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Investigation of melamine and cyanuric acid deposition in pig tissues using LC-MS/MS methods

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ABSTRACT

Four LC-MS/MS methods were developed to quantify melamine (MEL) and cyanuric acid (CYA) in various pig tissues at or above the level of concern (2.5 mg/kg). Pigs treated with 200 mg/kg bw/day CYA daily for 7 days did not accumulate significant residue concentrations in muscle, liver or kidney. Pigs treated with 200 mg/kg bw MEL daily for 7 or 28 days had MEL residues in muscles (3–13 ppm), liver (2.8–14.1 ppm) and kidney (9.4–27.2 ppm). Treatment with MEL and CYA at 100 mg/kg bw of each triazine daily for 7 days resulted in MEL (26–59 ppm in muscle, 30–49 ppm in liver and 367–6300 ppm in kidney) and CYA (1.8–5.8 ppm in muscle, 2.6–6.5 ppm in liver and 303–7100 ppm in kidney). Treatment with MEL and CYA at 1, 3 or 10 mg/kg bw/day for 7 days did not result in residues greater than the level of concern in all tissues tested. Pigs dosed with 33 mg/kg bw/day of MEL + CYA for 7 days contained residues above the level of concern only in kidney. Deposition of MEL and CYA depends on the tissue type (muscles, liver and kidney), dosage and whether the triazines are given alone or in combination.

1. Introduction

During the 2007 pet food recall in the United States, animal feeds destined for farm pigs, poultry and fish were contaminated with melamine and related compounds (MRC). In several states contaminated ingredients and scraps from the production of pet food were mixed with grain and bakery meal to make swine and chicken feed, with the scraps typically comprising up to 15% (USFDA, 2007). Edible tissues from animals that ate the contaminated feed in 2007 contained insignificant amounts of MRC. However, questions arose as to the depositions of MRC in edible tissues of animals if fed higher concentrations of the chemicals. During the 2008 infant formula recall in China, hundreds of tons of human food were found to be contaminated with melamine due to intentional adulteration of milk. Although Chinese authorities urged manufacturers and distributors to destroy all contaminated products, hundreds of tons of melamine-contaminated dairy products were still discovered years later according to Chinese and international public media (China Daily, 2010; CNN, 2011; New York Times, 2009, 2010, 2011; The Telegraph, 2011; Reuters, 2014). Discoveries of such large amounts of melamine-contaminated products after the fact suggest the possibility that some of those products may be diluted and fed to farm animals intentionally for economically motivated reasons.

The carry-over of MRC from feed to edible tissues of animals has been investigated in pig, chicken, lamb, sheep and fish (Andersen et al., 2011; Cruywagen et al., 2011; Li et al., 2010; Lv et al., 2010; USFDA, 2007; Wang et al., 2014). The data reported suggest that

Abbreviations: ACN, acetonitrile; ACUC, Animal Care and Use Committee; APCI, atmospheric pressure chemical ionization; bw, body weight; CYA, cyanuric acid; ESI, electrospray ionization; LC-MS/MS, liquid chromatography tandem mass spectrometry; LOD, limit of method detection; LOQ, limit of method quantitation; MEL, melamine; MRC, melamine and related compounds; MRM, multiple reaction monitoring; NOAEL, no observable adverse effect level; N/R, not reported; PVDF, polyvinylidene fluoride; PTFE, polytetrafluoroethylene; RPM, rotations per minute; RSD%, relative standard deviation percent; DOE, United States Department of Energy; USFDA, United States Food and Drug Administration; ZIC-HILIC, zwitterionic hydrophilic interaction liquid chromatography.

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multiple factors determine MRC depositions in tissues and more data are needed to fully evaluate human health risks associated with consumption of products derived from animals exposed to MRC.

In this study we investigated the deposition of melamine (MEL) and cyanuric acid (CYA) in various tissues of pigs dosed with various concentrations and combinations of these two chemicals. The major goal of this study is twofold. First, to develop LC-MS/MS methods that could be used by laboratories with various equipment platforms which are able to quantify MEL and CYA in various pig tissues at the level of concern (2.5 mg/kg; USFDA, 2008), and secondly, to determine deposition of MEL and CYA in the loin, ham, liver and kidney of pigs that had been exposed to these chemicals alone or in combination for 7 or 28 days during a previous NOAEL (No Observable Adverse Effect Level) study (Stine et al., 2011).

