Nondetectable or minimal detectable residue levels of N-(n-butyl) thiophosphoric triamide in bovine tissues and milk from a 28-d NBPT dosing study

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ABSTRACT: N-(n-butyl) thiophosphoric triamide (NBPT) (Figure 1) is an active ingredient in nitrogen stabilizer (urease inhibitor), which temporarily inhibits the action of the urease enzyme to improve the efficiency of urea-containing fertilizers. Given the potential for NBPT residues to be present in milk and tissues of dairy cattle, due diligence is needed to demonstrate the safety of NBPT in urea-based fertilizers used on forages and crops intended for consumption by Holstein dairy cows. This study used controlled dosing of NBPT in capsule form to dairy cattle for 28 d, followed by a 14-d depuration phase to assess the potential for residues to exist in milk and tissues of dairy cattle at exaggerated use levels. Fourteen lactating cows were selected for the dosing and depuration phases of the study, based on health, body weight (BW), and milk production. There were four treatment groups: 0 mg NBPT/kg BW (Control) (n = 2 cows), 1 mg NBPT/kg BW (1×) (n = 3 cows), 3 mg NBPT/kg BW (3×) (n = 3 cows), and 10 mg NBPT/kg/BW (10×) (n = 6 cows); levels were based on maximum tolerable amount of urea that a cow can ingest on a daily basis (1×) and the maximum concentration of NBPT commercially used when treating urea (0.1 wt% NBPT in urea). At the end of the 28-d dosing phase, cows were randomly selected for the 14-d depuration phase of the study (one control and three 10× cows). The results showed no NBPT residue is detectable at all dose levels, except that a residue level was above the lower limit of quantitation in a single milk and subcutaneous fat sample in the highest (10×) treatment group, which represents the level of NBPT that would be theoretically present in 10× the lethal dosing of daily consumable urea to a cow. Overall, the study demonstrated that it is unlikely for NBPT residues to be present in cattle milk or edible tissues or to cause negative effects on animal health under good agricultural practice.

Key words: cattle, milk, N-(n-butyl) thiophosphoric triamide, residue, tissue

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INTRODUCTION

N-(n-butyl) thiophosphoric triamide (NBPT; CAS No. 94317-64-3) is an active ingredient in nitrogen stabilizer (urease inhibitor), which inhibits the action of urease in soil to improve the efficiency of urea-containing fertilizers by delaying the conversion of urea to volatile ammonia (National Industrial Chemicals Notification and Assessment Scheme, 2011). NBPT has been the most successful urease inhibitor and has been the most widely used urea-based fertilizer since the mid-1990s (Cantarella et al., 2018).

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Urease enzymes in the gut produced by gut microorganisms play an important role in protein utilization efficiency and urea/ammonia toxicity in ruminants, with consumption of urease inhibitors such as NBPT-contaminated feed potentially affecting the health and quality of livestock production, as well as leading to a potential for human exposure (Patra and Aschenbach, 2018). Dairy cattle may be exposed inadvertently to NBPT through grazing on land to which treated urea has recently been applied or, to a lesser extent, through consumption of feedstuffs grown with the assistance of urea-containing fertilizers treated with NBPT. Therefore, there is a potential for NBPT to enter the food chain, although if it is used in accordance with Good Agricultural Practices (GAP) in dairy farming, this risk is very low.

The toxicity of NBPT was studied in a number of repeated oral dosing studies in rodents in which the no-observed-adverse-effect level (NOAEL) for male rats was identified as 74 mg/kg/d based on liver effects and neurobehavior and hematology parameter changes and 17 mg/kg/d in female rats based on histological changes observed in the uterus (reviewed in National Industrial Chemicals Notification and Assessment Scheme, 2011). However, only a low level of NBPT (0.038–0.064%) is present in the final fertilizer formulations; therefore, there is not a significant hazard concern associated with residues in food commodities because exposure is expected to be negligible (National Industrial Chemicals Notification and Assessment Scheme, 2011).

To investigate the possibility that ingested NBPT is transferred to milk or meat in cattle or other livestock populations, development and validation of a sensitive analytical method to detect NBPT residues in livestock tissues and milk is critically needed. A high-performance liquid chromatography (HPLC) method has been published for NBPT and its metabolites in soil samples (Douglass and Hendrickson, 1991); however, an analytical method for NBPT in animal milk and tissues was not identified. For this reason, a liquid chromatography–tandem mass spectrometry (LC–MS/MS) method was developed and validated to meet the study objective, ensuring sufficient sensitivity to detect low concentrations of NBPT residue in milk products (milk, skim milk, and cream) and selected tissues of cattle. This method can be modified to evaluate exposure in other species or within tissues of concern for human consumption. Due diligence related to the potential for NBPT residues to be detected in milk and tissues of dairy cattle was carried out to provide confidence that NBPT in urea-based fertilizers used on forages and crops intended for consumption by dairy cattle would not result in any significant residue levels in food products derived from dairy cattle intended for human consumption.

