JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

Cite This: J. Agric. Food Chem. 2018, 66, 6394−6401

pubs.acs.org/JAFC

Co-exposure of the Mycotoxins Lolitrem B and Ergovaline in Steers Fed Perennial Ryegrass (*Lolium perenne*) Straw: Metabolic Characterization of Excreta

Lia D. Murty,[†,∥] Jennifer M. Duringer,[*,‡] and A. Morrie Craig[§]

[†]Department of Pharmaceutical Sciences, Oregon State University, Corvallis, Oregon 97331, United States

[‡]Department of Environmental and Molecular Toxicology, Oregon State University, Corvallis, Oregon 97331, United States

[§]Department of Biomedical Sciences, College of Veterinary Medicine, Oregon State University, Corvallis, Oregon 97331, United States

Supporting Information

ABSTRACT: Past research showed a strong linear correlation between levels of the mycotoxins lolitrem B (LB, a tremorgen) and ergovaline (EV, an ergot alkaloid and potent vasoconstrictor) in perennial ryegrass (PRG) forage. The purpose of this study was to characterize the excretion of these two compounds in beef cattle consuming PRG straw and to utilize liquid chromatography−tandem mass spectrometry to investigate the metabolism of LB and EV in excreta. Four groups of steers (n = 6/group) were fed endophyte-infected PRG for 64 days (2256/638, 1554/373, 1012/259, or 247/<100 μg/kg LB/EV). Concentrations of LB and EV in both PRG straw and feces showed a linear relationship to each other. Feces reflected a dose−response for both mycotoxins, with values increasing most rapidly through 21 days then plateauing. Urine contained no detectable level of either compound or the ergoline lysergic acid. Screening for metabolites showed oxidation and reduction biotransformations for both toxins, with additional conjugation products detected for ergovaline.

KEYWORDS: *lolitrem B (LB), ergovaline (EV), perennial ryegrass, Lolium perenne, metabolism, excretion, cattle*

INTRODUCTION

Perennial ryegrass (PRG, *Lolium perenne*) is a hardy cool-season grass, whose seed is used to establish residential lawns, parks, and athletic fields and for erosion control. The fiber that remains after seed harvest is utilized for animal forage as a secondary product. Most varieties of PRG are naturally infected with the endophytic fungus *Epichloë lolii*, which enables the plant to be insect repellant and drought resistant, thereby decreasing the use of insecticides and fertilizers.[1,2] However, *E. lolii* can also produce lolitrem B (LB), which causes the tremorgenic neurotoxicity syndrome known as "ryegrass staggers" in livestock consuming forage that contains high levels of this compound.[3,4] Ergovaline (EV) is a vasoconstrictive ergot alkaloid normally associated with endophyte-infected tall fescue (*Festuca arudinacea*); it is also produced in endophytic PRG,[5,6] however, at approximately a 1:2−1:10 ratio with LB.[7−9]

In cattle, "ryegrass staggers" is observed when animals consume forage containing >1800 μg/kg LB.[10] Disease onset typically takes 1 to 2 weeks and is characterized by an increase in body temperature and respiration rate and impaired motor coordination (headshaking, staggering gait, and muscle spasms) due to the potent inhibition of lolitrem B on large conductance calcium-activated potassium (BK) channels.[11−13] LB accumulates primarily in the fat tissue of livestock,[14−17] yet toxicokinetic data on LB is limited, as are investigations into the enzymes responsible for its metabolism.[5,16]

Ergot alkaloid toxicity can elicit three clinical syndromes: first, during cold temperatures in winter months, "fescue foot" develops, which is a gangrenous condition in the extremities of affected animals; second, abdominal fat necrosis causes hard masses of necrotic fat to develop, which can compress gastrointestinal or reproductive organs, leading to digestive upset or calving problems; third, "summer slump" occurs during warm temperatures when animals show poor weight gain, intolerance to heat, rough hair coat, nervousness, lower milk production, and reduced conception rates.[18−20] The mechanism of toxicity for EV involves vasoconstriction through 5-HT_2 serotonin receptors[21] and as a D2 dopamine agonist.[22,23] In addition, one of the tools used to diagnose ergot poisoning is to measure serum prolactin levels, which has been shown to be decreased in controlled experiments where animals consumed endophyte-infected tall fescue.[24,25]

Ergot alkaloid metabolism was studied using radiolabeled compounds which showed biliary (fecal) excretion to be the primary route of elimination in monogastric models and humans, with a small amount detected in the urine.[26,27] A 28-day feeding trial in sheep with EV-containing tall fescue straw found 27% of recovered alkaloids excreted via the urine as lysergic acid (a smaller molecule making up the core ergoline ring structure of ergot alkaloids), while 73% were excreted in the feces as the parent molecule EV.[28] Both ergovaline and lysergic acid appeared in the ruminal fluid, indicating that the ruminal microbiome plays a role in both liberating ergovaline from feed and degrading it to lysergic acid. Ergot alkaloid

Received: February 21, 2018
Revised: May 30, 2018
Accepted: May 31, 2018
Published: May 31, 2018

DOI: 10.1021/acs.jafc.8b00963
J. Agric. Food Chem. 2018, 66, 6394−6401

metabolism in mouse liver microsomes demonstrated that the parent ergotamine was metabolized to mono- and dihydroxylated forms.[29] Another study also found extensive hydroxylation of ergocristine, ergotamine, ergometrine, and their respective epimers in human liver (HepG2), colon (HT-29), and primary renal cells (RPTEC).[30] CYP3A is the main subfamily of enzymes thought to be responsible for metabolizing ergot alkaloids, via N-dealkylation and mono- and dihydroxylation.[29,31−35]

The first objective of the present study was to quantify LB and EV excretion in bovine feces and urine from samples obtained during a 64-day feeding trial of 24 steers fed endophyte-infected perennial ryegrass and to determine if their concentrations were correlated in a dose−response manner to those in the feed. The second objective was to develop a liquid chromatography−tandem mass spectrometry (LC-MS/MS) screening method for biotransformation products of both LB and EV for analyzing excretory and other biomatrix samples. Studying the co-exposure of these two mycotoxins in PRG straw by defining their excretion, including metabolic products formed, mirrors real-world feeding regimens for animals consuming endophyte-infected plant material which could shed light on how they interact (alone or synergistically) to exert toxicity. Furthermore, the data produced are important from a food safety standpoint in terms of defining metabolic byproducts which can be used in future studies to determine if any risk to public health exists from residues in edible portions destined for human consumption.

MATERIALS AND METHODS

Chemicals and Reagents. HPLC and LC-MS/MS grade acetonitrile, methanol, dichloromethane, chloroform, and reagent grade ammonium carbonate were purchased from J.T. Baker (Phillipsburg, N.J.). Hexane (GC grade) was obtained from EMD Millipore (Billerica, MA) and ethyl acetate (analysis grade) from Arcos Organics (Thermo Fisher Scientific, Waltham, MA). Lolitrem B was purchased from AgResearch Limited, Ruakura Research Centre (Hamilton, New Zealand) and ergotamine tartrate from Sigma-Aldrich (St. Louis, MO), while ergovaline tartrate was procured from Dr. Forrest Smith, Department of Pharmaceutical Sciences, Auburn University (Auburn, AL). Ultrapure 18 mΩ cm^{-1} water was obtained from an Elga (Marlow, Buckinghamshire, U.K.) PURELAB Ultra Genetic system.

