Identification and Characterization of Toxicity of Contaminants in Pet Food Leading to an Outbreak of Renal Toxicity in Cats and Dogs

Roy L. M. Dobson,* Safa Motlagh,* Mike Quijano,* R. Thomas Cambron,* Timothy R. Baker,* Aletha M. Pullen,* Brian T. Regg,* Adrienne S. Bigalow-Kern,* Thomas Vennard,* Andrew Fix,* Renate Reimschuessel,† Gary Overmann,* Yuching Shan,* and George P. Daston*†

*The Procter & Gamble Company, Cincinnati, Ohio 45253; and †Center for Veterinary Medicine, U.S. Food and Drug Administration, Laurel, Maryland 20708

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This paper describes research relating to the major recall of pet food that occurred in Spring 2007 in North America. Clinical observations of acute renal failure in cats and dogs were associated with consumption of wet pet food produced by a contract manufacturer producing for a large number of companies. The affected lots of food had been formulated with wheat gluten originating from China. Pet food and gluten were analyzed for contaminants using several configurations of high-performance liquid chromatography (HPLC) and mass spectrometry (MS), which revealed a number of simple triazine compounds, principally melamine and cyanuric acid, with lower concentrations of ammeline, ammelide, ureidomelamine, and N-methylmelamine. Melamine and cyanuric acid, have been tested and do not produce acute renal toxicity. Some of the triazines have poor solubility, as does the compound melamine cyanurate. Pathological evaluation of cats and dogs that had died from the acute renal failure indicated the presence of crystals in kidney tubules. We hypothesized that these crystals were composed of the poorly soluble triazines, a melamine-cyanuric acid complex, or a combination. Sprague dawley rats were given up to 100 mg/kg ammeline or ammelide alone, a mixture of melamine and cyanuric acid (400/400 mg/kg/day), or a mixture of all four compounds (400 mg/kg/day melamine, 400 mg/kg/day cyanuric acid). Neither ammeline nor ammelide alone produced any renal effects, but the mixtures produced significant renal damage and crystals in nephrons. HPLC-MS/MS confirmed the presence of melamine and cyanuric acid in the kidney. Infrared microspectroscopy on individual crystals from rat or cat (donated material from a veterinary clinic) kidneys confirmed that they were melamine-cyanuric acid cocrystals. Crystals from contaminated gluten produced comparable spectra. These results establish the causal link between the contaminated gluten and the adverse effects and provide a mechanistic explanation for how two apparently innocuous compounds could have adverse effects in combination, that is, by forming an insoluble precipitate in renal tubules leading to progressive tubular blockage and degeneration.

Key Words: melamine; cyanuric acid; triazines; food safety.

There was an intensive recall of pet food in North America in March and April of 2007 following the discovery that some dogs and cats had become ill after eating certain lots of wet pet food. Although the recall involved a large number of pet food brands, all had been produced by a single manufacturing facility that was working under contract to a large number of pet food companies. This paper describes the research we conducted in the days and weeks following the recall to determine the nature of the contamination, and how these contaminants produced acute renal toxicity. The information described here was shared with the U.S. Food and Drug Administration on a daily basis while it was being generated, but has not yet been published.

The possible contamination of pet food first came to light through observations of acute renal failure in cats and dogs, beginning a few hours after consumption of the affected lots of food. An evaluation of the affected lots of food indicated that they were all prepared at the same contract facility at around the same period of time. The contract facility was able to determine that this period coincided with the introduction of a new supply of wheat gluten, sourced from China. Wheat gluten is added to wet pet foods as a thickening agent.

During the first days after the announcement of the recall, a number of different veterinary clinics, schools, and professional societies began to compile databases on the symptoms, clinical chemistry, and pathology of affected dogs and cats. The pattern of symptoms included anorexia, vomiting, lethargy, polyuria, and polydipsia. Clinical chemistry suggested renal failure: high blood urea nitrogen and creatinine levels. Histopathology of the kidneys of animals that died as the result of renal failure had yellowish-brown crystals present in the tubules (see www.avma.org/aa/petfoodrecall for a compilation of clinical and pathological observations).

We began an intensive analytical chemistry evaluation of the affected food and wheat gluten to characterize the possible contaminants so that we could ensure that we had correctly identified all of the affected lots and the source of the contamination. Known causes of renal toxicity were screened for in the affected pet food and wheat gluten, including metals,
mycotoxins, and pesticides: none were present at levels of concern. Therefore, we initiated a comprehensive analytical chemistry program to identify small-molecule contaminants. Melamine and other triazines (ammeline, ammelide, ureidomelamine, N-methylmelamine, cyanuric acid) were identified as the likely contaminants within the first week of investigation, but since melamine has low acute toxicity and no apparent renal toxicity, we were compelled to conduct toxicological investigation to make sure that we had identified the chemicals that were responsible for the poisonings.

