiple reaction monitoring mode (MRM) in the positive ESI mode for MEL and the negative ESI mode for CYA. The MS/MS parameters for two analytes were shown in **Table 2**.

The gradient elution used two solutions. Solution A was prepared by combining 50 mL of 5 mM aqueous ammonium acetate aqueous solution, 1 mL of formic acid, and 950 mL of acetonitrile. Solution B was constituted with 50 mL of acetonitrile, 950 mL of 5 mM aqueous ammonium acetate aqueous solution, and 1 mL of formic acid. The gradient elution program was as follows: solution A was changed from 100% at 0 min to 40% at 3 min; hold 2 min; changed from 40% at 5 min to 100% at 5.1 min; hold 6.9 min; total run time was 12 min. A flow rate of 0.3 mL/min was used in all steps.

Method Validation. The blank lamb tissues used for method validation were first analyzed by the above-described method, and no melamine or cyanuric acid residue was detected. To generate validation data, 1 mL of serum or 5.0 g portions of tissue homogenate were fortified by spiking with appropriate volumes of the intermediate standard mixture solution to produce samples (n = 5) containing 20, 40, and 80 ng/g of MEL and CYA. Samples were kept at room temperature for at least 15 min before proceeding with extraction. These samples were analyzed by LC-MS/MS, and the signal-nose (S/N) ratio was recorded. The limit of detection (LOD) and limit of quantification (LOQ) for each analyte were considered to be concentrations in tissues or serum sample that produced a signal-to-noise (S/N) ratio of 3 and 10, respectively.

RESULTS AND DISCUSSION

Method Performance. The techniques currently used to detect and quantify MEL and its analogues include ELISA (*25*), GC-MS (*26*), HPLC (*27*), GC-MS/MS (*28*), and LC-MS/MS (*29*, *30*). The LC-MS/MS-based method was selected in this work because as little as 2 μg/kg MEL was detected in trace analyses. In our LC-MS/MS experiments, calibration curves for MEL and CYA typically gave R^2 values > 0.989 for the serum sample, with lamb tissue samples consistently > 0.993. At the same time, MEL

Table 3. Recovery of MEL and CYA from Fortified Tissue

analyte	tissue	fortification level (μg/kg)	n	mean recovery (%)	RSD (%)
MEL	gluteal muscle	20	5	96.2	3.6
		40	5	90.6	3.8
		80	5	78.1	7.9
	liver	20	5	86.8	5.7
		40	5	81.4	7.3
		80	5	73.7	12.6
	kidney	20	5	91.6	4.2
		40	5	93.1	4.6
		80	5	76.9	6.5
	serum	20	5	98.3	2.1
		40	5	93.6	3.7
		80	5	90.7	3.9
CYA	gluteal muscle	20	5	87.5	6.7
		40	5	86.1	6.5
		80	5	70.3	8.2
	liver	20	5	80.7	4.6
		40	5	77.2	7.3
		80	5	66.8	12.9
	kidney	20	5	90.3	6.2
		40	5	92.1	5.7
		80	5	80.7	9.6
	serum	20	5	91.6	3.6
		40	5	88.4	8.5
		80	5	82.3	2.7

and CYA were extracted from fortified samples, and recoveries of each tissues and fortification level were presented in **Table 3**. The recoveries from fortified lamb serum or tissues for MEL were 73.7−98.3% over the concentration range of 20−80 μg/kg, and the recoveries of CYA were 66.8−91.6%. For each fortification level, RSD (relative standard deviation) values of MEL or CYA ranged from 2.1% to 12.6%. The LOQ for MEL and CYA in samples, defined as concentration produced a signal-to-noise ratio of 10, was 20 ng/g in lamb tissues and 40 ng/mL in serum. The LOD for MEL and CYA, defined as the concentration that produced a signal-to-noise ratio of 3, was 10 ng/g in animal tissues and 30 ng/mL in serum.