Animal Experiments. All procedures were approved by the Institutional Animal Care and Use Committee at Oregon State University (OSU) (IACUC #4031). Twenty-four steers (*Bos taurus*; breed, Angus cross) between 7 and 8 months of age with an average weight of 295 kg were purchased from a feedlot in Oregon. Animals were castrated and treated with anthelminthics prior to shipping. Upon arrival, steers were placed onto pasture for 10 days with open access to water and the barn for shelter. During this time, steers were examined by a veterinarian, and those affected were treated for dermatophytosis (ringworm), infectious bovine keratoconjunctivitis (IBK, pink eye), and bovine respiratory disease (also known as "shipping fever"). Following this initial acclimation period, animals were randomly separated into four pens of six steers each. Steers were allowed 2 weeks to adjust to a chopped perennial ryegrass straw diet (247/<100 μg/kg LB/EV) prior to the start of the study.

Endophyte-infected perennial ryegrass straw was chopped and mixed to formulate four rations as shown in Table 1 by combining different proportions of a no LB/EV and a high LB/EV straw lot. All values represent an average over 64 days, are based on dry matter, and were tested at the Endophyte Service Laboratory, OSU as detailed below.[10] Twice daily, straw for each pen was weighed; animals were fed double-blind from July−September and allowed to consume material ad libitum for the duration of the experiment with open access to water and salt blocks and were provided with a concentrate, CHS Beef Grower 20, at 0.9 kg/steer/day. Straw samples at the time of feeding and orts (leftover/refused feed) were taken each day from random locations in the feeding bunk of each group, dried, and stored at −20 °C until analysis for LB and EV concentrations, as described below. During the study, an error was made in the feed for group I, which was unintentionally given a lower ration ("washout") averaging 302/32 μg/kg LB/EV for days 19−26. Once the error was discovered, animals were placed back on group I feed (2256/638 μg/kg LB/EV) for the remainder of the trial (days 27−64).

Table 1. Concentration of Lolitrem B and Ergovaline in Perennial Ryegrass Diets Fed to Steers over 64 Days

group	LB (μg/kg)[a]	EV (μg/kg)[a]
I	2256 ± 166[b]	638 ± 77
II	1554 ± 213	373 ± 119
III	1012 ± 197	259 ± 53
IV[c]	247 ± 175	<100

[a]Values are based on samples taken each day for 64 days of the trial ± standard deviation and were analyzed via HPLC-fluorescence. [b]Near the established threshold of toxicity for lolitrem B.[10,40] [c]Control group.

Fecal samples were collected once per day on days −7, −1, twice daily on days 0−3, and once daily on days 4−7, then weekly for the duration of the trial (days 14−64) while steers were in a squeeze chute, from the rectum. Samples were immediately placed into freezer bags on ice, then transported to a chemical hood where they were spread on weigh boats and allowed to air-dry for 4−5 days at ambient temperature. After drying, samples were stored at −20 °C until analysis. Urine samples were collected from three randomly selected individuals per group by free-catch or induced for urination with furosemide in a urine specimen cup once daily on days −7, −1, and 0−7, then weekly for the duration of the trial.

Animals were clinically evaluated for the neurotoxicological end points defined for ryegrass staggers based upon previous work twice daily.[36,37] A score of 0 = no clinical signs; a score of 1 = resting tremors of the head and/or neck; and a score of 2 = resting tremors of the head, neck, and/or body, incoordination with handling, and marked stiffness of gait (humane end point).

Sample Preparation and Extraction. Dried feed, orts, and fecal samples were prepared for high performance liquid chromatography (HPLC)-fluorescence and LC-MS/MS analysis by grinding in a Cyclotec 1093 sample mill (Foss Tecator, Höganäs, Sweden), passing through a 0.5 mm screen. Straw and orts were evaluated for LB and EV every day for each group; fecal samples were pooled by group (n = 6/group) for each sampling time point. All samples were submitted to the Endophyte Service Laboratory, OSU for quantitation of LB and EV (methods evaluated by the Oregon Department of Agriculture using ISO 17025 standards as a reference).

The extraction method was performed according to previous work which utilized solid phase extraction (SPE) for sample cleanup.[10] For LB (HPLC-fluorescence) and LB metabolite (LC-MS/MS) determination, 0.200 g of dried, ground PRG straw, orts, or pooled feces was weighed in duplicate, then turned in the dark for 18−24 h at room temperature in 3 mL of extraction solution (chloroform/methanol 2:1 (v/v)). The tubes were then centrifuged at 650g for 5 min, and 1.6 mL of the supernatant was evaporated under a flow of nitrogen at ambient temperature. Two 1 mL additions of dichloromethane were used to dissolve the sample. CUSIL (United Chemical Technologies, Bristol, PA) SPE cartridges were loaded onto a positive pressure manifold and conditioned with 2 mL of dichloromethane. The sample was then applied, followed by a 2 mL dichloromethane wash. Next, a second wash of 0.5 mL of elution solution (dichloromethane/acetonitrile 4:1 (v/v)) was added. LB and its metabolites were eluted with 3.0 mL of elution solution, which was captured and sealed for analysis. After HPLC analysis, this extract was then dried under nitrogen and reconstituted for the LC-MS/MS assays detailed below with mobile phase. Dichloromethane was not used as a mobile phase solvent in the LC-MS/MS analysis; it was important to dry the sample extracts down

DOI: 10.1021/acs.jafc.8b00963
J. Agric. Food Chem. 2018, 66, 6394−6401

Table 2. Method Parameters for HPLC Separation of Ergovaline, Lolitrem B, and Lysergic Acid

	HPLC fluorescence				LC-MS/MS EMS-IDA-EPI, MRM					
	ergovaline[b,10]		lolitrem B[10]		ergovaline		lolitrem B[41]		lysergic acid[38]	
injection volume (μL)	10		20		10		50		50	
flow rate (mL/min)	1.0		0.5		0.65		0.65		0.5	
analytical separation column[a]	Gemini 4.6 × 150 mm, 5-μm particle size[d]		Zorbax RX-SIL 4.6 × 250 mm, 5-μm particle size[c]		Gemini 4.6 × 150 mm, 5-μm particle size[d]		Prodigy ODS (30) 150 × 4.6 mm, 5-μm particle size[d]		Luna phenyl-hexyl (250 × 2 mm, 5-μm particle size)[d]	
gradient (given in % A)	time (min)	% A[e]	time (min)	% A[f]	time (min)	% A[e]	time (min)	% A[i]	time (min)	% A[k]
	0–3.5	99	0	100	0–2	99	0–10	100	0–0.5	90
	3.5–5.5[f]	35	15	100	2–62[g]	35	10–65[g]	0	0.5–1	90
	5.5–7.5	30			62–67[h]	35	65–75[j]	0	1–5[g]	30
	7.5–8.5	99			67–72	99	75–80	100	5–8[l]	30
	8.5–12	80							8–9	90
									9–15	90

[a]Guard columns of similar composition were used for both analytes and columns. [b]Ergotamine internal standard elution at 9 min. [c]Agilent, Santa Clara, CA. [d]Phenomenex, Torrance, CA. [e]A, 2.5 mM ammonium carbonate in 35% deionized water/65% acetonitrile; B, acetonitrile. [f]Dichloromethane/acetonitrile/purified water (400:100:2 (v/v/v)). [g]Decreased linearly. [h]Held for 5 min at 35% A. [i]A, 40% acetonitrile and 0.1% acetic acid; B, acetonitrile and 0.1% acetic acid. [j]Held for 5 min at 0% A. [k]A, 2.5 mM ammonium carbonate in water; B, acetonitrile. [l]Held for 3 min at 30%A.