Melamine is widely used in plastics, and is perhaps best known as the plastic in colorful, inexpensive dinnerware. The toxicity of melamine has been extensively evaluated. Pharmacology studies in rats and dogs indicate that high doses of melamine have diuretic properties, but do not produce renal toxicity (Lipschitz and Stokey, 1945). The U.S. National Toxicology Program (NTP, 1983) evaluated the subchronic and chronic toxicity of melamine in mice and rats. Again, there was no indication of renal toxicity. The principal effect was the occurrence of calculi in the urinary bladder with associated bladder tumors. Cyanuric acid has also been evaluated for acute, subchronic and chronic toxicity in rat, mice, and dogs (Hammond et al., 1986; Hodge et al., 1965), and produced a similar spectrum of effects as melamine: diuresis, and at high concentrations bladder calculi in subchronic and chronic studies. There were no indications of renal toxicity or the formation of precipitates in kidney tubules. The acute LD₅₀ of these compounds is > 1 g/kg body weight.

Less was known about the toxicity of the other contaminants found at lower levels. Methylmelamine has a published acute LD₅₀ of 270 mg/kg and is not demethylated by microsomal metabolizing systems (Rutty and Connors, 1977). Therefore, it is unlikely that this contaminant could have produced the observed effects. There were no reports of ammeline and ammelide in the toxicology literature, but reports from the nutrition literature indicated that feeding ammeline to sheep at a dosage of approximately 100 mg/kg/day produced kidney stones after several weeks (Mackenzie and van Rensburg, 1968). The effect was sufficiently severe that a number of sheep in the experimental group died.

Since renal crystals had also been reported in animals ingesting the contaminated pet food, this result suggested to us two hypotheses: first, that ammeline or ammelide was sufficiently toxic to dogs and cats that it produced adverse effects at lower doses and after fewer doses than in sheep; or second, that a mixture of the melamine-related compounds was responsible for the toxicity.

We considered the possibility that melamine or one of the other triazines was being biotransformed into a more toxic compound. However, metabolism studies on melamine and cyanuric acid indicate that they are not metabolized by mammals (Arnes et al., 1979; Hammond et al., 1986; Worzalla et al., 1973), although they are metabolized by bacteria (Cook et al., 1985; Jutzi et al., 1982). We also considered the possibility that one or more of the compounds (particularly those that had not been evaluated for toxicity) were directly toxic to cat or dog kidney cells. We evaluated that hypothesis by conducting cytotoxicity studies in established cell lines from each species. Finally, we conducted in vivo studies in rodents when it became clear that the most likely hypothesis was an interaction between two or more of the triazines within the renal tubules that was responsible for the toxicity.

Our results indicate that the principal contaminants in the wheat gluten and pet food were melamine and cyanuric acid. These compounds, when coingested form an insoluble precipitate in kidney tubules that is of sufficient severity to cause renal failure via physical blockage.

MATERIALS AND METHODS

Chemicals. Ammeline and ammelide were obtained from TCI America (Portland, OR). Melamine and cyanuric acid were obtained from Sigma-Aldrich (Milwaukee, WI). Ureidomelamine and methylmelamine could not be obtained from a commercial source and were synthesized by P&G synthetic chemists. Chemical purity of the commercially obtained materials, as determined by P&G, was: melamine, 99.4%, cyanuric acid, 97.9%, ammeline, 86.9%, ammelide, 97.8%. In each case the impurities were one or more of the other triazines. The stable-isotope-labeled internal standard used in the quantitative HPLC-MS/MS methods, [15N₅]-melamine (99 atom%), was obtained from ICON Services, Inc. (Summit, NJ). All other chemicals and solvents utilized in the analytical procedures were reagent grade or better.

Analytical chemistry procedures. Several combinations of HPLC and mass spectrometry (MS) configurations were employed to discover, identify and ultimately quantify small-molecule causative agents in the affected pet food and wheat gluten, as well as to quantify these compounds in biological tissues. In addition, Fourier transform infrared (FTIR) spectroscopy techniques were used to evaluate the chemical identity of crystals found in kidney and in wheat gluten samples. The following provides additional details of the analytical procedures and instrumentation utilized.

Discovery and identification of chemical adulterants by liquid chromatography-MS. Reversed-phase HPLC and hydrophilic interaction chromatography (HILIC, a variation of normal-phase HPLC) (Alpert, 1990), separations were carried out on aqueous and organic extracts of pet food and wheat gluten, using both positive (+) and negative (-) electrospray ionization (ESI), in conjunction with high resolution time-of-flight (TOF) MS detection. Prior to extraction, wet pet food was finely ground using a high speed food processor, and 1 g extracted in 10 ml of 1% formic acid (aq) or acetonitrile. Samples were vortexed for 5 min and 2 ml of the resulting suspension centrifuged (21,000 × g, 5 min) and 0.5 ml of the supernatant fluid passed through a Nanosep Omega 30,000 MWCO ultrafiltration device ( Pall Corp., East Hills, NY). Wheat gluten (0.1 g) was likewise extracted, but without preprocessing. An aliquot of each filtered extract was then diluted 20:1 in water or acetonitrile prior to HPLC or HILIC analysis, respectively. HPLC and HILIC separations were carried out using an Alliance 2965 system (Waters, Milford, MA), interfaced to an LCT TOF-MS (Waters-Micromass, Beverly, MA), for screening and initial identification experiments. A Q-TOF-II hybrid quadrupole-TOF MS/MS instrument (Waters-Micromass) was used for more-definitive structure assignments (fragmentation data) and for comparative (versus reference compounds) structure confirmation experiments. For the HILIC separations, an Atlantis HILIC Silica, 2.1 x 250 mm (3 μm), column was employed, using a broad acetonitrile:20mM ammonium formate (aq) gradient, over 20 min, at 0.3 ml/min flow rate.