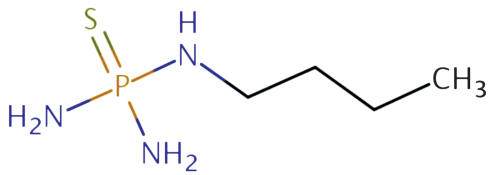

Figure 1. Structure of NBPT.

A cattle feeding study was conducted using a controlled daily dosing protocol of NBPT in capsule form for 28 consecutive days, with dose levels ranging from 1 mg NBPT/kg body weight (BW) to 10 mg NBPT/kg/BW, producing possible exposure levels up to 10× the lowest exposure, to assess the potential for residues to accumulate in milk and tissues of dairy cattle at exaggerated use levels. In addition, a depuration or withdrawal study phase was included to determine the length of time needed for residue dissipation in milk and tissues in the event that NBPT residue accumulation occurred during the dosing phase of the study.

MATERIALS AND METHODS

The animal portion of this study was conducted in compliance with the U.S. Environmental Protection Agency's (U.S. EPA) Good Laboratory Practices Standards (40 CFR Part 160) and Residue Chemistry Test Guidelines OPPTS 860.1480 (U.S. EPA, 1996) Meat/Milk/Poultry/Eggs, EU Guidelines—Doc. 7031/VI/95 rev4, Livestock Feeding Studies OECD Guideline 505. In addition, animals were treated in a humane manner, received a prestudy health examination by a licensed veterinarian, and were humanely euthanized at the end of the study in accordance with accepted American Veterinary Medical Association practices.

The in-life portion of this study was conducted at Genesis Midwest Laboratories (Neillsville, WI), and the analysis of milk, tissues, and dose was conducted at Frontage Laboratories, Inc., (formerly Ricerca Biosciences, Concord, OH), as referenced in the final study report (McClanahan, 2016).

The test substance NBPT was supplied by Shangyu Sunfit Chemical Co. Ltd (Technical grade, Lot 140620, with a reported purity of ≥97%). All other chemicals and solvents, including acetonitrile, acetone, phosphate-buffered saline, HPLC-grade water, formic acid, and ammonium hydroxide, were

supplied by Fisher Scientific Company (Pittsburgh, PA) or Sigma-Aldrich, Inc. (St. Louis, MO) and were of HPLC grade or better, unless otherwise noted. Bovine tissues, including liver, kidney, muscle, and fat tissues, were obtained from a local butcher shop (Barb and Patty's, Mentor, OH), and unpasteurized whole milk was obtained from control animals from the in-life animal laboratory (Genesis MidWest Laboratory).

STUDY DESIGN

Animals and Study Design

A total of 16 lactating Holstein dairy cows were purchased and, on arrival at the testing facility, underwent a physical examination including exterior organs and extremities (for bruising and disorders), body temperature, respiratory and digestive systems (for congestion and irregularities), heart rate, udder (for mastitis), abdominal organs, and reproductive tract (for fetal presence). No cows were excluded based upon initial examination or subsequent daily examinations during the acclimation phase of 21 d before the start of the experimental phase. Following the acclimation phase, two cows were excluded from the study due to bad legs or poor behavior.

The remaining 14 lactating Holstein dairy cows were included in the 28-d dosing phase of the study, based on health, BW, and milk production and were assigned randomly to one of four treatment groups: 0 mg NBPT/kg BW/d (Control), 1 mg NBPT/kg BW/d (1×), 3 mg NBPT/kg BW/d (3×), and 10 mg NBPT/kg BW/d (10×). The 1× dose level for NBPT was based on the maximum tolerable amount of urea that a cow can ingest on a daily basis (approximately 1 g/kg BW) and the maximum concentration of NBPT commercially used when treating urea (0.1 wt% NBPT in urea; NICNIA, 2011). Ruminants that are not acclimated to ingesting urea may experience adverse effects at intakes of 0.3–0.5 g of urea/kg BW, with death occurring at urea levels of 1–1.5 g/kg BW (Thompson, 2019). These levels of NBPT represent exaggerated dietary burden levels (on a dry-matter basis) in the event of inadvertent exposure to NBPT through grazing on land to which treated urea has recently been applied or, to a lesser extent, through consumption of feedstuffs grown with the assistance of urea-containing fertilizers treated with NBPT.

The cows were 3–5 years of age, approximately 400–700 kg BW, and mid- to late lactation. In compliance with Residue Chemistry Test Guidelines OPPTS 860.1480 Meat/Milk/Poultry/Eggs and humane animal practices for studies requiring slaughter of animals, animal numbers were limited, such that the Control treatment group consisted of two cows, the 1× group of three cows, the 3× group of three cows, and the 10× group of six cows. At the end of the 28-d dosing phase, one Control cow and three 10× cows were randomly selected to complete the 14-d depuration phase of the study (Table 1) to assess any residues in tissue and milk.