Table 3. MS/MS Method Parameters for Detection of Lolitrem B, Ergovaline, and Lysergic Acid

	LB	EV	LB	Lysergic acid
	EMS-IDA-EPI	EMS-IDA-EPI	MRM	MRM
ionization type	APCI (+)	ESI (+)	APCI (+)	ESI (+)
EMS scan range	150–630; 625–1500	100–280; 275–1500	N/A[a]	N/A[a]
EMS cell exit potential (CEP)	5.54 V	4.74; 7.54	N/A[a]	N/A[a]
EPI (MS/MS) threshold	>1000 counts	>1000 counts	N/A[a]	N/A[a]
EPI collision energies	47, 61, 63	47, 61, 63	N/A[a]	N/A[a]
EPI cell exit potential (CEP)	3.62 V	3.62 V	N/A[a]	N/A[a]
curtain gas (psi)	30	30	30	30
nebulizer current (μA)	6	N/A[a]	6	NA
ion spray voltage (V)	N/A[a]	5000	NA	3500
temperature (°C)	450	550	450	350
gas 1 (psi)	65	55	65	35
gas 2 (psi)	0	55	0	35
declustering potential (V)	25	20	61	45
entrance potential (V)	10	10	7	10
cell exit potential (V)	N/A[a]	N/A[a]	4	2
collision energy (V)	10	10	51	30.5

[a]Parameter not required in this method of analysis.

and reconstitute in solvents similar to those used for the LC-MS/MS analysis to obtain the best results.

For EV (HPLC-fluorescence) and EV metabolite (LC-MS/MS) examination, SPE of dried, ground PRG straw, orts, and pooled feces was accomplished using the method from Craig et al.[10] Briefly, 1 g of sample was added to 10 mL of chloroform, 1 mL of internal standard (0.661 mg/L ergotamine tartrate), and 1 mL of 0.001 N NaOH. The tubes were capped and mixed for 18–24 h in the dark. The tubes were then centrifuged at 650*g*, and 5 mL of the supernatant was loaded onto Ergosil (United Chemical Technologies, Bristol, PA) SPE columns. The column was washed with 2 mL of acetone/chloroform (4:1 (v/v)) solution, and EV and its metabolites were eluted with 2.5 mL of methanol. The eluent was collected, dried under nitrogen at 50 °C, and reconstituted with 0.5 mL of methanol. After sonication and mixing, the extract was transferred to an amber HPLC vial and sealed for analysis. This extract was then dried under nitrogen and reconstituted with 50:50 (v/v) mobile phase for LC-MS/MS EMS-IDA-EPI screening (see below). Methanol was not used as a mobile phase in the LC-MS/MS method, so it was vital to dry the sample and reconstitute with solvents similar to those used for the LC-MS/MS analysis.

Urine was pooled by group (n = 3 per group) for each day. Samples were prepared for lolitrem B quantitation via SPE using Waters (Milford, MA) Oasis HLB cartridges (60 mg/3 cc), modified from Miyazaki et al.[14] Two milliliters of urine were acidified with 2 mL of 0.1 M phosphoric acid and allowed to sit at room temperature for 15 min. SPE columns were conditioned with 2 mL of H_2O; samples were vortexed, then loaded onto the column. After the sample passed through, the column was washed with 2 mL of H_2O. The sample was eluted with 6 mL of 1:9 (v/v) hexane–ethyl acetate, dried under nitrogen, then reconstituted with 0.5 mL of acetonitrile, vortexed, and sonicated for 10 s. Vortexing and sonication were repeated until the residue was fully dissolved. The eluent was filtered with Whatman 1.2 μm syringe filters into an HPLC vial for analysis by LC-MS/MS.

Urine was extracted for lysergic acid according to a previously established, validated method.[38] Briefly, samples were acidified, centrifuged, then applied to an SPE column (Discovery DSC-SCX, Supelco, Bellfonte, PA) that had been preconditioned with methanol, 0.1 M HCl, and water. The SPE column was washed twice with 3 mL

DOI: 10.1021/acs.jafc.8b00963
J. Agric. Food Chem. 2018, 66, 6394–6401

of water, then allowed to dry under vacuum for 2 min. The sample was eluted with 3 mL of 95:5 (v/v) methanol/ammonium hydroxide, dried under nitrogen, then reconstituted in 0.2 mL of 1:1 (v/v) methanol/0.05 M phosphate buffer (pH 8.5). This was sonicated for 30 s and filtered using 1.7 mL of centrifuge tubes with filter inserts (Nanosep, Pall Life Sciences, East Hills, NY) at 13,200 rpm for 5 min. The eluent was placed in an amber HPLC vial for analysis by LC-MS/MS.

HPLC-Fluorescence Analysis. Analyses were performed using a Series 200 autosampler and pump (PerkinElmer Instruments, Shelton, CT) coupled to an LS 40 fluorescence detector (PerkinElmer). Data collection was completed using a PC-based data system which consisted of a 900 Series Interface and Total Chrom Workstation (PerkinElmer).

LB was visualized using an excitation wavelength of 268 nm and an emission wavelength of 440 nm; method details can be found in Table 2.[10] A calibration curve (range was constructed from reference PRG material, 500−4000 ng/mL) then a linear regression fit of the peak height versus the amount of analyte injected were used to quantitate LB in unknown samples. Limit of detection (LOD) and limit of quantitation (LOQ) were established as 30 and 100 ng/mL, respectively. Precision evaluation for this method showed day to day variation of 7.3% and within day variation of 3.9%. Spiked recovery for LB was 91.5%.

For EV, separation details via HPLC are given in Table 2. EV was visualized using an excitation wavelength of 250 nm and an emission wavelength of 420 nm. A calibration curve (range 150−1100 ng/mL) was constructed from tall fescue reference material, and a linear regression fit of the peak area versus the amount of analyte injected was used to quantitate ergovaline in unknown samples. LOD and LOQ were established as 31 and 100 ng/mL, respectively. Precision evaluation for this method showed day to day variation to be 5.7%, within day precision to be 3.8%, and spiked recovery to be 91%.

Liquid Chromatography−Mass Spectrometry Analysis. The LC-MS/MS system consisted of a PerkinElmer Series 200 autosampler, PerkinElmer LC 200 micropump, a Peltier Series 200 cooling tray, and an AB SCIEX 3200 QTRAP system (Applied Biosystems, Foster City, CA). LB was analyzed using positive atmospheric chemical ionization (APCI), while EV and lysergic acid were analyzed with positive electrospray ionization (ESI). The HPLC settings for MS/MS detection of lolitrem B, ergovaline, and lysergic acid are given in Table 2. Multiple reaction monitoring (MRM) transitions were obtained using the quantitative optimization tool in Analyst 1.4.2; mass spectrometer settings are shown in Table 3. MRM methods were used to quantify lolitrem B and lysergic acid in urine.