Quantification of triazines by HILIC-MS/MS. Quantification of targeted triazine compounds in either wheat gluten or kidney extracts was accomplished.
using HILIC-MS/MS. HILIC separation was carried out employing a 10ADVp
ing pumping system (Shimadzu, Columbia, MD) interfaced to a Sciex API 4000
triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA),
operated in the posESI or negESI mode and employing selected reaction
monitoring (SRM), for selective detection of each compound of interest. The
separation was carried out on an Atlantis HILIC Silica, 4.6 x 50 mm (5 μm),
column, using a rapid acetonitrile: 20mM ammonium formate (aq) gradient
(6 min run time), at 0.85 ml/min flow rate. Each sample extract (20 μl injected)
was analyzed twice; first using +ESI and then using −ESI modes. SRM
detections schemes were: +ESI Mode: melamine (m/z 127–85), [15N2]-
melamine internal standard (m/z 133–89), ammeline (m/z 128–86), ammelide
(m/z 129–87) and (when monitored) ureidomelamine (m/z 170–127); −ESI Mode:
cyanuric acid (m/z 128–42) and ammelide (m/z 127–84). Because
ammelide was readily detected in both ionization modes, the mean of +ESI and
−ESI results was calculated.

Sample preparation for the wheat gluten was as described in the
identification work, with the following exceptions: 2.5% (vol/vol) HCl (aq)
as the extraction solvent; addition of the internal standard; and an additional
100-fold dilution in posESI mode. Kidney samples were prepared at
about 40 mg of tissue with 10 ml of 2.5% (vol/vol) HCl (aq), sonicating for 1 h
at 50°C, adding internal standard, and diluting an aliquot of each extract 200:1
(50,000© total dilution of tissue mass).

Characterization of crystals in kidney by FTIR.
The chemical identity of crystals in cat and rat kidney tissue was determined by FTIR microspectroscopy. A Hyperion 3000 infrared microscope coupled to an IFS 66/s
spectrometer (Bruker Optics, Billerica, MA) was utilized for spectral
acquisition. Spectra were collected using a combination of focal-plane array
spectral imaging (Lewis et al, 1995) and an ATR infrared microscope objective
coupled to a single element detector. Frozen tissue samples were sectioned
with a microtome to produce a thickness of 5 μm and mounted on low-e microscope
slides for infrared spectroscopy (Kevely Technologies, Chesterland, OH).
The number of scans coaveraged was 64 and 128 for ATR and focal-plane array
configurations, respectively.

Cytotoxicity experiments. Cytotoxicity of individual contaminants was
determined using Madin-Darby canine kidney cells and Crandell feline kidney
cells (ATCC, Manassas, VA). Cells were seeded into six-well plates
(approximately 13,000 cells per plate) and maintained on 10% fetal bovine
serum in Dulbecco’s modified Eagle’s medium. When the cells had reached
semiconfluence test compounds were added at multiple concentrations at levels
up to 500 μM and the solubility limit for the compound. Cytotoxicity was
determined after 24 h of exposure to the test agents using the MTT assay for
cell viability (Sigma Assay Kit M3655, St Louis, MO). Mercaptoethanol was
used as a positive control. The full range of concentrations tested for each
chemical is given in Figure 2.

Animals. Female Sprague-Dawley rats (Charles River CD:BR/VAFPlus,
Portage, MI) were used in this study. The rats were 240–285 g at the time of
the study. Animals were housed individually in plastic shoebox cages with wood
shavings and allowed ad libitum access to tap water and lab chow (Purina 5001,
St Louis, MO) until used for experiments. During the course of experiments
animals were maintained in polycarbonate metabolism cages that allowed for
the quantitation of water consumption and the quantitative collection of urine.
Animal rooms were maintained at a constant temperature (22 ± 1°C, relative
humidity 50% ± 10%) and photoperiod (12 h light/day). All experimental
procedures were approved by P&G’s IACUC and the need for animal
experiments was evaluated by an internal bioethics review which determined that
in vivo experiments were necessary. All animal care was conducted in
accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Animal experiments. Experiments were conducted to determine the tox-
icity of ammeline, ammelide, and two mixtures of triazines. Ammeline and
ammelide were administered as a single dose of either compound given by oral
gavage at dose levels of 0, 10, 30, or 100 mg/kg as a suspension in 1%
carboxymethylcellulose (CMC). Two sets of mixtures were used. The first was
mixture of melanime/cyanuric acid given by oral gavage at a dosage of 400/
400 mg/kg/day, the second was melamine/ammelene/cyanuric acid,
400/400/400 mg/kg/day. Both were suspensions in 1% CMC and both were
given daily for three consecutive days.