Cows were offered approximately 6 kg Genesis Midwest Dairy Mix (Northside Elevator, Loyal, WI), 20 kg corn silage, and 4 kg baled hay per day, divided into two feedings corresponding to the morning and afternoon milking. The nutrient content of the Genesis Midwest Dairy Mix is presented in Table 2. Cows were offered ad libitum access to fresh potable water via automatic waterers. Individual feed consumption was measured daily prior to the morning feeding. Individual BWs were measured during the acclimation phase and weekly thereafter. Cows were observed twice daily for clinical abnormalities. Any changes in overall appearance or behavior, and abnormalities such as trauma, injuries, excreta disorders, discharges, and

Table 1. Animal number per treatment per study phase

Treatment	Target NBPT dose levels (mg/ kg BW)[a]	Dosing phase	Depuration phase
Control	0	2 cows	1 cow
1×	1	3 cows	—
3×	3	3 cows	—
10×	10	6 cows	3 cows
Total		14 cows	4 cows

[a]The amount of NBPT for each animal's daily dose was calculated based on the average BW of the dose group from the previously completed week.

Table 2. Nutrient composition of genesis midwest dairy mix for dairy cattle

Nutrient	Minimum	Maximum
Crude protein	19.2%	
Crude fat	4.0%	
Crude fiber		4.5%
Acid detergent fiber		5.5%
Calcium	1.2%	1.4%
Phosphorus	0.4%	
Salt	1.1%	1.3%
Sodium	0.02%	0.02%
Selenium	0.96 ppm	
Vitamin A	11,200 IU/lb	
Vitamin D	3,200 IU/lb	
Vitamin E	48 IU/lb	

respiratory problems, were noted. If daily observation revealed any health-related issues, cows were reexamined by a licensed veterinarian.

Cows were housed in individual 4' × 7' concrete stalls with rubber mats and stanchions and were grouped per treatment. Untreated wood shavings were used as bedding, which was changed daily. Ventilation was provided by two 24" window and two 36" floor fans. Daily temperature and humidity were recorded throughout the study. A photoperiod of 14 h light and 10 h darkness was used. Feces and urine were collected via a concrete gutter at the end of each stall, which was cleaned daily.

Test Substance

The certified NBPT test substance (lot#140620) was supplied from Shangyu Sunfit Chemical Co. Ltd and stored frozen under temperatures ranging from −2.5 to −18.5 °C. The NBPT was encapsulated in gelatin capsules (Torpac "Lock Ring" capsules, size 11) prior to dose administration. Prepared test capsules were stored frozen under temperatures ranging from −2.5 to −23.5 °C. Test capsules were prepared weekly based on each group's average BW from the previous week. The dose of the NBPT (mg of NBPT) was adjusted for the purity of the NBPT (97.6%). The dose calculations are summarized in Table 3.

Oral administration of NPBT was selected to evaluate the possibility of milk and tissue accumulation in the event of NBPT ingestion. Oral administration of the encapsulated NBPT was accomplished via a balling gun once per day for 28 d following the morning milking. Dosing began with the Control group (receiving empty capsules) followed by treatment groups in ascending order to minimize cross contamination. On day 28, dosing was delayed so that necropsy, on day 29, could be performed within 24 h of the last dose.

Milk Collection and Handling

Milk samples were collected twice daily via DeLaval milking machines, placed into labeled collection containers, and weighed. Milk collection began on receipt of the animals and continued throughout the duration of the study. Separate milking machines were used for each treatment group to minimize the potential for cross contamination.

Beginning on study day 1, and continuing throughout the dosing and depuration phases, composite daily milk samples were prepared for each cow based on the proportion of milk collected at each of the two daily milk collections. Individual cow composite daily milk samples were not pooled across treatment groups. Following the morning milking, approximately 500 g of milk from each animal was retained and refrigerated. Following the evening milking, the correct amount of milk to be added from each milking to prepare a 250-g composite sample was calculated based on the

Table 3. Dose calculations

Treatment	Target NBPT dose levels (mg/ kg BW)[a]	Dose week	Group average BW (kg)[b]	Total dose per day (mg a.i)[c]	Total dose per day (mg)
1×	1	1	532.5	532.5	545.6
		2	538.5	538.5	551.7
		3	543.2	543.2	556.6
		4	553.0	553.0	566.6
3×	3	1	524.5	1,573.5	1,612.2
		2	529.0	1,587.0	1,626.0
		3	526.3	1,578.9	1,617.7
		4	535.8	1,607.4	1,646.9
10×	10	1	552.7	5,527.0	5,662.9
		2	553.6	5,536.0	5,672.1
		3	551.4	5,514.0	5,649.6
		4	548.3	5,483.0	5,617.8

a.i., actual intake; TDL, target dose level.