For LB, the MRM transitions used were 686.4 → 628.2 (for quantitation), and 686.4 → 238.1 and 686.4 → 196.1 (for qualitative confirmation). LOD was established as 4 ng/mL, while LOQ was 12 ng/mL. The extraction method showed an average recovery of 73%. Precision was evaluated at 50, 100, and 500 ng/mL and showed an intraassay variation of 7.1, 7.3, and 2.5%, respectively. The method showed an interassay variation of 10, 8, and 14% for 50, 100, and 500 ng/mL, respectively. LB was quantitated using a linear calibration curve of 50−1000 ng/mL.

For lysergic acid, the transition of 269.2 → 44.0 was used for quantitation against spiked urine standards that were extracted using the same SPE procedure as the samples; 269.2 → 182.0 and 192.0 were used as qualitative transitions. Lysergic acid extraction was validated as in Lodge-Ivey et al.[38] Briefly, recovery was 87 and 85% for 150 ng/mL and 30 ng/mL spiked bovine urine. Lysergic acid was quantitated using the MRM method over a linear regression of 200−1000 ng/mL. The LOQ was 50 ng/mL, and the LOD was 20 ng/mL.

Screening for metabolites of LB and EV in feces was completed using the enhanced mass spectrum mode with independent data acquisition and enhanced product ion scans (EMS IDA EPI) with the same equipment and mobile phases mentioned previously for the MRM methods (Table S1). The EMS-IDA-EPI method parameters for LB and EV are shown in Table 3. HPLC conditions were the same except for the gradient used (Table 2); the gradient was extended in an attempt to separate out all components in feces to identify potential metabolites. An EPI scan of 1.46 μM LB in ACN and 100 nM EV in MeOH standard using the respective LC-MS/MS method are shown in Figure 1, which illustrates where the main fragments detected are generated for each molecule.

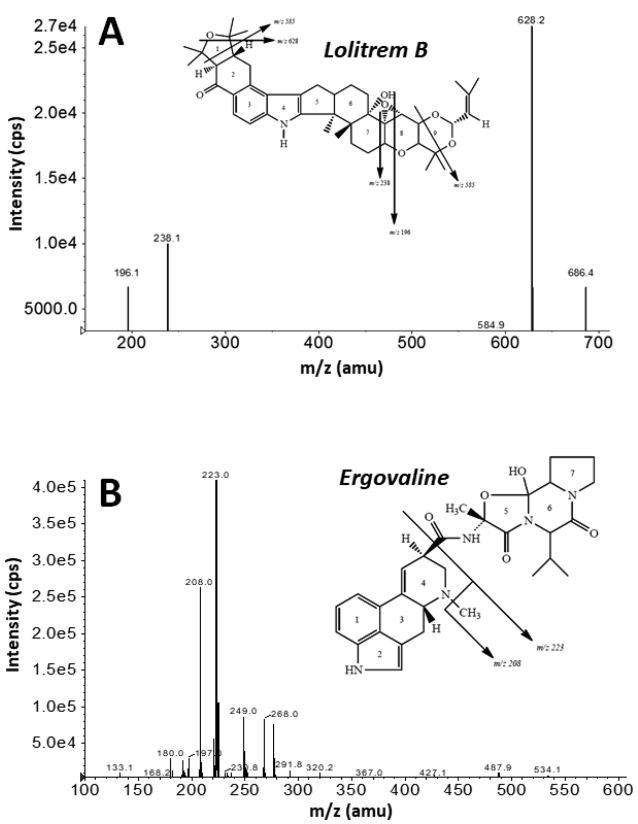

Figure 1. Enhanced product ion (EPI, MS/MS) scan for lolitrem B (A, 1.46 μM in ACN) and ergovaline (B, 100 nM in MeOH) standards.

Metabolite screening and identification were conducted using LightSight Software, version 2.0 (Applied Biosystems). Metabolite identification used LightSight software to compare the fragmentation pattern of EV and LB to other MS/MS scans obtained using the EMS-IDA-EPI method and matched the parent compound with a known biotransformation based upon mass. Mass fragments associated with LB fragmentation were 628.4, 237.0, and 196.3 *m/z*, while mass fragments associated with EV were 223.1, 208.1, and 207.0.

Table S2 provides a summary of the methods used for detection, screening, and quantitation for lolitrem B, ergovaline, and lysergic acid.

Statistical Analysis. Analysis of all HPLC-fluorescence data was performed using one-way analysis of variance (ANOVA), followed by Bonferroni's test (GraphPad Prism 5.0, GraphPad Software, La Jolla, CA). For determination of a linear relationship between LB and EV, the concentrations of LB and EV in feed and feces were compared across all groups using Pearson's test of correlation to determine coefficients (GraphPad Prism 5.0). Simpler statistical variables such as average, standard deviation, coefficient of variation, and the two-tailed Student's *t* test were analyzed assuming equal variance using Excel (Microsoft, Redmond WA).

RESULTS AND DISCUSSION

Investigation into the linear relationship of LB and EV in PRG straw from this study was repeated according to Hovermale et al.[7] Briefly, LB concentration was treated as the X variable, and EV concentration was treated as the Y variable. Daily values for PRG straw given to groups I−III throughout the 64-day study (group IV was near or below the detection limit for most samples) were evaluated via linear regression (r^2) and Pearson