Deposition of MEL in Lamb Tissues for Treatments with Different Doses of MEL in the Basal Diet. The concentrations of measured MEL are presented in **Figure 1**. In the control treatment (**Figure 1A**), the levels of MEL in the tissue samples were below the detection limit and could not be accurately quantified. This may be a result of a low concentration of MEL (30 μg/kg) in the control basal diet. The MEL concentrations measured in tissues from the Mel-2 treatment (with 2 mg of MEL per kg of diet) were below 40 μg/kg (**Figure 1B**), which was consistent with reports from the US-FDA and US-FSIS, where trace levels of MEL were detected in tissues from some hogs that consumed a suspect feed product containing rice proteins (*31*). The maximum MEL concentrations in lamb tissues for the Mel-2 treatment during the 60-day feeding studies were 22 μg/kg in muscle, 21 μg/kg in liver, and 32 μg/kg in kidney.

Parts **C**, **D**, and **E** of **Figure 1** present MEL concentrations in lamb tissues when the MEL concentration in the basal diet was increased to 10, 30, and 100 mg per kg of diet, respectively. At the first sampling at day 7, the MEL concentration in tissues was generally higher when the MEL dose in the diet was increased. At later time points, there was an accumulation of MEL in gluteal muscle, longissimus dorsi muscle, kidney, and liver, although it was found to be dose-independent. MEL concentrations in gluteal muscle ranged from 33 to 49 μg/kg for lambs from the Mel-10 treatment, from 59 to 131 μg/kg for lambs from the Mel-30 treatment, and from 227 to 374 μg/kg for lambs from the Mel-100 treatment. MEL concentrations in longissimus dorsi muscle were found to be similar to those in gluteal muscle. MEL concentrations in the liver ranged from 36 to 65 μg/kg for lambs from the Mel-10 treatment, from 84 to 154 μg/kg for lambs from the Mel-30 treatment, and from 158 to 412 μg/kg for lambs from the Mel-100 treatment. MEL concentrations in the kidney were higher than those for other tissues and ranged from 80 to 117 μg/kg for the Mel-10 treatment, from 180 to 329 μg/kg for the Mel-30 treatment, and from 347 to 808 μg/kg for the Mel-100 treatment. MEL concentrations in the serum were affected by the time of sampling. In this study, the lamb blood was consistently taken from the vein of the neck after withdrawing the diet for 16 h. The MEL concentrations in the serum ranged from 23 to 62 μg/kg for lambs from the Mel-10 treatment, from 52 to 97 μg/kg for lambs from the Mel-30 treatment, and from 131 to 267 μg/kg for lambs from the Mel-100 treatment.

Deposition of CYA in Animal Tissues When Coadministered with MEL. CYA may be present in animal feed products in the USA as a result of the addition of feed-grade biuret (*32*), but there is little information regarding the deposition of MEL in ruminant animals that are simultaneously administered with CYA and MEL. We found that MEL concentrations in lamb tissues were similar for the Mel-100 (the 1000 g diet had 100 mg of MEL) and Mel-Cya (the 1000 g diet contained 100 mg of MEL and 100 mg of CYA) treatments (**Figure 1E** vs Figure 2**A**). Under the treatment conditions, the formation of complexes of MEL and CYA had little effect on MEL accumulation in tissues, which is different from the case of a literature study based on fish (*13*). The CYA concentrations in lamb tissues from the Mel-Cya treatment ranged from 44 to 62 μg/kg in gluteal muscle, from 33 to 73 μg/kg in longissimus dorsi muscle, from 59 to 120 μg/kg in liver, and from 128 to 191 μg/kg in kidney (**Figure 2B**). Our results indicated that MEL accumulation in lamb tissues was more favored than CYA when the two compounds were fed at the studied conditions. A literature study also showed that the MEL concentration in tissues was generally 1 order of magnitude higher than that of CYA when the two compounds were coadminstered by fish (*13*). Moreover, no weight loss and mortality of lambs were noted when lambs consumed the complex of MEL and CYA during the 60-day feeding studies.

Clearance of MEL in Lambs Post-MEL-treatments. After switching to the control diet at day 60 for the lambs subjected to the Mel-100 treatment, the decrease in MEL levels in all tissues was observed immediately after withdrawal of the MEL (**Figure 3A**). The MEL contents in lamb tissues were 38, 15, 14, and 29 μg/kg in gluteal muscle, longissimus dorsi muscle, liver, and kidney, respectively, at 84 h after the MEL withdrawal (**Figure 3A**). Although the highest MEL level found in kidney was 2-fold higher than that in gluteal muscle, the MEL concentrations decreased to below 20 μg/kg in all tissues 108 h after the MEL withdrawal. Furthermore, we found that the MEL concentration was lower than the detection limit of 10 μg/kg for lamb tissues after 6 days of MEL withdrawal. Our study was in agreement with the findings of the US-FSIS that reported undetectable levels of MEL in muscle and kidney tissues in hogs 24 and 48 h after exposure to contaminated feed (*31*).