Total dose per day (mg a.i.) = [BW (kg)] × [TDL (mg NBPT/kg BW)]. Total dose per day (mg) = [BW (kg)] × [[TDL (mg NBPT/kg BW)]/purity (97.6%)].

[a]The amount of NBPT for each animal's daily dose was calculated based on the average BW of the dose group from the previously completed week.

[b]Values taken from Table 8.

[c]Based on 97.6% purity in the test substance.

percentage of the sum of the morning and evening milk production for each animal on that study day. The calculated amounts of the morning and evening milk were then combined and thoroughly mixed to form the 250-g composite sample. This composite daily sample was then divided into two 125-g samples, one of which was used as the analytical sample and one stored frozen until completion of study reporting. All unused milk was discarded.

In addition, milk from study day 25 was separated into milk and cream for the Control, 3×, and 10× treatment groups to evaluate the potential partitioning in these fractions. As with the whole-milk samples, samples were collected in proportion to production to provide composite daily samples of skim milk and cream each weighing about 100 g. Proportioned whole milk was separated into skim milk and cream using a cream separator (DeLaval, New York #519). Composite daily samples of skim milk and cream were divided into 50-g samples, one of which was used as the analytical sample and one stored frozen until completion of study reporting. All unused skim milk and cream were discarded.

Animal Termination, Necropsy, and Tissue Collection

At the end of the 28-d dosing phase, the 10 cows not selected for the 14-d depuration phase were euthanized on the morning of day 29 in the presence of a licensed veterinarian using a captive bolt pistol, followed by immediate exsanguination. The Control animal was euthanized prior to treatment animals to prevent cross contamination. At necropsy, tissues were dissected and examined by a licensed veterinarian for gross pathology. Following gross pathology examination, approximately 1 kg each of liver (representative samples from each lobe), kidney (representative from center and both ends), muscle (composite with equal portions from flank, loin, and round), and 500 g each of subcutaneous, mesenterial, and perirenal fat was collected approximately 19.5 h after the last dose of NBPT was administered. If less than 500 g was present in any region, all available fat was collected. Individual animal tissues were stored frozen until homogenization with dry ice using a Robot Coupe (Model BXV4) homogenizer. Tissue homogenates were stored frozen at temperatures ranging from −18.0 to −29.5 °C.

The animals used in the depuration phase were euthanized, followed by gross pathology examination and tissue collection and homogenization as described. During the depuration phase, one 10× animal randomly selected during prestudy randomization was euthanized on each of depuration phase days 4, 8, and 15. The remaining control animal was euthanized on day 15 immediately prior to the final 10× animal.

Laboratory Analysis

Sample preparation and analytical method for quantitating NBPT in milk and tissues. The test substance, NBPT, was used to prepare calibration standards. All other reagents and solvents used are described in the Supplementary Information. Bovine milk was extracted using acetonitrile with water used as the reagent blank. For liver, kidney, muscle, and fat samples, tissues were ground up, sublimed in a freezer with dry ice, homogenized with 0.1 M phosphate-buffered saline (pH 7.2), and extracted using acetonitrile. Complete details of the extraction method used are provided in the Supplementary Information.

LC–MS/MS analysis of NBPT in milk and tissue extracts. Analysis for determination of NBPT residues in cattle milk and tissues was conducted using LC–MS/MS analysis via an Agilent 1200 HPLC system (Agilent Technologies, Inc., Santa Clara, CA) coupled to an AB SCIEX API 4000 (SCIEX, Framingham, MA). Chromatographic separation was carried out on a Phenomenex Luna C8(2) column (50- × 2.00-mm ID, 3-µm particle) with Phenomenex SecurityGuard C18 guard column (4- × 2-mm ID), operated at 35 °C at an initial flow rate of 0.7 mL/min, with a mobile phase system consisting of 0.2% formic acid and 0.012% NH_4OH in water (solvent A) and acetonitrile (solvent B). The solvent gradient program, together with the time-dependent changes in flow rate, is shown in Supplementary Table S1. The systems were run using the operating software Analyst (version 1.4.2). The mass spectrometer was operated in the positive ionization mode with turbo-ion spray and in the multiple reaction monitoring (MRM) scan mode. The instrument was optimized to monitor the ionic transition from the precursor ion at *m*/*z* 168.1 to the product ion at *m*/*z* 74. Both Q1 and Q3 quadrupoles mass filters were tuned for unit resolution. NBPT residues were quantitated using 8-point calibration curves, prepared with the NBPT test substance, with varying concentrations used for milk and all other tissues to reflect the different degrees of dilution during the extraction. Chromatographic peak integrations were conducted with the LC–MS/MS data system software Analyst. Either Analyst or Watson LIMS (Thermo Fisher Scientific, Waltham,

MA) was used for linear regression analysis, calculation of sample concentrations, and acceptance or rejection of the analytical runs. A detailed description of the validated method is presented in the Supplementary Information.