DOI: 10.1021/acs.jafc.8b00963
J. Agric. Food Chem. 2018, 66, 6394−6401

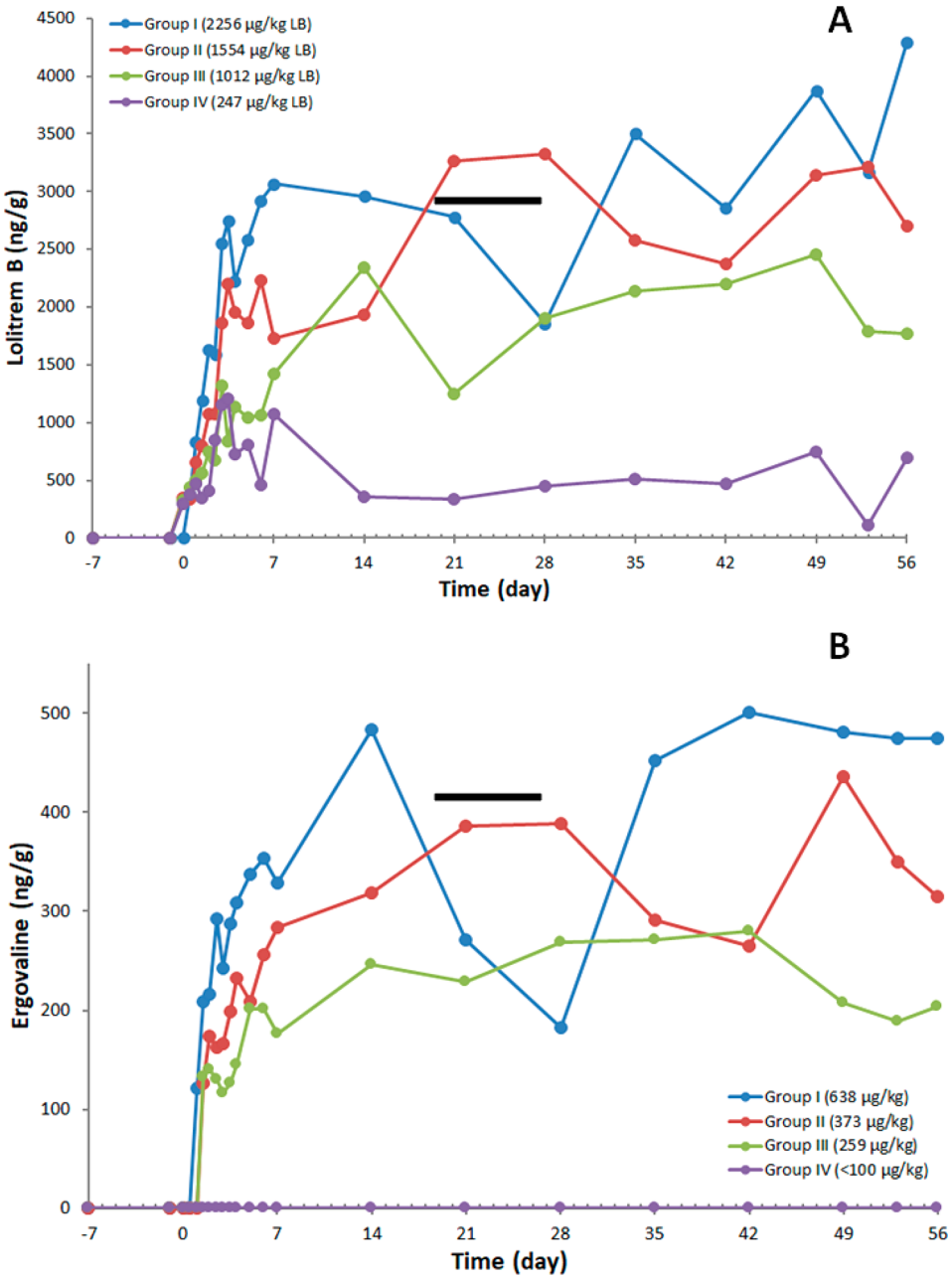

Figure 2. Concentration (ng/g) of lolitrem B (A) and ergovaline (B) in fecal material collected from steers fed varying levels of endophyte-infected perennial ryegrass straw over 64 days. Groups are identified by the average concentration of toxicant given over the 64 days of the experiment. Values represent pooled feces for each group and day. The black bar shows the washout period, which occurred on days 19−26 for group I where they were fed the control feed (group IV).

correlation coefficient tests. Groups I, II, and III showed r^2/Pearson coefficient values of 0.7965/0.834, 0.4213/0.902, and 0.3731/0.776, respectively. This demonstrates that the relationship between the two toxins grew stronger the higher the concentration of EV and LB; the r^2 value calculated previously was 0.7335,[7] which aligns most closely with Group I. Overall, the ratio of LB to EV in groups I−III was 3.98 ± 1.08, within the range of other studies which have examined this relationship.[7−9] The Pearson coefficients were all very close to +1, indicating that a linear equation describes the relationship of EV and LB most accurately in these PRG straw samples that were harvested in the summer. Repussard et al.[9] conducted a study on perennial ryegrass sampled over three years in southern France and found a strong correlation between lolitrem B and ergovaline as well; however, they found that the ratio of the two toxicants varied depending on the time of year due mainly to abiotic factors.

Feces quantitation by HPLC-fluorescence did show a dose−response pattern to the amount of LB and EV consumed in the feed, with the amount increasing rapidly, then plateauing around 21 days for groups II and III and 35 days for group I due to the unintentional washout from days 19−26 (Figure 2). LB and EV for group I were not completely eliminated during the washout but did decline to a level near group III, recovering to previous levels around day 35. LB concentration in group II reached those of group I toward the latter part of the study (>3000 ng/g LB), which is likely correlated to the incidence of ryegrass staggers, whose appearance was observed after day 51

DOI: 10.1021/acs.jafc.8b00963
J. Agric. Food Chem. 2018, 66, 6394−6401

in both groups (assignment of 1 or greater on clinical scale used). The washout influenced the date of initial disease incidence in group I, as previous studies have observed clinical signs manifesting after approximately 14 days.[39,40] Animals in groups III and IV, which remained generally at or below 2000 ng/g fecal LB, did not have any discernible clinical signs of toxicity. There was no observation of clinical signs associated with fescue toxicosis in any animals. Samples showed significant differences between groups for both LB and EV concentration throughout the study (ANOVA, P value <0.001). Investigation into the linear relationship between EV and LB in feces using HPLC-fluorescence data was performed as it was in the feed. Groups I, II, and III showed R^2/Pearson coefficients of 0.8193/0.834, 0.8727/0.902, and 0.6778/0.776, respectively; the linear relationship between these two toxicants appears to be even stronger in the feces than it was in the feed. Overall, the ratio of LB to EV in the feces of groups I−III was 7.85 ± 1.83. This is an increase from the feed, suggesting more excretion of LB and/or absorption of EV. The ratio of the concentration of LB in the feces/feed averaged 1.23, 1.51, and 1.61 for groups I−III, respectively, then increased to 2.85 for group IV (control). It appears that excretion rate is held relatively constant to consumption for lolitrem B at higher levels; a t test was significant only for comparisons between groups I and III and IV (p = 0.04 and 0.03).

Using the EMS-IDA-EPI LC-MS/MS method described above for LB and fecal samples from group I, possible metabolites were identified using daughter ions found in fragmentation of the LB standard, specifically m/z 196 and 238 (Figure 1A). Investigation done using LightSight analysis determined three possible metabolites (Table 4, which also shows suggestions for what the possible biotransformation reactions are that could produce these metabolites), the most interesting being the possible oxidation metabolite with a parent m/z of 702.4. In addition to demethylation plus oxidation and/or hydrogenation for the metabolite consistent with an m/z of 688, this peak could be lolitrem E, as identified in previous studies on endophyte isolates examining the *LTM* locus (specifically LtmK, a P450 monooxygenase) which show it to be a precursor in lolitrem B biosynthesis.[41] In a recent survey of perennial ryegrass pastures associated with staggers in Australia, lolitrem E is present at about 2−5% the concentration of lolitrem B.[6] To our knowledge, no studies describing LB metabolism from the parent compound into identified metabolites and their eventual excretion in livestock exist. Perennial ryegrass seed and hay containing 2 ppm lolitrem B fed to horses for 2 weeks resulted in detectable amounts in the plasma but none in the urine via an ELISA test.[42] The urine results are congruent with what we determined in the current study with cattle (no lolitrem B was identified in the urine samples using the MRM method) and the fact that lolitrem B is a lipophilic molecule. The authors noted that plasma concentration of lolitrem B did not correlate with severity of clinical signs, which varied substantially among the seven horses evaluated. Another study in sheep found rapid elimination of lolitrem B from the serum after IV administration, with a peak around 5 min and drop to 22% of peak dose by 15 min.[43] Yet the tremoring symptoms continued for 16 h afterward, suggesting that lolitrem B is stored in a compartment (adipose tissue) and released slowly until eliminated.[44] In every study that has examined fat, lolitrem B has been detected;[14,16,17] this compartment even reflects an increase or decrease in lolitrem B concentration if fluctuated in the feed.[15] Thus, the main excretion and metabolism route of LB in cattle appears to be through the GI tract via fecal elimination, with deposition/storage occurring in the fat for slow release if large enough quantities are consumed.