Previous studies suggested that the plasma half-life of MEL was approximately 4 h in hogs (*33*, *34*) and the plasma half-life was approximately 2.7 h in rats (*35*). In our study, the MEL concentration in the lamb serum for the Me-100 treatment was 1010 ± 65 μg/L 1 h after the MEL withdrawal in the diet. The average MEL concentration in serum decreased to 25 ± 5 μg/L after the MEL withdrawal for 84 h (**Figure 3B**). To determine the apparent half-life of melamine in the serum of the lambs,

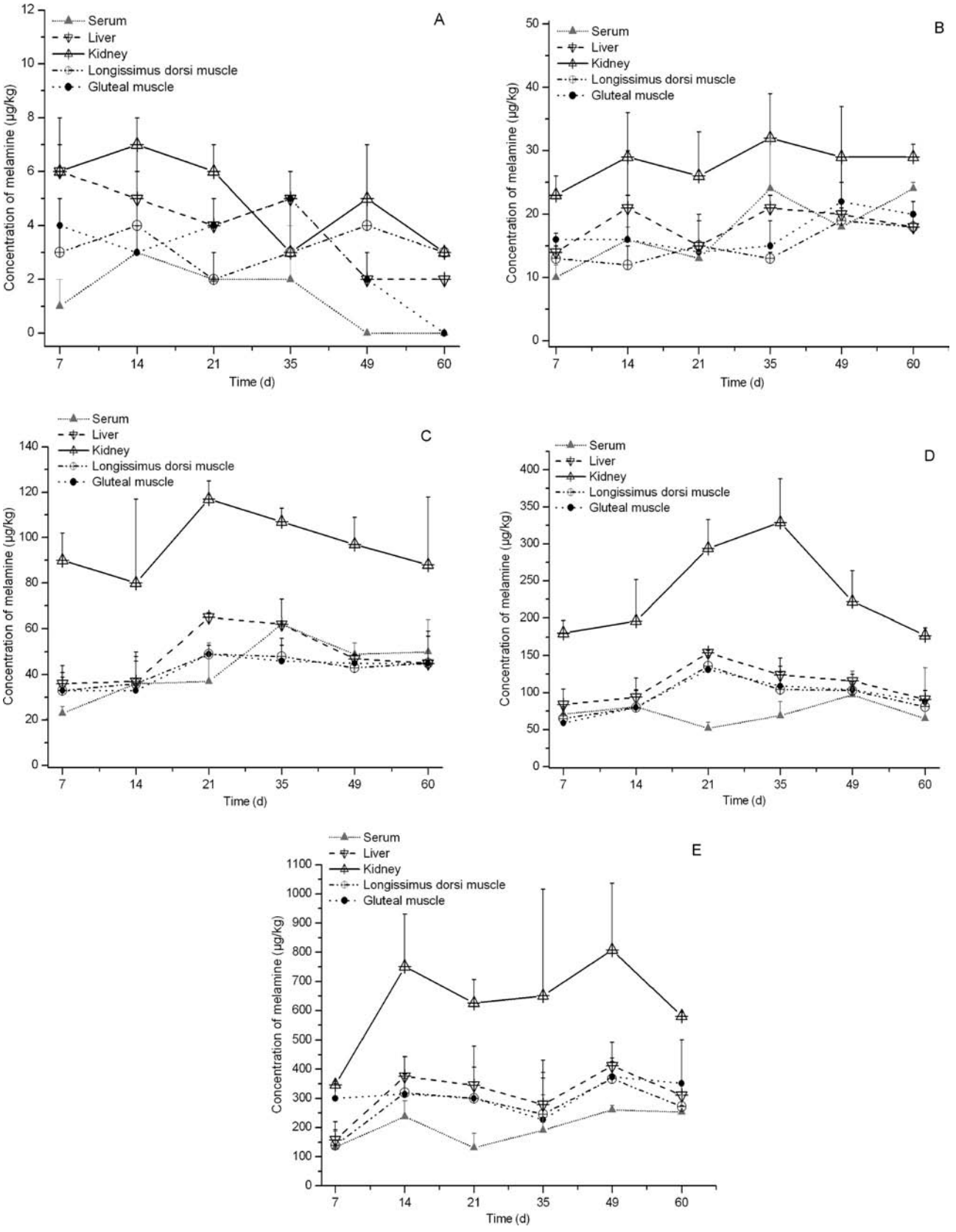

Figure 1. MEL accumulation in lamb tissues during 60-day feeding treatments with a basal diet supplemented with different levels of MEL: (**A**) 0, (**B**) 2, (**C**) 10, (**D**) 30, and (**E**) 100 mg of MEL per kg of diet. Error bars are standard deviations from three sample replicates.