Doses were selected based on the analytical chemistry results available at the
time. The 400 mg/kg/day dosage of melamine was based on the observation
of a 5–6% concentration in the contaminated gluten, 10-fold dilution of the
gluten in cat food, and the package directions that 5 kg cats be fed 4–3 ounce
(approx. 90 g) packages per day, or 360–430 mg/kg/day. The other compounds
in the complex mixture were initially all believed to be present at approximately
ten-fold lower concentration than melamine, hence the 400/400/400 dose ratio.
It was subsequently determined through use of a stronger acid (2.5% HCl (aq))
evacuation of the gluten and a further-refined HILIC-MS/MS methodology
(including the availability of synthetic reference compounds and incorporation
of the stable-isotope-labeled internal standard) that the cyanuric acid concen-
tration was comparable to that of melamine; therefore, the second mixture was
simplified to include only melamine and cyanuric acid, at equivalent dosage.
Rats were placed in metabolism cages after dosing and water consumption
and urine volume were measured over each 24-h period. Urine pH was also
measured for each 24-h urine sample. Rats were euthanized by carbon dioxide
inhalation 24 h after the final dose. Blood samples were obtained from the
inferior vena cava after euthanasia and evaluated for clinical chemistry changes
using a Hitachi 717 automated analyzer. Analyses included a range of indi-
cators of renal and hepatic damage, but for the sake of brevity only indicators of
renal damage are reported here. Urine samples were evaluated for the presence
of crystals, and for creatinine, Na, K, Cl protein, and specific gravity using the
automated analyzer. Blood and urine chemistry assessments were carried out by
the Cincinnati Veterinary Laboratory (Cincinnati, OH). Internal organs were
re-weighed, and samples were preserved in 10% neutral buffered formalin
for histopathology. Samples of kidney were frozen immediately for frozen sections.

Data interpretation. Data are expressed as means ± SD. Differences be-
tween individual treatment groups and controls were determined using
Student’s t-test.

RESULTS

Identification of Contaminants in Pet Food and Wheat Gluten

The strategy to utilize (in parallel) a combination of: organic and aqueous extractions; gradient reversed-phase HPLC and
HILIC separation methodologies; and positive and negative ESI-MS detection modes, provided comprehensive profiling
and detection. This approach assured appropriate coverage of the broadest range of possible adulterant chemical classes, in the
shortest possible time. Detailed comparisons between profiles derived from control versus tainted products drew attention to
several suspected contaminants since the aqueous extracts of
tainted wheat gluten and wet pet food. The corresponding
accurate-mass MS data yielded elemental composition assign-
ments for each of these small-molecule contaminants. Sub-
sequent reanalysis of contaminant-containing extracts, with the
hybrid quadrupole-TOF tandem MS instrument, provided characteristic fragments for each compound of interest, leading
to final structure assignments.

Owing to the highly polar nature of the observed contami-
ants, the HILIC-(TOF)MS profiling approach yielded the
clarest data supporting the presence of six triazine analogs in
the tainted extracts (Fig. 1). Five of these compounds were
readily detected as protonated molecular ions, in the + ESI

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mode. While the sixth triazine (cyanuric acid) was weakly detected in this mode, it was much more evident as a deprotonated molecular ion with $/C0/ESI$ detection (not shown).

Synthetically prepared forms of each of these triazine analogs were similarly analyzed by HILIC-(TOF)MS and MS/MS to provide confirmation of structural assignments. These materials subsequently served as reference standards in the development of HILIC-MS/MS-based methods for the quantification of triazine contaminants in various matrices (gluten and biological specimens). For example, using this general methodology, approximate levels of triazine analogs found in one tainted gluten lot were determined (Table 1).

Cytotoxicity

Cytotoxicity of each triazine was determined in cell lines derived from dog and cat kidney epithelium. Results indicate little cytotoxicity of any of the triazines even at very high concentrations (Fig. 2). Some of the compounds were not very soluble, limiting the concentration that could be tested. Still, it was possible to conclude that direct cytotoxicity was unlikely to have been the cause of the adverse renal effects observed in the affected animals.

In Vivo Toxicity of Ammeline and Ammelide

We evaluated the acute toxicity of these two triazines individually. It was evident from histopathological evaluation of affected dogs and cats that crystalline matter was present in kidney tubules and the comparatively low solubility of these two compounds (approx. 60–70 mg/l) suggested the possibility that these compounds were precipitating out in the kidney or initiating the precipitation of endogenous substances. We evaluated the toxicity of a single gavage dose of ammeline or

![FIG. 1. HILIC-(ESI)TOF-MS selected ion (m/z) profiles from analysis of aqueous extracts of control (left) and tainted (right) cat food. The high resolution ($R \approx 10,000$) of TOF-MS permits $m/z$ displays with tight tolerances ($m/z \pm 0.01$ in this case). The above $m/z$ values were selected to precisely correspond to protonated molecules (MH$^+$) of elemental compositions consistent with each of the six triazines, to the exclusion of a myriad of usual pet food components. The result is a striking representation of these contaminants in profiles from the tainted sample, relative to their absence in the corresponding control cat food profiles (For comparison, both sets of profiles are locked to the same intensity scales.). Note that absolute response (peak intensity) is not directly indicative of the relative levels of these components. Each compound has a characteristic response, which also differs greatly between −ESI and +ESI detection modes. In fact, in −ESI (not shown), cyanuric acid and ammelide yield the more-predominant signals among these compounds.]}
Ammelide at 10, 30, or 100 mg/kg, in rats. The two lower doses were reasonable approximations of the amount of these triazines found in a single feeding or daily consumption of the contaminated food, respectively. Rats were evaluated 24 h after dosing for any effects on clinical chemistry or histopathology. No toxicity was observed (Table 2).