This method was determined to have an accuracy of 70–110%, and the limit of quantitation (LOQ) determined to be 0.05 ppm for milk and 0.04 ppm for tissues. A detailed description of the method and its validation is presented in the Supplementary Information and was conducted in accordance with the U.S. Environmental Protection Agency's "Good Laboratory Practice Standards," published in 40 CFR Part 160.

Dosing capsules Two replicates of encapsulated NBPT doses per dose group were extracted and analyzed weekly. Acetonitrile (100–1000 mL) was added to the dosing capsule in a glass bottle of appropriate size. The mixture was stirred at ambient temperature with a magnetic stir bar until the NBPT dissolved. Each sample except the Control was then diluted two more times based on dose level (Table 4). The NBPT level was quantified by LC–MS/MS using the validated method described briefly in the Supplementary Information.

Milk and tissue samples Milk, skim milk, cream, and tissue samples were received frozen and analyzed for NBPT using methods validated for each matrix (see Supplementary Information). Storage stability samples representing the five matrices (milk and homogenates of liver, kidney, muscle, and fat) were fortified with NBPT at 1 and 10 ppm. Stability assessments in milk were conducted at 33 °C (time 0 and 0–5 min and 0.5, 1, and 4 h), ambient temperature (22 °C; time 0, 4, and 24 h), refrigerator (~5 °C), and freezer (~−20 and ~−80 °C) temperature (time 0, 4, and 24 h and 7, 14, and 28 d) with three replicates at each time interval. Stability assessments in tissue homogenates were conducted at 39 °C (time 0 and 0–5 min and 0.5, 1, and 4 h) with three replicates at each time interval. NBPT in milk was stable stored in a ~−80 °C freezer for 28 d. NBPT was found to be stable in tissue homogenates at 39 °C for up to 4 h and, as a result, stability assessments in tissue homogenates at lower temperature were not conducted with tissue samples stored between −18 and −29.5 °C for up to 21 d prior to analysis; stability at this temperature and for period of storage was determined to be adequate based on reaction rates decreasing by 50% for every 10° decrease in temperature (Arrhenius Equation; reviewed by Laidler,1987). Milk, skim milk, cream, and tissue samples were analyzed in the 10× dose group first for all matrices. If residues were detected in excess of the lower LOQ, samples from the next-lower dose group were analyzed.

Statistical Analysis

Statistical analysis was limited to simple measures of central tendency and/or dispersion. Due to the heterogeneous population of animals (different ages, parity, and stage of lactation), further analyses were not employed. The heterogeneous population met the criteria for meat and milk residue testing.

RESULTS AND DISCUSSION

Dosing

The average amount of NBPT administered over the dosing phase was equivalent to 0.99, 3.09, and 10.14 mg/kg BW for the 1×, 3×, and 10× groups, respectively (Table 5). Overall, the capsule analysis, resulting in 88.9% of the expected levels, with an overall SD of 5.8%, which met the acceptable study criteria (±15%; 85–115%) for dose formulation verification. NBPT prepared in capsules for dosing was found to be stable when stored frozen over the course of the dosing period (stored frozen from 58 to 79 d since time of preparation), the variability in the expected dose concentration varied less than 7%.

Table 4. Dose dilution for capsule analysis

Treatment	Target dose[a]	First dilution with acetonitrile	Second dilution with acetonitrile	Third dilution with acetonitrile
Control	0	1:100		
1×	1	1:100	1:3.6–3.8	1:200
3×	3	1:300	1:3.6–3.8	1:200
10×	10	1:1000	1:3.6–3.8	1:200

[a]Values reported as mg NBPT per kg BW for each animal calculated based on the average BW of the dose group from the previously completed week.

Animal Husbandry and Health

Daily temperature averages are presented in Table 6, with a total experimental phase minimum of 1.6°C and a maximum of 18.8°C. At initial examination and during daily observations, all cows were observed to be healthy and normal, with no indications of treatment-related effects. In addition, abnormal behavioral reactions that have been observed at extremely high dose levels in exposed rodent studies (reviewed in National Industrial Chemicals Notification and Assessment Scheme, 2011) were not expected in this study and were not observed at the low NBPT dose levels utilized.

Table 6. Average barn temperature and humidity per study phase

	Temperature		Relative humidity	
Phase[a]	Min	Max	Min	Max
	(°C)	(°C)	(%)	(%)
Acclimation	6.5	12.5	56	98
Dosing	8.0	12.3	52	97
Depuration	10.1	14.7	47	72

Min, minimum; Max, maximum.