we performed a nonlinear regression analysis (GraphPad Prism 4) on the melamine concentrations in serum. Based on the equation ($Y = 1064 \times \exp(-0.1367 \times X) + 44.17$; $R^2 = 0.9632$), the half-life of melamine in the serum of lambs was 5.8 h.

Maximum Concentrations of MEL in Lamb Tissues. The maximum MEL level found in the lamb tissues from the Mel-100 treatment was 416 ± 15 μg/kg in gluteal muscle, 435 ± 12 μg/kg in longissimus dorsi muscle, 469 ± 13 μg/kg in liver, and 1067 ±

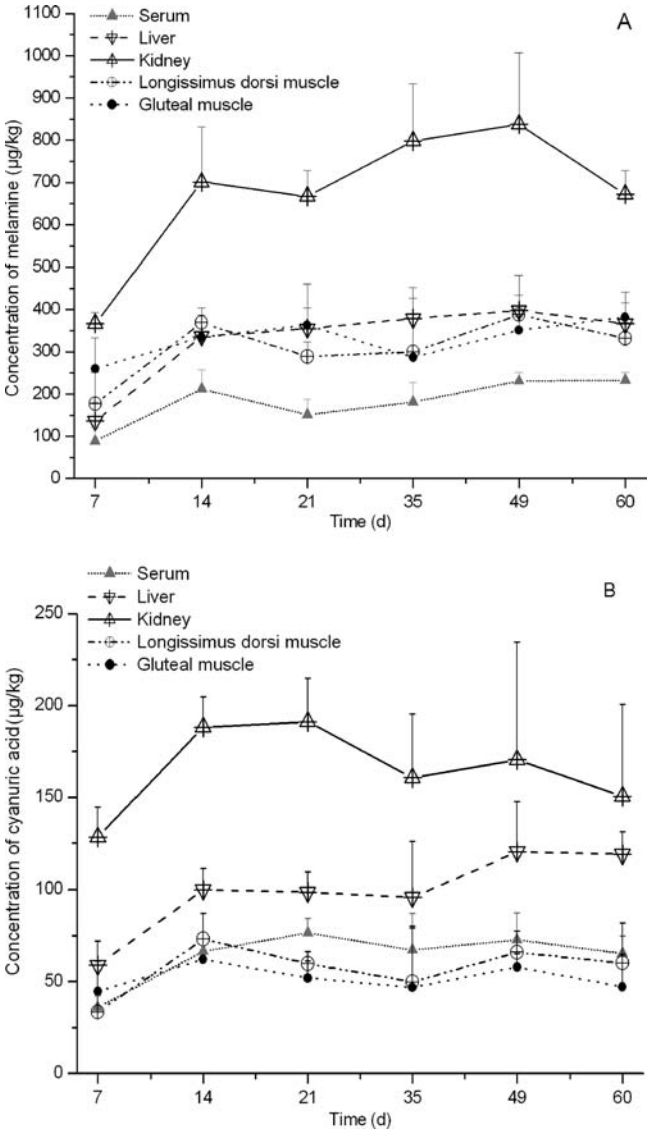

Figure 2. Concentrations of MEL and CYA in lamb tissues during 60-day feeding studies using a basal diet containing 100 mg of MEL and 100 mg of CYA per kg of diet. Error bars are standard deviations from three sample replicates.