In Vivo Toxicity of Mixtures of Triazines

We evaluated the toxicity of two mixtures, one containing melamine, ammeline, ammelide and cyanuric acid in a 10/1/1/1 ratio, and the other containing melamine and cyanuric acid in a 1/1 ratio. Both mixtures produced toxicity. The initial signs included diuresis in most animals, but by the end of the three day dosing period many of the animals, particularly in the melamine/cyanuric acid group, were oliguric (Fig. 3). Some of these animals were also hematuric. These effects were accompanied by decreased food consumption and decreased body weight. The responses tended to be variable, indicating that some animals progressed more rapidly into renal failure than others. Clinical chemistry results showed a marked increase

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>Water Solubility (mg/l)</th>
<th>Approximate concentration in Gluten (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melamine</td>
<td><img src="" alt="N3C3.png" /></td>
<td>3240</td>
<td>8.4</td>
</tr>
<tr>
<td>Ammeline</td>
<td><img src="" alt="O3N3.png" /></td>
<td>75</td>
<td>1.7</td>
</tr>
<tr>
<td>Ammelide</td>
<td><img src="" alt="O3N3C3.png" /></td>
<td>77</td>
<td>2.3</td>
</tr>
<tr>
<td>Cyanuric acid</td>
<td><img src="" alt="O3C3.png" /></td>
<td>2000</td>
<td>5.3</td>
</tr>
<tr>
<td>Melamine cyanurate</td>
<td><img src="" alt="N2O4N2.png" /></td>
<td>2 (measured as individual components)</td>
<td></td>
</tr>
<tr>
<td>Ureidomelamine</td>
<td><img src="" alt="N3N3O2.png" /></td>
<td>Not determined</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Methylmelamine</td>
<td><img src="" alt="N3C1.png" /></td>
<td>Not determined</td>
<td>&lt; 1</td>
</tr>
</tbody>
</table>

aSolubility data from Syracuse Research Corp. Physical Properties Database, available as part of the National Library of Medicine’s ChemID Plus program.

bApproximate % by weight, determined for a single lot of tainted gluten. Contaminant concentration varied from lot to lot (based on comparison of results from FDA and other labs), with melamine usually present as the most abundant of the triazine contaminants in tainted materials.
FIG. 2. Cytotoxicity data for six triazines and a positive control (mercuric chloride) in Madin-Darby canine kidney cells (diamonds) and Crandell feline kidney cells (squares). Compounds were tested up to 1000 μg/ml or their solubility limit. The highest concentrations of methylmelamine may have elicited some cytotoxicity, but this level of methylmelamine was probably not achieved in the rat experiments or poisonings. Given the lack of cytotoxic potency it is unlikely that direct cytotoxicity was primarily responsible for the adverse renal effects.
in blood urea nitrogen levels and decrease in creatinine clearance, both indicators of renal impairment (Table 2). Urine pH was also significantly decreased by both mixtures in all three 24-h urine samples (data not shown).

At necropsy, the kidneys of the treated groups were found to be edematous, with kidney weight significantly higher than the controls (Table 2). The kidney tubules were streaked with brownish-yellow precipitate (Fig. 4). Histological examination in frozen sections revealed these to be crystals present in a large fraction of renal tubules, particularly in the medulla (Fig. 4). The crystals appeared to be sufficiently abundant and large to block tubular flow. Examination of sections of formalin-fixed tissue (which does not preserve the crystals) revealed extensive tubular dilatation in distal tubules as well as basophilic debris in distal tubules and loops of Henle. This was present in both treatment groups but was more severe in the melamine/cyanuric acid group, an observation that was consistent with the greater severity in renal functional parameters.

We were able to obtain frozen sections and formalin-fixed sections from cat kidney specimens donated by clinical veterinarians from animals that had died after eating the tainted food. The frozen sections indicated the presence of crystalline material that appeared to be identical to that obtained from cat kidney specimens of animals that had died after eating the tainted food. The frozen sections revealed these to be crystals present in crystals that appeared to be identical to that obtained from cat kidney specimens of animals that had died after eating the tainted food. The frozen sections revealed these to be crystals present in the renal tubules, particularly in the medulla (Fig. 4). The crystals appeared to be sufficiently abundant and large to block tubular flow. Examination of sections of formalin-fixed tissue (which does not preserve the crystals) revealed extensive tubular dilatation in distal tubules as well as basophilic debris in distal tubules and loops of Henle. This was present in both treatment groups but was more severe in the melamine/cyanuric acid group, an observation that was consistent with the greater severity in renal functional parameters.