Additionally, no direct studies on incubation with lolitrem B and liver microsomes, cytochrome P450s, or other relevant enzymes have been performed. A study which fed endophyte-infected perennial ryegrass hay to lactating ewes made an initial attempt, examining liver microsomes from treated sheep for drug metabolizing enzyme activities.[16] They found that O-dealkylation increased in the liver but decreased in the kidneys, that glutathione *S*-transferase activity decreased, and that uridine diphosphate glucuronosyltransferase activity increased. However, the hay used in that study contained both lolitrem B and ergovaline, so it is difficult to determine which toxin (or both) is responsible for these changes. The compounds we identified suggest oxidation and reduction pathways as the major source of metabolism and excretion. Microarray analysis performed on liver biopsies from animals in this study found that genes involved in lipid/steroid biosynthesis/metabolism and oxidation−reduction were altered.[42] Zbib[16] also found changes in enzymes that ameliorate oxidative damage (superoxide dismutase in plasma and kidneys and catalase in kidneys) in ryegrass-fed lactating ewes. Thus, research exploring hepatic or other target organ metabolism of lolitrem B would provide information that could help tie together the metabolites detected in the feces and compartments in which they were formed with past work.

Using the EMS-IDA-EPI method described above for EV and fecal samples from group I, potential metabolites were identified using the common and most abundant ergot alkaloid daughter ions of m/z 208 and 223 (see Figure 1B for sites of fragmentation).[45] Secondary and less abundant ions used for identification were m/z 249 and 516. Lysergic acid was tested via LC-MS/MS in urine but was below the limit of quantitation (LOQ) (50 ng/mL) for all samples; group I had trace levels for most time points but never exceeded the LOQ.

Table 4. Lolitrem B and Ergovaline Metabolites Identified in Excreta from Steers Fed Varying Levels of Mycotoxin-Contaminated Perennial Ryegrass Straw over 64 Days

	parent[a]	daughter ions[a]	proposed biotransformation(s)[b]
LB	687.4	629.3, 238.9, 237.9	deamination, reduction AND/OR primary amide hydrolysis
	688.4	630.0, 237.9	demethylation plus oxidation AND/OR hydrogenation
	702.4	644.2, 237.9, 196.1	oxidation
EV	516.0	248.9, 223.0, 208.0	dehydration
	532.0	248.9, 221.1, 208.0	dehydrogenation
	535.9	223.1, 208.0	demethylation, oxidation AND/OR hydrogenation
	550.0	248.9, 221.0, 208.0	oxidation
	802.9	363.0, 292.1, 226, 207	loss of cyclohexyl ring and bis-glucuronidation
	816.9	562.0, 256.1	loss of cycohexyl ring, dioxidation, CO, and glutathione conjugation
	824.7	726.0, 563.0	loss of NH_3 and glutathione conjugation

[a]Identified using enhanced mass spectra; independent data acquisition with enhanced product ion scan (EMS IDA EPI) experiments. [b]Proposed biotransformation obtained using LightSight version 2.0.

DOI: 10.1021/acs.jafc.8b00963
J. Agric. Food Chem. 2018, 66, 6394−6401

The possible oxidation and reduction products identified in the feces from ergovaline (Table 4) correspond with previous toxicokinetic studies on ergot alkaloid metabolism, which concluded that hepatic cytochrome P450 enzymes (namely, CYP3A) are the primary catalysts responsible for their formation.[31,33,35,46,47] Hydroxylation (mono and di-) is the dominant metabolic pathway that has been described for ergot alkaloids thus far;[29−31,33,48−50] the metabolite with an m/z of 550 represents a mass consistent with hydroxylation for ergovaline. The metabolite consistent with an m/z of 516 suggests loss of water (dehydration); this transformation was also seen in incubations with mouse liver microsomes and ergotamine.[29] Proposed biotransformations for masses of m/z 532, 535, and 536 are suggested in Table 4 and target Phase I reactions, while masses of m/z 802.9, 816.9, and 824.7 are thought to be derived from processes coupled to Phase II enzymes including glutathione and glucuronide conjugation.

In summary, this study delineated a better understanding of LB and EV metabolism by evaluating the consumption of these mycotoxins at levels both below and above the threshold of toxicity in bovine species, as well as considering the possible synergistic effects that might occur when consumed together. Fecal and urine sample analysis showed that the majority of the LB and EV excreted in cattle occurs through the GI tract. Future work is needed for definitive identification of the metabolic products seen here, specifically analysis via high resolution mass spectrometry to generate accurate chemical structures. Samples should be generated from both in vitro incubation experiments as well as toxicokinetic research, with applicable livestock species. This will aid in follow-up studies designed to quantify these compounds in edible tissues in order to gauge their relative risk to public health.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.8b00963.

Methods of analysis and quantitation for analytes of interest and metabolite transitions identified by enhanced mass spectrum mode with independent data acquisition and enhanced product ion scans (EMS IDA EPI) based upon fragmentation of the parent molecule using LightSight software (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*Tel: 541-737-4568. Fax: 541-737-8160. E-mail: Jennifer.Duringer@oregonstate.edu.

ORCID

Jennifer M. Duringer: 0000-0002-0742-4805

Present Address

‖L.D.M.: 5314 Claymore Meadow Lane, Spring, TX 77389.

Funding

This work was jointly supported by the Oregon Agricultural Experiment Station (ORE00871) and the United States Department of Agriculture (Cooperative Agreement number 58-6227-8-044).

Notes

Any opinions, findings, conclusions or recommendations expressed here are those of the authors and do not necessarily reflect the view of the United States Department of Agriculture. The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Anita Holman and the employees of the Endophyte Service Laboratory for support with sample extraction and analyses; Drs. Charles Estill and Linda Blythe for providing veterinary advice and services; and Matt Kennedy and the OSU Farm Services team for their excellent assistance in the care of all animals used in this project.

■ ABBREVIATIONS USED

LB, lolitrem B; EV, ergovaline; PRG, perennial ryegrass; LC-MS/MS, liquid chromatography−tandem mass spectrometry; OSU, Oregon State University; HPLC, high performance liquid chromatography; APCI, atmospheric pressure chemical ionization; ESI, electrospray ionization; MRM, multiple reaction monitoring; V, volts

■ REFERENCES

(1) Ravel, C.; Courty, C.; Coudret, A.; Charmet, G. Beneficial Effects of Neotyphodium Lolii on the Growth and the Water Status in Perennial Ryegrass Cultivated under Nitrogen Deficiency or Drought Stress. *Agronomie* **1997**, *17*, 173−181.

(2) Bacon, C. W.; Richardson, M. D.; White, J. F. Modification and Uses of Endophyte-Enhanced Turfgrasses: A Role for Molecular Technology. *Crop Sci.* **1997**, *37*, 1415−1425.

(3) Gallagher, R. T.; White, E. P.; Mortimer, P. H. Ryegrass Staggers: Isolation of Potent Neurotoxins Lolitrem A and Lolitrem B from Staggers-Producing Pastures. *N. Z. Vet. J.* **1981**, *29* (10), 189−190.