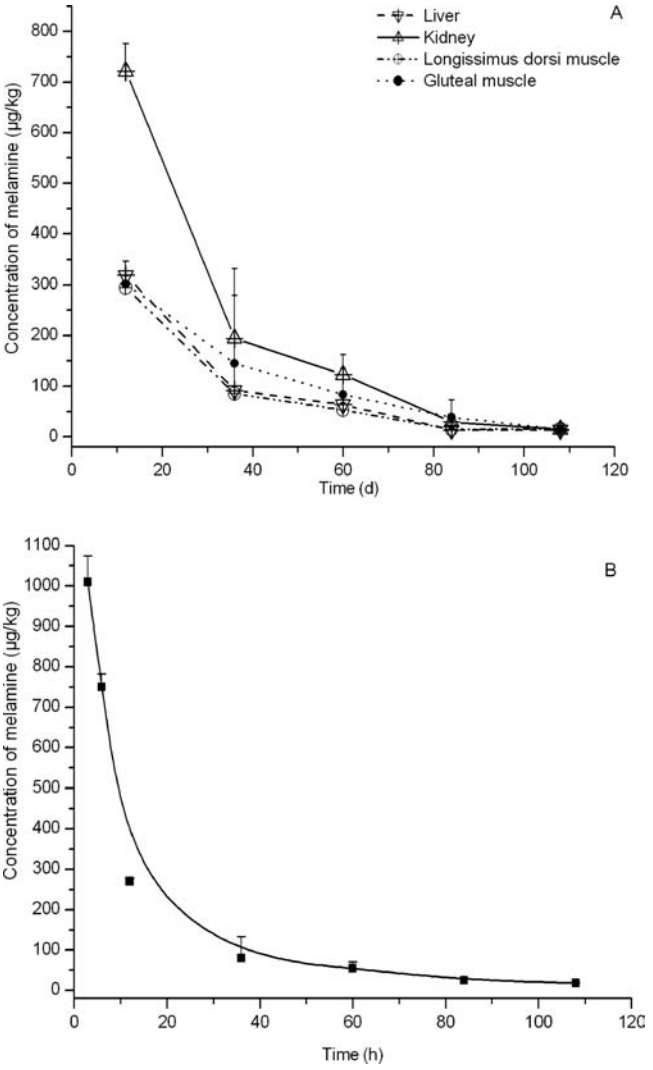

Figure 3. Clearance of MEL in (**A**) tissues and (**B**) serum of lambs after withdrawing MEL from the diet that was previously used at a level of 100 mg of MEL per kg of diet to continuously feed lambs for 60 days. Error bars are standard deviations from two sample replicates.

26 μg/kg in kidney. After the maximum MEL concentration was reached in lamb tissues, a fluctuation in MEL levels was observed. Although the highest MEL level in muscle and liver was reached on day 49, the maximum MEL concentration in the kidney was found on day 53 after the administration. The observation of higher MEL levels found in kidney compared with those in muscles is probably because MEL is mainly eliminated by the kidney, also reported in a previous study (*35*).

In summary, we have evaluated the deposition and elimination of MEL in lambs consuming 2−100 mg/kg MEL in a basal diet for a period of 60 days. Meat samples from lambs exposed to 2 mg/kg of MEL were below the 50 μg/kg level that was used in the US-FDA human health risk assessment for MEL. In addition, we showed that CYA, present in the diet at a 1:1 mass ratio to MEL at 100 mg/kg, did not affect the accumulation of MEL and accumulated at a much lower level than MEL. After stopping MEL in the diet, MEL contents in the tissues and serum decreased to below 20 μg/kg in all tissues 108 h after the MEL withdrawal. Our results may provide some information for further work on human health risk assessment for MEL toxicology.

ABBREVIATIONS

CYA, cyanuric acid; ELISA, enzyme-linked immunosorbent assay; GC-MS, gas chromatography−mass spectrometry; GC-MS/MS, gas chromatography−tandem mass spectrometry; HILIC, hydrophilic interaction liquid chromatography; HPLC, high-performance liquid chromatography; LC-MS/MS, liquid chromatography−tandem mass spectrometry; MEL, melamine; US-FDA, United States Food and Drug Administration; US-FSIS, United States Food Safety Inspection Service.

ACKNOWLEDGMENT

This study was supported by the *Special Fund for Establishment of Maximum Residue Limit* (*MRL*) *of MEL in Feed* (*Ministry of Agriculture, P.R. China*). The authors are grateful to Shuming Yang and Yonglin Zhu for technical assistance. The authors also wish to acknowledge Qixin Zhong for his useful suggestions and English editing.

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Received for review July 28, 2009. Revised manuscript received December 7, 2009. Accepted December 08, 2009.