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We were able to obtain frozen sections and formalin-fixed sections from cat kidney specimens donated by clinical veterinarians from animals that had died after eating the tainted food. The frozen sections indicated the presence of crystalline material that appeared to be identical to that observed in the rat experiments, and the formalin-fixed sections revealed comparable histological effects (Fig. 5).

**Chemical Characterization of the Renal Tubular Crystals**

The chemical identity of the crystal in the cat and rat kidney tissue was determined by FTIR microspectroscopy techniques. The location of crystals in the tissue was determined by operating the infrared microscope in the optical microscopy

**FIG. 3.** Urine volume (measured on a daily basis) in rats receiving either a mixture of melamine/ammelide (400 mg/kg/day) or melamine/cyanuric acid (400/400 mg/kg/day) for three days. Each symbol represents the urine volume for one animal. There was a tendency towards increased urine volume initially, which may have been the earliest indication of toxicity or may have been the result of the osmotic diuretic properties of melamine and cyanuric acid. There was a tendency towards oliguria/anuria at later time points, especially in the melamine/cyanuric acid group. The values were highly variable in the complex mixture group, probably indicating that the tubular blockage was not as severe in this group and therefore the progression to renal failure less likely.

**TABLE 2**

Effects of Ammeline, Ammelide, a Triazine Mixture, or a Mixture of Melamine and Cyanuric Acid on Kidney Weight and Renal Function in Rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>Paired kidney weight (g)</th>
<th>Kidney/body weight (%)</th>
<th>BUN (mg/dl)</th>
<th>Serum creatinine (mg/dl)</th>
<th>Creatinine clearance (ml/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>2.05 ± 0.16</td>
<td>0.80 ± 0.08</td>
<td>18.5 ± 4.7</td>
<td>0.52 ± 0.08</td>
<td>88.60 ± 24.15</td>
</tr>
<tr>
<td>Ammeline: 10 mg/kg</td>
<td>6</td>
<td>1.87 ± 0.14</td>
<td>0.77 ± 0.05</td>
<td>19.8 ± 2.3</td>
<td>0.57 ± 0.05</td>
<td>75.37 ± 11.12</td>
</tr>
<tr>
<td>30 mg/kg</td>
<td>6</td>
<td>2.00 ± 0.22</td>
<td>0.80 ± 0.08</td>
<td>19.8 ± 4.3</td>
<td>0.53 ± 0.05</td>
<td>77.38 ± 6.02</td>
</tr>
<tr>
<td>100 mg/kg</td>
<td>6</td>
<td>2.07 ± 0.14</td>
<td>0.84 ± 0.04</td>
<td>17.6 ± 2.6</td>
<td>0.50 ± 0.00</td>
<td>86.38 ± 6.58</td>
</tr>
<tr>
<td>Control (single dose)</td>
<td>10</td>
<td>2.07 ± 0.15</td>
<td>0.82 ± 0.06</td>
<td>22.1 ± 3.0</td>
<td>0.50 ± 0.00</td>
<td>87.90 ± 6.30</td>
</tr>
<tr>
<td>Triazine mixture (single dose)</td>
<td>10</td>
<td>2.45 ± 0.22</td>
<td>1.02 ± 0.08*</td>
<td>39.1 ± 19.7*</td>
<td>0.82 ± 0.26*</td>
<td>49.06 ± 21.07*</td>
</tr>
<tr>
<td>Control (three doses)</td>
<td>10</td>
<td>2.15 ± 0.21</td>
<td>0.84 ± 0.06</td>
<td>21.5 ± 1.4</td>
<td>0.51 ± 0.03</td>
<td>95.40 ± 13.00</td>
</tr>
<tr>
<td>Triazine mixture (three doses)*</td>
<td>10</td>
<td>2.62 ± 0.36</td>
<td>1.14 ± 0.26*</td>
<td>63.3 ± 52.0*</td>
<td>1.57 ± 1.45*</td>
<td>46.52 ± 37.51*</td>
</tr>
<tr>
<td>Melamine + cyanuric acid (three doses)*</td>
<td>10</td>
<td>3.61 ± 0.68</td>
<td>1.60 ± 0.23*</td>
<td>169.0 ± 51.3*</td>
<td>3.82 ± 2.10*</td>
<td>8.50 ± 5.31*</td>
</tr>
</tbody>
</table>

*This mixture consisted of melamine (400 mg/kg/day), ammeline, ammelide, and cyanuric acid (each at 40 mg/kg/day).

Melamine and cyanuric acid were each dosed at 400 mg/kg/day.  

*p < 0.05 by Student’s t-test.
mode with the sample between crossed polarization viewing through the ATR infrared microscope objective. Once the location of a crystal in the tissue was confirmed, the ATR infrared microscope objective was lowered onto the location and an infrared spectrum was collected. Spectra collected from crystals in the tissue samples were compared to infrared spectra collected from reference compounds. Similarly, spectra collected from crystals isolated from tainted wheat gluten were compared to reference compounds. These analyses confirmed spectra from crystals in kidney tissue from both species and crystals isolated from tainted wheat gluten matched the reference spectrum of a melamine-cyanuric acid cocrystal reference material (Fig. 6).