(4) Ball, O. J. P.; Barker, G. M.; Prestidge, R. A.; Sprosen, J. M. Distribution and Accumulation of the Mycotoxin Lolitrem B in Neotyphodium Lolii-Infected Perennial Ryegrass. *J. Chem. Ecol.* **1997**, *23*, 1435−1449.

(5) Philippe, G. Lolitrem B and Indole Diterpene Alkaloids Produced by Endophytic Fungi of the Genus Epichloë and Their Toxic Effects in Livestock. *Toxins* **2016**, *8* (2), 47.

(6) Reed, K. F. M.; Mace, W. J.; Walker, L. V.; Fletcher, L. R. Endophyte Metabolites Associated with Perennial Ryegrass Toxicosis. *Anim. Prod. Sci.* **2016**, *56* (5), 895−907.

(7) Hovermale, J. T.; Craig, A. M. Correlation of Ergovaline and Lolitrem B Levels in Endophyte-Infected Perennial Ryegrass (Lolium Perenne). *J. Vet. Diagn. Invest.* **2001**, *13*, 323−327.

(8) Auldist, M. J.; Thom, E. R. Effects of Endophyte Infection of Perennial Ryegrass on Somatic Cell Counts, Mammary Inflammation, and Milk Protein Composition in Grazing Dairy Cattle. *N. Z. J. Agric. Res.* **2000**, *43*, 345−349.

(9) Repussard, C.; Zbib, N.; Tardieu, D.; Guerre, P. Ergovaline and Lolitrem B Concentrations in Perennial Ryegrass in Field Culture in Southern France: Distribution in the Plant and Impact of Climatic Factors. *J. Agric. Food Chem.* **2014**, *62* (52), 12707−12712.

(10) Craig, A. M.; Blythe, L. L.; Duringer, J. M. The Role of the Oregon State University Endophyte Service Laboratory in Diagnosing Clinical Cases of Endophyte Toxicosis. *J. Agric. Food Chem.* **2014**, *62* (30), 7376−7381.

(11) Imlach, W. L.; Finch, S. C.; Dunlop, J.; Meredith, A. L.; Aldrich, R. W.; Dalziel, J. E. The Molecular Mechanism of "Ryegrass Staggers," a Neurological Disorder of K+ Channels. *J. Pharmacol. Exp. Ther.* **2008**, *327* (3), 657−664.

(12) Mortimer, P. H. Perennial Ryegrass Staggers in New Zealand. In *Effects of Poisonous Plants on Livestock*;Keeler, R. F., Van Kampen, K. R., James, L. F., Eds.; Academic Press: London, 1978; pp 353−361.

(13) Gallagher, R. T.; Hawkes, A. D.; Steyn, P. S.; Vleggaar, R. Tremorgenic Neurotoxins from Perennial Ryegrass Causing Ryegrass Staggers Disorder of Livestock: Structure Elucidation of Lolitrem B. *J. Chem. Soc., Chem. Commun.* **1984**, *9*, 614−616.

(14) Miyazaki, S.; Ishizaki, I.; Ishizaka, M.; Kanbara, T.; Ishiguro-Takeda, Y. Lolitrem B Residue in Fat Tissues of Cattle Consuming