[a]Acclimation phase = 21 d (late Dec through mid-January); dosing phase = 28 d (mid-January through mid-February); depuration phase = 14 d (mid-February through early March).

Feed Intake, BW, and Milk Production

Feed intake is shown in Table 7, BW in Table 8, and milk production in Table 9. Overall, feed consumption, BW changes, and milk production were within normal ranges for mid- to late-lactation Holstein cows in this age range (National Research Council 2001); appeared to be consistent throughout the acclimation, dosing, and depuration

Table 5. Summary of actual NBPT dose administration by treatment

		Treatment[a]			
NBPT dose administration		Control	1×	3×	10×
Dose average (mg/kg BW)	Week 1	0	0.99	3.08	10.10
	Week 2	0	0.98	3.05	10.17
	Week 3	0	0.98	3.03	10.03
	Week 4	0	1.02	3.18	10.24
Study average (mg/kg BW)		0	0.99	3.09	10.14
Study average (mg/kg in feed on DM basis)[b]		0	42.19	123.35	430.95

[a]Treatments correspond to target NBPT doses as follows: Control = 0 mg NBPT per kg BW; 1× = 1 mg NBPT per kg BW; 3× = 3 mg NBPT per kg BW; 10× = 10 mg NBPT per kg BW. Target dose values calculated based on the average BW of the dose group from the previously completed week.

[b]Values reported as mg in feed on a dry-matter (DM) basis (ppm or mg/kg).

Table 7. Daily feed consumption summary

		Daily feed intake							
		kg dry weight				% of BW			
Phase of study	Week within phase	Control[a]	1×	3×	10×	Control	1×	3×	10×
Acclimation	1	13.4	13.3	13.3	13.3	2.2	2.5	2.5	2.4
	2	13.4	13.4	13.4	13.4	2.2	2.5	2.6	2.4
	3	12.9	12.8	12.8	12.8	2.1	2.4	2.4	2.3
	Phase average	13.2	13.1	13.1	13.1	2.2	2.5	2.5	2.4
Dosing	1	12.9	12.9	12.8	12.8	2.1	2.4	2.4	2.3
	2	12.9	12.9	12.8	12.8	2.2	2.3	2.4	2.3
	3	12.9	12.9	12.8	12.8	2.2	2.3	2.4	2.3
	4	12.9	12.9	12.8	12.8	2.2	2.3	2.4	2.4
	Phase average	12.9	12.9	12.8	12.8	2.2	2.3	2.4	2.3
Depuration	1	12.8	—	—	12.9	2.0	—	—	2.3
	2	12.9	—	—	12.9	2.0	—	—	2.4
	Phase average	12.9	—	—	12.9	2.0	—	—	2.4

[a]Treatments correspond to target NBPT doses as follows: Control = 0 mg NBPT per kg BW; 1× = 1 mg NBPT per kg BW; 3× = 3 mg NBPT per kg BW; 10× = 10 mg NBPT per kg BW. Target dose values calculated based on the average BW of the dose group from the previously completed week.

phases of the study; and did not appear to be affected by dosing procedures or treatment with test substance. Due to highly variable feed intake, BW, and milk production in dairy cows of different ages and stages of lactation, statistical evaluation was not conducted. However, no major differences in performance parameters were noted between treatments.

Milk, Skim Milk, and Cream Residue Analysis

Milk, skim milk, and cream samples were analyzed within 30 d of sample collection since a storage stability study identified the NBPT was stable at −80 °C up to this time. For the individual composite daily milk samples, NBPT residues in the 10× dose group were below the lower LOQ (below lower limit of quantitation [BQL]) of 0.05 ppm for days 0–27 of the dosing phase (Table 10). Thus, the 3× and 1× dose group samples were not analyzed. For day 28 of the dosing phase, NBPT was detected in the composite daily milk sample from one cow from the 10× group at a level 0.053 ppm, which was above the lower LOQ. Therefore, individual composite daily milk samples from the 1× and 3× group on day 28 were analyzed for NBPT. During the depuration

Table 8. Body weight summary

Average body weight (kg)					
		Treatment[a]			
Phase of study	Week within phase	Control	1×	3×	10×
Acclimation	1	609.5	535.5	534.2	547.9
	2	599.8	532.5	524.5	552.7
	3	604.3	538.5	529.0	553.6
	Phase average	604.5	535.5	529.2	551.4
Dosing	1	599.3	543.2	526.3	551.4
	2	594.0	553.0	535.8	548.3
	3	592.5	556.3	538.8	554.0
	4	576.5	547.3	526.8	541.3
	Phase average	590.6	550.0	532.0	548.8
Depuration	1	636.5[b]	—	—	553.2
	2	643.5[b]	—	—	548.5
	Phase average	640.0[b]	—	—	544.4

[a]Treatments correspond to target NBPT doses as follows: Control = 0 mg NBPT per kg BW; 1× = 1 mg NBPT per kg BW; 3× = 3 mg NBPT per kg BW; 10× = 10 mg NBPT per kg BW. Target dose values calculated based on the average BW of the dose group from the previously completed week.