2. Methods

2.1. Animals, husbandry and experimental design

Loin, ham, liver and kidney used in this study were obtained from animals used in a previous NOAEL study where the formation of renal crystals was used as the key parameter to evaluate the toxic effect of the melamine and cyanuric acid exposure (Stine et al., 2011). The reader is referred to the NOAEL study communication for a detailed description of animal treatment and the experimental design. Briefly, weanling cross-bred barrows (20–26 kg body weight) were obtained from a local producer and fed a standard corn and soybean diet during a 2-week acclimatization period. In the 7 day study, pigs were randomly assigned to treatment groups of 0, 1.0, 3.3, 10, 33, or 100 mg/kg bw/day of MEL and CYA each or 200 mg/kg bw/day of either compound individually (MEL or CYA; n = 2 each treatment). Pigs were given the test compound(s) for 7 consecutive days and sacrificed on the 8th day. In the 28 day study, pigs were randomly assigned to 0, 1.0, or 3.3 mg/kg bw/day of MEL + CYA or 200 mg/kg bw/day MEL alone (n = 12 control, n = 8 other treatments). Pigs were given the test compound(s) for 28 consecutive days and sacrificed on the 29th day. MEL and/or CYA were mixed with retail chocolate pudding in amounts tabulated from daily weight measurements to ensure accurate dosing. The number of pigs used in the study was based on both the need to address objectives of the preliminary risk assessment and the need to adhere to the Animal Care and Use Committee (ACUC) guidelines for reducing the number of large mammals used in research. Animal research was conducted at a laboratory certified by the Association for Assessment and Accreditation of Laboratory Animal Care International. The experimental protocol was approved by the ACUC at the Office of Research, Center for Veterinary Medicine, USFDA, and all procedures were conducted in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals (1996) and the Animal Welfare Act of 1966 (P.L. 89–544), as amended.

2.2. Preparation and analysis of tissues

The kidney, stomach, bladder, urine and blood were analyzed for presence of crystals, blood urea nitrogen, urine pH, blood creatinine, kidney weight and other parameters, and the results were reported in a previous communication (Stine et al., 2011). Loin, ham and liver were pre-cut into 5–10 g cubes and divided into four portions (70–150 g) for LC-MS/MS analysis in each collaborating laboratory. Loin, ham and liver from pigs with IDs 1466 and 1470 were excluded from the study as the labels had come off in the freezer. A portion (approx. 50%) of kidney from each experimental pig was archived. The rest of each kidney was homogenized with dry ice using a blender (Waring Commercial) to obtain a homogeneous powder and divided into four equal portions (15–50 g) for LC-MS/MS analysis in each of four collaborating laboratories.

2.3. LC-MS/MS analysis

2.3.1. Laboratory #1

The procedure was modified from previously reported method (Heller and Nochetto, 2008). Loin or ham (2 g) was weighed into a 50 mL polypropylene centrifuge tube. Then, 20 mL of 2.5% aqueous formic acid was added and the mixture was homogenized for 20 s with a homogenizer (Omni International) using a metal probe. Each sample was spiked with internal standards to obtain 400 ng/g each of melamine-$^{13}C^{15}N$ and cyanuric acid-$^{13}C^{15}N$ and sonicated for 30 min. Samples were mixed on a multi-tube vortex mixer for 30 min and centrifuged at 3750 *g* for 30 min at 4 °C. Supernatant (1.8 mL) was transferred in a 2 mL tube with 0.2 mL of hexane, vortexed for 20 s and centrifuged at 16,000 *g* for 30 min. Top (hexane) layer was removed by pipetting and approximately 0.5 mL aliquot was filtered through a glass fiber syringe filter (0.7 or 1.0 μm pore size). Filtrate (0.05 mL) was added to 0.95 mL of acetonitrile (ACN), vortexed for 15 s and centrifuged for 30 min at 16,000 *g* to precipitate particulate matter. Supernatant (0.9 mL) was filtered through a polyvinylidene fluoride (PVDF) syringe filter (0.2 μm pore size) in such a way that the first 0.80 mL was discarded (to wash out possible contaminants) and only the last 0.1 mL was placed into a 350 μL autosampler vial for LC-MS/MS analysis. Liver and kidney were processed using the same procedure as for loin and ham, but the defatting step with hexane was omitted.

Extracts were automatically injected (5 μL) and chromatographed on a guarded analytical column (SeQuant, ZIC-HILIC, 150 × 2.1 mm 5 μm) at 30 °C using Shimadzu Prominence LC coupled with an AB Sciex API-4000 triple-Q mass spectrometer. CYA and MEL eluted at 4.2 min and 7.5 min, respectively, under gradient elution of mobile phase A (95% of ACN and 5% of 0.1% aqueous formic acid) and B (50% of 20 mM ammonium formate and 50% of ACN). Mobile phase A was 100% from 0 to 4 min. Concentration of mobile phase B (50% of 20 mM ammonium formate and 50% of ACN) gradually changed from 0 to 75% for the next 5 min (from 4 to 9 min). Flow was diverted to waste between 0–3.5 min and 10–14 min. Mass spectral data were acquired in electrospray ionization (ESI) with multiple reaction monitoring (MRM) mode using negative polarity for CYA and positive for MEL. Peaks were integrated and values corrected to internal standards to compensate for matrix effect, losses during extraction and instrument variability using Analyst 1.5.1 software. Concentration of analytes was calculated based on calibration curve standards prepared in solvent. Linear regression was used to quantify MEL for up to 2.5 μg/g and CYA for up to 3.5 μg/g in samples. An exponential regression was used for a calibration curve to quantify MEL between 2.5 and 3.5 μg/g in samples.