Endophyte-Infected Perennial Ryegrass Straw. *J. Vet. Diagn. Invest.* **2004**, *16*, 340−342.
(15) Finch, S. C.; Fletcher, L. R.; Babu, J. V. The Evaluation of Endophyte Toxin Residues in Sheep Fat. *N. Z. Vet. J.* **2012**, *60*, 56−60.
(16) Zbib, N.; Repussard, C.; Tardieu, D.; Priymenko, N.; Domange, C.; Guerre, P. Toxicity of Endophyte-Infected Ryegrass Hay Containing High Ergovaline Level in Lactating Ewes. *J. Anim. Sci.* **2015**, *93* (8), 4098.
(17) Shimada, N.; Yoshioka, M.; Mikami, O.; Tanimura, N.; Yamanaka, N.; Hanazumi, M.; Kojima, F.; Miyazaki, S. Toxicological Evaluation and Bioaccumulation Potential of Lolitrem B, Endophyte Mycotoxin in Japanese Black Steers. *Food Addit. Contam., Part A* **2013**, *30* (8), 1402−1406.
(18) Stuedemann, J. A.; Hoveland, C. S. Fescue Endophyte: History and Impact on Animal Agriculture. *J. Prod. Agric.* **1988**, *1*, 39−44.
(19) Porter, J. K.; Stuedemann, J. A.; Thompson, F. N.; Lipham, L. B. Neuroendocrine Measurements in Steers Grazed on Endophyte-Infected Fescue. *J. Anim. Sci.* **1990**, *68*, 3285−3292.
(20) Thompson, F. N.; Stuedemann, J. A. Pathophysiology of Fescue Toxicosis. *Agric., Ecosyst. Environ.* **1993**, *44* (1−4), 263−281.
(21) Dyer, D. C. Evidence That Ergovaline Acts on Serotonin Receptors. *Life Sci.* **1993**, *53* (14), PL223−228.
(22) Larson, B. T.; Samford, M. D.; Camden, J. M.; Piper, E.; Kerley, M. S.; Paterson, J. A.; Turner, J. T. Ergovaline Binding and Activation of D2 Dpoamine Receptors in GH4ZR7 Cells. *J. Anim. Sci.* **1995**, *73*, 1396−1400.
(23) Strickland, J. R.; Cross, D. L.; Birrenkott, G. P.; Grimes, L. W. Effect of Ergovaline, Loline, and Dopamine Antagonists on Rat Pituitary Cell Prolactin Release in Vitro. *Am. J. Vet. Res.* **1994**, *55*, 716−721.
(24) Hurley, W. L.; Convey, E. M.; Leung, K.; Edgerton, L. A.; Hemken, R. W. Bovine Prolactin, TSH, T4 AND T3 Concentrations as Affected by Tall Fescue Summer Toxicosis and Temperature. *J. Anim. Sci.* **1980**, *51*, 374−379.
(25) Schillo, K. K.; Leshin, L. S.; Boling, J. A.; Gay, N. Effects of Endophyte-Infected Fescue on Concentrations of Prolactin in Blood Sera and the Anterior Pituitary and Concentrations of Dopamine and Dopamine Metabolites in Brains of Steers. *J. Anim. Sci.* **1988**, *66* (3), 713−718.
(26) Maurer, G.; Schreier, E.; Delaborde, S.; Nufer, R.; Shukla, A. P. Fate and Disposition of Bromocriptine in Animals and Man. II: Absorption, Elimination and Metabolism. *Eur. J. Drug Metab. Pharmacokinet.* **1983**, *8* (1), 51−62.
(27) Nimmerfall, F.; Rosenthaler, J. Ergot Alkaloids: Hepatic Distribution and Estimation of Absorption by Measurement of Total Radioactivity in Bile and Urine. *J. Pharmacokinet. Biopharm.* **1976**, *4* (1), 57−66.
(28) De Lorme, M. J.; Lodge-Ivey, S. L.; Craig, A. M. Physiological and Digestive Effects of Neotyphodium Coenophialum-Infected Tall Fescue Fed to Lambs. *J. Anim. Sci.* **2007**, *85* (5), 1199−1206.
(29) Duringer, J. M.; Lewis, R.; Kuehn, L.; Fleischmann, T.; Craig, A. M. Growth and Hepatic in Vitro Metabolism of Ergotamine in Mice Divergently Selected for Response to Endophyte Toxicity. *Xenobiotica* **2005**, *35* (6), 531−548.
(30) Mulac, D.; Grote, A. K.; Kleigrewe, K.; Humpf, H. U. Investigation of the Metabolism of Ergot Alkaloids in Cell Culture by Fourier Transformation Mass Spectrometry. *J. Agric. Food Chem.* **2011**, *59*, 7798−7807.
(31) Althaus, M.; Retzow, A.; Castell, J. V.; Gómez-Lechón, M.-J.; Amalou, Z.; Rose, T.; Appel, K. In Vitro Identification of the Cytochrome P450 Isoform Responsible for the Metabolism of α-Dihydroergocryptine. *Xenobiotica* **2000**, *30*, 1033−1045.
(32) Ball, S. E.; Maurer, G.; Zollinger, M.; Ladona, M.; Vickers, A. E. M. Characterization of the Cytochrome P-450 Gene Family Responsible for the N-Dealkylation of the Ergot Alkaloid CQA 206−291 in Humans. *Drug Metab. Dispos.* **1992**, *20*, 56−63.
(33) Moubarak, A. S.; Rosenkrans, C. F. J. Hepatic Metabolism of Ergot Alkaloids in Beef Cattle by Cytochrome P450. *Biochem. Biophys. Res. Commun.* **2000**, *274*, 746−749.
(34) Peyronneau, M.-A.; Delaforge, M.; Riviere, R.; Renaud, J.-P.; Mansuy, D. High Affinity of Ergopeptides for Cytochromes P450 3A: Importance of Their Peptide Moiety for P450 Recognition and Hydroxylation of Bromocryptine. *Eur. J. Biochem.* **1994**, *223*, 947−956.
(35) Zbib, N.; Repussard, C.; Tardieu, D.; Priymenko, N.; Domange, C.; Guerre, P. Ergovaline in Tall Fescue and Its Effect on Health, Milk Quality, Biochemical Parameters, Oxidative Status, and Drug Metabolizing Enzymes of Lactating Ewes. *J. Anim. Sci.* **2014**, *92* (11), 5112−5123.
(36) Fisher, M. J.; Bohnert, D. W.; Ackerman, C. J.; Schauer, C. S.; DelCurto, T.; Craig, A. M.; Vanzant, E. S.; Harmon, D. L.; Schrick, F. N. Evaluation of Perennial Ryegrass Straw as a Forage Source for Ruminants. *J. Anim. Sci.* **2004**, *82*, 2175−2184.
(37) Galey, F. D.; Tracy, M. L.; Craigmill, A. L.; Barr, B. C.; Markegard, G.; Peterson, R.; O'Connor, M. Staggers Induced by Consumption of Perennial Ryegrass in Cattle and Sheep from Northern California. *J. Am. Vet. Med. Assoc.* **1991**, *199* (4), 466−470.
(38) Lodge-Ivey, S. L.; Walker, K.; Fleischmann, T.; True, J. E.; Craig, A. M. Detection of Lysergic Acid in Ruminal Fluid, Urine, and in Endophyte-Infected Tall Fescue Using High-Performance Liquid Chromatography. *J. Vet. Diagn. Invest.* **2006**, *18*, 369−374.
(39) Tor-Agbidye, J.; Blythe, L. L.; Craig, A. M. Correlation of Endophyte Toxins (Ergovaline and Lolitrem B) with Clinical Disease: Fescue Foot and Perennial Ryegrass Staggers. *Vet. Hum. Toxicol.* **2001**, *43* (3), 140−146.
(40) Blythe, L. L.; Estill, C.; Males, J.; Craig, A. M. Determination of the Toxic Threshold of Lolitrem B in Cattle Eating Endophyte-Infected Perennial Ryegrass. In *Fungal Endophytes of Grasses*;Popay, A. J., Thom, E., Eds.; New Zealand Grassland Association, 2007; Vol. *13*, pp 399−402.
(41) Young, C. A.; Tapper, B. A.; May, K.; Moon, C. D.; Schardl, C. L.; Scott, B. Indole-Diterpene Biosynthetic Capability of Epichloë Endophytes as Predicted by Ltm Gene Analysis. *Appl. Environ. Microbiol.* **2009**, *75* (7), 2200−2211.
(42) Johnstone, L. K.; Mayhew, I. G.; Fletcher, L. R. Clinical Expression of Lolitrem B (Perennial Ryegrass) Intoxication in Horses. *Equine Vet. J.* **2012**, *44* (3), 304−309.
(43) Munday-Finch, S.; Garthwaite, I. Toxicology of Ryegrass Endophyte in Livestock. *Grassl. Res. Pract. Ser.* **1999**, *7*, 63−67.
(44) Li, L.; Tanaree, D. D.; Estill, C. E.; Duringer, J. M.; Blythe, L. L.; Galen, A.; Livesay, S.; Craig, A. M.; et al. Bovine Hepatic Differential Gene Expression in Response to Perennial Ryegrass Staggers. *World Mycotoxin J.* **2015**, *8*, 351−360.
(45) Lehner, A. F.; Craig, M.; Fannin, N.; Bush, L.; Tobin, T. Electrospray[+] Tandem Quadrupole Mass Spectrometry in the Elucidation of Ergot Alkaloids Chromatographed by HPLC: Screening of Grass or Forage Samples for Novel Toxic Compounds. *J. Mass Spectrom.* **2005**, *40*, 1484−1502.
(46) Moubarak, A.; Rosenkrans, C. J.; Johnson, Z. Modulation of Cytochrome P450 Metabolism by Ergonovine and Dihydroergotamine. *Vet. Hum. Toxicol.* **2003**, *45*, 6−9.
(47) Settivari, R. S.; Evans, T. J.; Rucker, E.; Rottinghaus, G. E.; Spiers, D. E. Effect of Ergot Alkaloids Associated with Fescue Toxicosis on Hepatic Cytochrome P450 and Antioxidant Proteins. *Toxicol. Appl. Pharmacol.* **2008**, *227*, 347−356.
(48) Bauermeister, A.; Aguiar, F. A.; Marques, L. M. M.; Malta, J. D. S.; Barros, F.; Callejon, D. R.; de Oliveira, A.; de Lopes, N. In Vitro Metabolism Evaluation of the Ergot Alkaloid Dihydroergotamine: Application of Microsomal and Biomimetic Oxidative Model. *Planta Med.* **2016**, *82* (15), 1368−1373.
(49) Bicalho, B.; Giolo, J. M.; Lilla, S.; De Nucci, G. Identification and Human Pharmacokinetics of Dihydroergotoxine Metabolites in Man: Preliminary Results. *Biopharm. Drug Dispos.* **2008**, *29* (1), 17−28.
(50) Maurer, G.; Frick, W. Elucidation of the Structure and Receptor Binding Studies of the Major Primary, Metabolite of Dihydroergotamine in Man. *Eur. J. Clin. Pharmacol.* **1984**, *26* (4), 463−470.

DOI: 10.1021/acs.jafc.8b00963
J. Agric. Food Chem. 2018, 66, 6394−6401