[b]This value represents a single animal that averaged 647.9 kg BW during the dosing phase.

Table 9. Milk production summary

Daily milk production (kg)					
		Treatment[a]			
Phase of study	Week within phase	Control	1×	3×	10×
Acclimation	1	17.2	15.9	17.9	15.6
	2	17.0	14.8	18.0	15.0
	3	15.8	14.7	17.9	15.4
	Phase average	16.4	14.8	18.0	15.2
Dosing	1	15.6	14.1	18.6	15.5
	2	16.4	14.2	18.6	15.5
	3	16.3	13.8	18.5	15.3
	4	15.8	13.5	18.1	14.9
	Phase average	16.0	13.9	18.4	15.3
Depuration	1	11.9	—	—	16.0
	2	11.0	—	—	15.7
	Phase average	11.5	—	—	16.0

[a]Treatments correspond to target NBPT doses as follows: Control = 0 mg NBPT per kg BW; 1× = 1 mg NBPT per kg BW; 3× = 3 mg NBPT per kg BW; 10× = 10 mg NBPT per kg BW. Target dose values calculated based on the average BW of the dose group from the previously completed week.

Table 10. Summary of NBPT residue in milk

Average NBPT concentration in milk (ppm)[a]				
		Treatment[b]		
Phase of study	Day within phase	1×	3×	10×
Dosing	0	NA	NA	BQL[c]
	1	NA	NA	BQL
	2	NA	NA	BQL
	3	NA	NA	BQL
	4	NA	NA	BQL
	5	NA	NA	BQL
	6	NA	NA	BQL
	7	NA	NA	BQL
	8	NA	NA	BQL
	9	NA	NA	BQL
	10	NA	NA	BQL
	11	NA	NA	BQL
	12	NA	NA	BQL
	13	NA	NA	BQL
	14	NA	NA	BQL
	17	NA	NA	BQL
	21	NA	NA	BQL
	24	NA	NA	BQL
	25	NA	NA	BQL
	26	NA	NA	BQL
	27	NA	NA	BQL
	28	BQL	BQL	0.009
Depuration	3	NA	NA	BQL
	7	NA	NA	BQL
	14	NA	NA	BQL

NA, not analyzed.

[a]Mean of data from three cows from the 1× and 3× groups and mean of data from six cows from the 10× group during the dosing phase (days 0–28). In the depuration phase, mean of data from three cows from day 3, two cows from day 7, and one cow from day 14.

[b]Treatments correspond to target NBPT doses as follows: 1× = 1 mg NBPT per kg BW; 3× = 3 mg NBPT per kg BW; 10× = 10 mg NBPT per kg BW. Target dose values calculated based on the average BW of the dose group from the previously completed week.

[c]BQL = Below lower limit of quantitation (0.05 ppm). BQL values from individual samples were set to 0 for calculation of mean values

phase, concentrations of NBPT in milk were BQL in all 10× individual composite daily milk samples, so 3× and 1× samples were not analyzed.

For the skim milk and cream samples prepared on day 25 of the dosing phase, individual composite daily milk samples were analyzed, and NBPT residues in the 10× dose group were BQL for all days (Table 11). Thus, the 3× and 1× dose samples were either not analyzed or not collected, respectively. No abnormal reactions were observed in any of the animals treated with NBPT. Because NBPT is present at extremely low levels in fertilizer formulations (0.038–0.064%), residues of NBPT in food products derived from dairy cattle intended for human consumption are expected to be negligible (National Industrial Chemicals Notification and Assessment Scheme, 2011).

Tissue Residue Analysis

Tissues were analyzed within 21 d of sample collection and stored at −18 to −29.5 °C prior to analysis. The LOQ for NBPT in tissue samples was 0.04 ppm (Supplementary Information). NBPT was detected at 0.368 ppm in the subcutaneous fat of one animal from the 10× group on day 28 of the dosing phase (Table 12). Therefore, all subcutaneous fat samples from the 3× and 1× groups for day 28 were analyzed. For the remaining matrices (liver, kidney, muscle, and mesenterial and perirenal fat), NBPT residues in the 10× group were BQL and, as a result, tissue samples from the 1× and 3× groups were not analyzed. During the depuration phase, all tissue samples from the 10× group were analyzed and found to be BQL.

Although a single milk and subcutaneous fat sample in the 10× treatment group indicated a residue above the lower LOQ; this level of NBPT exposure could only be achieved at urea levels toxic

Table 11. NBPT residue in skim milk and cream summary

Average NBPT concentration in milk (ppm)[a]				
		Treatment[b]		
Day of dosing phase	Product	1×	3×	10×
Day 25	Skim milk	NC	NA	BQL[c]
	Cream	NC	NA	BQL

NC = not collected; NA = not analyzed.