2.3.2. Laboratory #2

The procedure was adapted in part from the previously reported method (Filigenzi et al., 2008). Muscles, liver and kidney were homogenized using a Robot Coupe after freezing with liquid nitrogen or dry ice. Each sample (1 g) was weighed out in a polypropylene centrifuge tube and spiked with internal standards at a final concentration of 500 ng/g each of melamine-^{13}C and cyanuric acid-^{13}C. An aliquot of 25 mL of extraction solvent (50% ACN, 40% water and 10% diethylamine) was added and the mixture was homogenized for 1 min using an Ultra-Turrax T-25 tissue homogenizer. The extract was centrifuged at 800 RPM (5 min, 25 °C) and 2.5 mL of the supernatant was transferred to 5.5 mL of ACN. After vortex mixing (20 s), the mixture was centrifuged at 1500 RPM for 5 min at 25 °C. Approximately 4.5 mL of supernatant was filtered through a 1.0 μm PTFE syringe filter and 4 mL of filtrate was transferred to a glass tube for evaporation to dryness under a stream of nitrogen in the TurboVap water bath at 60 °C. The residue was reconstituted with 1 mL of ACN/water (9:1) mixture, vortexed for 20 s and sonicated for 2 min. The extract was then filtered through a 0.45 μm PVDF syringe filter into an autosampler vial and analyzed on Agilent 1100 LC coupled with an AB Sciex API-4000 triple-Q mass spectrometer. A 5 μL aliquot was injected and chromatographed on the guarded analytical column (Phenomenex, HILIC 100Å, 2.6 μm, 2.1 mm × 100 mm) at 30 °C. CYA eluted at 0.7 min and MEL at 2.1 min under gradient elution from 100% to 90% of mobile phase A over the first 3 minutes. Mobile phase A comprised 10 mM aqueous ammonium acetate buffer (pH 5) and mobile phase B comprised 100% of ACN. Mass spectral data were acquired in atmospheric pressure chemical ionization (APCI) MRM mode with negative polarity for CYA and positive for MEL. The ratio of quantitation and confirmation ions for unknown samples was ± 30% of the mean ratio for the calibration curve standards prepared in solvent. Peaks were integrated and values were corrected to internal standards to compensate for matrix effect, losses during extraction and instrument variability using Analyst 1.5.1 software.

2.3.3. Laboratory #3

The procedure was adapted from previously reported methods (Filigenzi et al., 2008; Turnipseed et al., 2008). Loin, ham or liver was pre-cut into chunks, blended using a Waring blender followed by a Tekmar tissumizer, weighed (2 g) into a polypropylene centrifuge tube and spiked with internal standards to obtain 1 μg/g each of melamine-^{13}C and cyanuric acid-^{13}C. The mixture was vortexed after adding 1 mL of water. An aliquot of 7 mL of extraction solvent (5:5:0.1, ACN:water:diethylamine) was added and the mixture was centrifuged at 5000 RPM after shaking for 10 min. Supernatant (3 mL) was mixed with 3 mL of water and 3 mL of hexane, shaken for 10 min and centrifuged at 5000 RPM for 10 min. The top layer, containing hexane, was removed and 500 μL of the aqueous extract was filtered through Millipore membrane (3000 nominal molecular weight limit units), using an Amicon Ultra-0.5 filtration unit at 14,000 RPM for 30 min. The filtrate (300 μL) was dried in a glass tube at 55°C, dissolved in 300 μL of mobile phase A, and injected (2 μL) for LC-MS/MS analysis.

The kidney was processed using the same procedure as for loin, ham and liver, but only 1 g of kidney was used. Extraction solvents used for kidney processing were reduced in equal proportions. Extracts were analyzed on a guarded analytical column (Nest Group, ZIC-HILIC, 150 × 2.1 mm 5 μm) at 30 °C. Mobile phase A was a mixture of ACN (95%) and 0.1% aqueous formic acid (5%). Mobile phase B was a mixture of ACN (50%) and 20 mM ammonium formate (50%). Mass spectral data were acquired using Varian 1200-MS (for some loin) or Waters Quattro Micro triple-Q (for some loin, ham, liver and kidney) mass spectrometers in ESI mode with positive polarity for MEL and negative for CYA.

Calibrator and quality control (QC) spiked samples were prepared in blank matrices purchased from local supermarkets or butcher shops. Calibrator values and QC samples were required to be ± 20% of nominal (spiked) value. The ratio of quantitation and confirmation ions for unknown samples was ± 20% of the mean ratio for the calibration standards. If the lowest or highest calibrator did not pass the