HILIC-MS/MS was used to quantify the levels of individual triazine compounds in renal tissue. A representative set of SRM chromatographic profiles, resulting from analysis of one rat kidney extract, is provided in Figure 7. Even upon a 50,000:1 net dilution of the kidney specimen, these profiles clearly indicate that this method has sufficient sensitivity and selectivity to measure the compounds of interest in this study. A summary of results from kidney analyses (Table 3) confirms the presence of high levels of melamine and cyanuric acid in rat and cat kidney. The concentration of these two triazines in the three cat samples we were able to obtain were in the range of 3000–21,000 µg/g wet tissue, with the rat samples being on the order of 2000–3000 µg/g. Some of the related contaminants, notably ammelide, were also present in the tissue at measurable concentrations.

**DISCUSSION**

Several triazines, but predominantly melamine and cyanuric acid, were identified as the contaminants in a batch of wheat gluten that had been used as an ingredient in a number of brands of wet pet food. The total fraction of triazines in the contaminated gluten was on the order of 10–13%. Assuming that wet cat foods contained up to 10% gluten and feeding according to manufacturer’s guidance, this would have resulted in dosages of melamine and cyanuric acid in the 360–430 mg/kg/day range, comparable to the 400 mg/kg/day used in the rodent experiments.

We were able to reproduce the manifestations of toxicity observed in dogs and cats that had eaten the tainted food by administering a mixture of melamine and cyanuric acid, with or without the other melamine-related contaminants, to rats. Findings in rats included the development of a crystalline precipitate in kidney tubules along with clinical indicators of acute renal failure. We believe that the acute renal failure was attributable to physical blockage of the tubules. Chemical analysis of...
renal tubular crystals from the rat experiments and from samples obtained from cats that had died after consuming the contaminated food confirmed that these deposits were primarily composed of melamine and cyanuric acid. A small study in which cats were fed increasing amounts of melamine and cyanuric acid also reported renal failure and the presence of renal crystals (Brown et al., 2007; Puschner et al., 2007). Our study provides the definitive analytical chemistry that supports that melamine and cyanuric acid were the contaminants that had produced toxicity, and our toxicology work comprehensively demonstrates the link between exposure to these two triazines and toxicity.

Melamine and cyanuric acid form a highly ordered structure held together by multiple hydrogen bonds between each pair of molecules. The molecules assemble into a lattice consisting of alternating units of cyanuric acid and melamine (Fig. 8, adapted from Whitesides et al., 1991). This complex is practically insoluble in water. In fact, the lack of solubility initially hampered our ability to detect cyanuric acid in the mix of contaminants present in gluten as it was almost entirely complexed with melamine in a form that was not readily extractable by water alone. Therefore, we were initially only able to estimate levels of free melamine base, along with the other contaminants. The lattice can be disrupted with acid and heat. Given that the lattice structure depends on hydrogen bonding between corresponding hydroxyl and amino groups of paired triazines, it is likely that ammelide could substitute, albeit imperfectly, for cyanuric acid in the lattice when melamine is present in excess. This may explain the significant retention of ammelide in the cat renal tissue we analyzed, as melamine was present in a greater concentration than cyanuric acid. Similarly,
one might expect ammeline to substitute for melamine if cyanuric acid were present in excess. It may also be possible for endogenous molecules with similar chemical structure, such as uric acid, to bind in such a crystalline lattice, although we did not test this hypothesis. It is of interest to note that ammelide and cyanuric acid are sufficiently similar to uric acid that they (and ammeline, to a lesser extent) are competitive inhibitors of hepatic uric acid oxidase (Fridovich, 1965).

Our working model to explain the toxicity of a mixture of melamine and cyanuric acid is as follows: (1) The complex was stable in the gluten and during processing in pet food manufacture. This notion is supported by the fact that we were able to visually detect the presence of crystals in the wheat gluten that were identified by IR spectroscopy as being predominantly composed of melamine and cyanuric acid. It is also supported by the observation that aqueous and organic extraction of the tainted pet food failed to disrupt the melamine-cyanuric acid complex; the true concentration of these compounds in pet food or gluten was only determined after acid extraction. (2) The melamine-cyanuric acid complex was ingested intact and was dissociated in the low pH of the gastric lumen after ingestion. (3) Because the two compounds have different pKa’s (6.9 for cyanuric acid, 5 for melamine), it is likely that the acid is preferentially absorbed in the stomach and the base in the small intestine. (Otherwise it would have been possible for the compounds to re-establish a crystalline structure upon exiting the stomach.) (4) The compounds were both present in renal filtrate and re-formed an insoluble complex in the kidney tubules. This produced a physical blockage, leading to renal impairment. It is worth noting that we observed the presence of the crystals in the tubules in the rat experiments before any cellular damage could be observed. Cytotoxicity evaluations of melamine, cyanuric acid and the other triazines to Madin-Darby canine kidney cells and Crandell feline kidney cells showed no toxicity, a result that supports the conclusion that the adverse effect was initially a physical blockage.