[a]Mean of data from three cows the 10× group.

[b]Treatments correspond to target NBPT doses as follows: 1× = 1 mg NBPT per kg BW; 3× = 3 mg NBPT per kg BW; 10× = 10 mg NBPT per kg BW. Target dose values calculated based on the average BW of the dose group from the previously completed week.

[c]BQL = Below lower limit of quantitation (0.05 ppm). BQL values from individual samples were set to 0 for calculation of mean values.

Table 12. NBPT residue summary in edible tissues

Average NBPT concentration in tissues (ppm)[a]					
			Treatment[b]		
Phase of study	Day within dosing phase	Tissue type	1×	3×	10×
Dosing	28	Liver	NA	NA	BQL
		Kidney	NA	NA	BQL
		Muscle	NA	NA	BQL
		Mesenterial fat	NA	NA	BQL
		Perirenal fat	NA	NA	BQL
		Subcutaneous fat	BQL	BQL	0.123
Depuration	3	Liver	—	—	BQL
		Kidney	—	—	BQL
		Muscle	—	—	BQL
		Mesenterial fat	—	—	BQL
		Perirenal fat	—	—	BQL
		Subcutaneous fat	—	—	BQL
	7	Liver	—	—	BQL
		Kidney	—	—	BQL
		Muscle	—	—	BQL
		Mesenterial fat	—	—	BQL
		Perirenal fat	—	—	BQL
		Subcutaneous fat	—	—	BQL
	14	Liver	—	—	BQL
		Kidney	—	—	BQL
		Muscle	—	—	BQL
		Mesenterial fat	—	—	BQL
		Perirenal fat	—	—	BQL
		Subcutaneous fat	—	—	BQL

NA = not analyzed.

[a]Mean of data from three cows from the 1×, 3×, and 10× groups during the dosing phase (days 28). In the depuration phase, each day of study represent a single cow from the 10× group.

[b]Treatments correspond to target NBPT doses as follows: 1× = 1 mg NBPT per kg BW; 3× = 3 mg NBPT per kg BW; 10× = 10 mg NBPT per kg BW. Target dose values calculated based on the average BW of the dose group from the previously completed week.

[c]BQL = Below lower limit of quantitation (0.04 ppm). BQL values from individual samples were set to 0 for calculation of mean values

to cattle. The 10× treatment group represented a level 10× the maximum tolerable amount of urea that a cow can ingest on a daily basis and the maximum amount of commercially applied NBPT to the urea as described in the methods. Overall, the study showed negligible effects of NBPT dosing at exaggerated levels on animal health or residues in milk or edible tissues.

As predicted, NBPT residue levels were not detectable in milk or tissues from cattle administered NBPT at levels that represent exaggerated dietary burden levels (on a dry-matter basis) in the event of inadvertent exposure to NBPT through grazing on land to which treated urea has recently been applied or, to a lesser extent, through consumption of feedstuffs grown with the assistance of urea-containing fertilizers treated with NBPT. The NBPT dose levels administered to cattle in this study were 1, 3, or 10 mg/kg BW per day for 28 d, with milk and tissue levels found to be below the limit of detection (<0.05 ppm) in all but two samples at the highest dose level. Based on studies in rodents that are used to assess potential human health effects, the lowest NOAEL was identified to be 17 mg/kg/d based on an NBPT reproductive toxicity in female rats (National Industrial Chemicals Notification and Assessment Scheme, 2011). To provide a sense of the tissue levels in rats administered a dose ~16× higher than the NOAEL (269 mg/kg BW), the highest tissue concentration of 12.64-µg equivalent of NBPT/g of tissue was measured in liver at 168 h postadministration. This level was estimated to be 0.29% of the administered dose, with no other tissues in the rat identified as containing NBPT levels above 0.10% of the administered dose (National Industrial Chemicals Notification and Assessment Scheme, 2011). Based on the assumption that the kinetics of NBPT is linear at a dose of 17 mg/kg, the NOAEL, the level estimated in liver following this dose would be 0.79 µg/g of tissue, or

~0.79 ppm, 6.4 and 88 times higher than the level of NBPT identified in only one fat sample (Table 12) and one milk sample (Table 10), respectively, of the administered dose to cattle in the current study. This strongly suggests that the only two samples in which NBPT was identified in cattle tissues following exaggerated relevant exposure levels contained amounts that were far below the identified NOAEL in rodents.

In conclusion, this study provides confidence that NBPT in urea-based fertilizers used on forages and crops intended for consumption by dairy cattle does not produce detectable residue levels in these food commodities, eliminating the concern of NBPT entering the human food chain.

SUPPLEMENTARY DATA

Supplementary data are available at *Translational Animal Science* online.

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CONFLICTS OF INTEREST

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