It is not clear to us why melamine and cyanuric acid do not re-form a crystalline structure until they reach the kidney tubules. The volume of distribution for the two compounds is equivalent (Hammond et al., 1986; Lipschitz and Stokey, 1945): they distribute to total body water. We were able to measure ppm levels of both compounds in nonrenal tissues in our rat studies 24 hours after the last dose (vs. parts per thousand levels in kidney) but did not observe the presence of crystals in any other tissue (data not shown). A possible explanation is that the two compounds do not recombine unless their concentration exceeds a critical point, which might occur as they progress down the osmotic gradient in the kidney. It is also possible that the compounds interfere with uric acid metabolism, which may precipitate in the tubules, providing a seed for melamine and cyanuric acid precipitation. Ammelide, ammeline, and cyanuric acid are inhibitors of hepatic uric acid oxidase (Fridovich, 1965), an effect that would increase circulating uric acid levels. We did observe more than a
TABLE 3
Concentration of Melamine and Related Compounds in Kidneys of Cats Accidentally and Rats Experimentally Exposed

<table>
<thead>
<tr>
<th>Sample or experimental group</th>
<th>Melamine (μg/g tissue)</th>
<th>Ammeline (μg/g tissue)</th>
<th>Ammelide (μg/g tissue)</th>
<th>Cyanuric Acid (μg/g tissue)</th>
<th>Ureidomelamine (μg/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat A</td>
<td>21,300 ± 4780a</td>
<td>1110 ± 176</td>
<td>11,300 ± 2130</td>
<td>12,600 ± 3130</td>
<td>1140 ± 227</td>
</tr>
<tr>
<td>Cat B</td>
<td>3640 ± 1280</td>
<td>46 ± 13</td>
<td>145 ± 34</td>
<td>2850 ± 1160</td>
<td>68 ± 20</td>
</tr>
<tr>
<td>Cat C</td>
<td>6850 ± 2130</td>
<td>219 ± 64</td>
<td>3230 ± 490</td>
<td>5210 ± 1840</td>
<td>197 ± 45</td>
</tr>
<tr>
<td>Rat, mixtureb—1 day</td>
<td>323 ± 220</td>
<td>3 ± 2</td>
<td>230 ± 143</td>
<td>184 ± 118</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>Rat, mixturec—3 days</td>
<td>517 ± 646</td>
<td>6 ± 10</td>
<td>316 ± 492</td>
<td>159 ± 246</td>
<td>2 ± 3</td>
</tr>
<tr>
<td>Rat, melamine/cyanuric acidd—3 days</td>
<td>2360 ± 671</td>
<td>5 ± 1f</td>
<td>188 ± 79f</td>
<td>2790 ± 738</td>
<td>&lt; 1</td>
</tr>
</tbody>
</table>

Values for the cats are means ± SD for three or four regions of each kidney. Cat tissue was donated from veterinary clinics. Values for rats are means ± SD for 6 animals in the group dosed for 1 day, and 10 animals in the groups dosed for 3 days.

This mixture was a suspension of melamine, ammeline, ammelide, and cyanuric acid at a dosage of 400, 40, 40, and 40 mg/kg/day, respectively.

This mixture was a suspension of melamine and cyanuric acid, each dosed at 400 mg/kg/day.

Ammeline and ammelide are present in these samples because the dosed compounds had minor levels of these compounds as impurities (0.2–0.4% ammeline, and 0.4–1.8% ammelide). The calculated dosages from the impurities were 2.4 mg/kg/day of ammeline and 8.8 mg/kg/day ammelide.

doubling in serum uric acid concentration 24 h after a single dose of the complex mixture, although this did not persist after three days of dosing. It is also possible that one or more of the compounds could compete for the renal uric acid transporter, although we have no data to address this.

The nature of the toxicity may pose a challenge for traditional risk assessment methods, which are based on assessment of the toxicity of individual compounds. In this case, the toxicity of the melamine cyanurate complex is qualitatively different from that of melamine or cyanuric acid administered alone. The dose-response curve and no-observed adverse effect level for the mixture is almost certainly different from that of either compound alone. Recent press reports indicate that the use of melamine may be more widespread than thought in the animal feed industry. Given that ammeline, ammelide, and cyanuric acid are bacterial breakdown products of melamine, and could also be formed during melamine synthesis, it is plausible that exposures to mixtures of melamine and related compounds could happen again. Therefore, improving the risk assessment for melamine mixtures is a significant need.

The intentional contamination of a food ingredient has also posed a challenge to the food industry and regulatory agencies involved in food safety. The information that is available from the news media indicates that melamine was added to wheat gluten to boost the nitrogen content, making it appear that the protein content was much higher than it really was, and that the gluten was of much higher quality (New York Times, 4/28/2007, “Filler in animal feed is open secret in China”). Industry and regulatory agencies have procedures in place to prevent triazine contamination in the future, but will have to be diligent in identifying other links in the food chain that are vulnerable to deliberate tampering of this sort.

In summary, we have identified melamine, cyanuric acid, and several other triazines as the contaminants in wet cat and dog foods manufactured and sold in early 2007 under a large number of brand names. We also identified the mode of action by which a combination of melamine and cyanuric acid produced toxicity. Our toxicology results can serve as the foundation for further work on appropriate therapeutic interventions.

ACKNOWLEDGMENTS

We are grateful to a large number of people who set aside everything they were doing to help on this project: Harold...
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REFERENCES


