Quantitative Analyses of Bovine Urine and Blood Plasma for Loline Alkaloids

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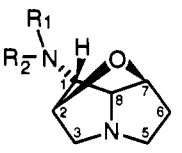
Capillary gas chromatographic methods for the routine analysis of the loline alkaloids in bovine blood plasma and urine have been developed. Urine samples diluted with MeOH were suitable for direct GC analysis. Plasma samples, following protein precipitation, were also suitable for direct GC analysis. *N*-Methylloline was used as an internal standard for these analyses. Peak identities were verified by mass spectrometry and comparison to known standards. The methods should prove to be useful in toxicological studies concerning the role of loline alkaloids in fescue toxicosis.

Tall fescue (*Festuca arundinacea* Schreb), the prominent cool-season pasture grass in the southeastern United States, is estimated to grow on over 35 million acres annually (Defelice and Henning, 1990). Recent surveys suggest that over 95% of all tall fescue pastures are infected with the endophytic fungus, *Acremonium coenophialum* Morgan Jones and Gams (Hill et al., 1990). The presence of the endophyte in tall fescue causes grazing animals to perform poorly; endophyte-related production losses to cattle producers have been estimated to be between $200 and $800 million annually (Siegel et al., 1984; Hoveland, 1990).

Through comparative studies using endophyte-infected (EI) and endophyte-free (EF) tall fescue, both the loline and the ergot-type alkaloids (Figures 1 and 2, respectively) have been implicated as chemical factors in fescue toxicosis (Sanchez, 1987). Reliable analytical methods have been reported for the quantitation of both groups of alkaloids in plant materials [for examples, see Yates et al. (1990) and Rottinghaus et al. (1991)]. However, few toxicological studies identifying and quantifying fescue metabolites in animal fluids and tissues have been reported [an exception is Savary et al. (1990)], even though such studies are necessary to determine the role of individual metabolites in fescue toxicosis.

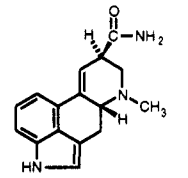
The loline alkaloids (Figure 1) have been isolated from the genera *Lolium*, *Festuca*, *Poa*, *Stipa*, *Adenocarpus*, and *Hordeum* (Siegel et al., 1990; Petroski et al., 1989, 1992; Powell and Petroski, 1992). *N*-Formylloline (NFL) and *N*-acetylloline (NAL) are the primary naturally occurring loline alkaloids in tall fescue (Kennedy and Bush, 1983), but loline (L), norloline (NL), *N*-methylloline (NML), and *N*-acetylnorloline (NANL) also occur naturally. *N*-Formylnorloline (NFNL) has been reported as a synthetic derivative (Yates et al., 1990; Petroski et al., 1989). Some of the loline alkaloids have been reported to have insecticidal (Eichenseer et al., 1991) and herbicidal properties (Petroski et al., 1990).

The loline alkaloids are structurally unique saturated pyrrolizidines that contain an amino substituent at C-1 and an ether linkage between C-2 and C-7; they do not appear to occur naturally as *N*-oxides (Mattocks, 1986). Importantly, the lolines do not cause classical pyrrolizidine

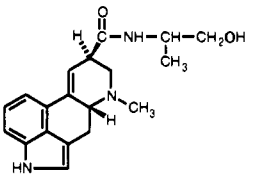


	R_1	R_2
Norloline	H	H
Loline	H	CH_3
N-Methylloline	CH_3	CH_3
N-Formylnorloline	H	HCO
N-Acetylnorloline	H	CH_3CO
N-Formylloline	CH_3	HCO
N-Acetylloline	CH_3	CH_3CO

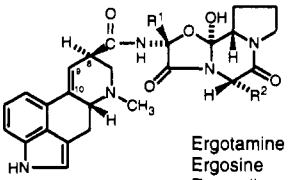
Figure 1. Loline alkaloids (saturated pyrrolizidine alkaloids).



Lysergic acid amide



Ergonovine



	R^1	R^2
Ergotamine	CH_3	$PhCH_2$
Ergosine	CH_3	i-Bu
Ergovaline	CH_3	i-Pr
Ergocryptine	i-Pr	i-Bu

Figure 2. Ergot-type alkaloids isolated from endophyte-infected tall fescue.

toxicity since they lack the requisite unsaturation between C-1 and C-2 and the esterified acid moiety. However, the direct or indirect mammalian toxicity of the loline alkaloids and their role in fescue toxicosis are currently unknown (Bush et al., 1993).

To our knowledge the loline alkaloids have never been quantitated in animal fluids; however, they have been detected in horse urine (Takeda et al., 1990). Presented here are convenient capillary GC methods to tentatively identify each of the loline alkaloids present in bovine urine and blood plasma and relate their concentration to an internal standard. Analyte identities may be confirmed by mass spectrometry or cochromatography with standards. These methods should assist toxicologists determine the role of the loline alkaloids in fescue toxicosis.

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EXPERIMENTAL PROCEDURES

Instruments. Gas chromatography was performed with a Hewlett-Packard 5980A instrument equipped with flame ionization detectors. The oven temperature was held at 100 °C for 0.50 min and then programmed to 250 °C at 10 °C/min. The injector temperature was 250 °C, and the detector temperature was 270 °C. The helium flow for columns was 20 mL/min, and the auxiliary gas flow was 10 mL/min. The hydrogen flow was 30 mL/min, and the air flow was 400 mL/min. Hewlett-Packard Ultra-2 columns (12 m × 0.2 mm), with a film thickness of 0.33 μm, were used. All injections were splitless.

For peak identity confirmation, mass spectra were recorded in the EI mode at 70 eV in a Hewlett-Packard MSD 5970 mass spectrometer with sample introduction through a gas chromatograph.

Samples. Four blank bovine blood plasma and four plasma samples were collected from cattle on EF and EI tall fescue seed-supplemented feed at the University of Missouri in June 1992. Feed samples were prepared from 15% mixtures of EI or EF *F. arundinacea* (KY-31) seed, corn (40.6%), cotton seed hulls (25%), soybean meal (14.4%), cane molasses (3.75%), $CaCO_3$ (0.94%), and NaCl (0.22%). Representative seed and seed-supplemented feed samples were analyzed as described below.

The blank bovine urine sample was supplied by Dr. Jack W. Oliver and was collected from a steer at the time of slaughter in April 1992. The bovine urine sample collected from a 5-year-old, 1100-lb Simmental cow feeding on EI tall fescue pasture (percent infection unknown) and the associated forage materials were supplied by Mr. Stanley O. Jones. These collections were made in September 1991.

Standard Compounds. Saturated pyrrolizidine alkaloids native to EI tall fescue were prepared from loline (Petroski et al., 1989). 2-Phenylmorpholine (PM) was purchased from Aldrich Chemical Co., Milwaukee, WI.

Sample Preparations. *Forage and Seed Samples.* The urine-related forage materials were air-dried at ambient temperature and were powdered to pass through a No. 20 mesh prior to extraction. The plasma-related seed and feed were powdered to pass through a No. 20 mesh. Extraction solvent consisted of CH_2Cl_2/MeOH/NH_4OH (75:25:0.5). PM standard (50.0 μg/mL and extraction solvent were added to the powdered materials (10 mL/g) in a screw-cap glass centrifuge tube, the foil-lined cap was tightly sealed, and the tube was placed on an orbital shaker (170 rpm) overnight at room temperature. The mixture was centrifuged at 2500 rpm for 25 min, and 1 mL was removed and placed in a screw-cap vial (1.5 dram). The sample was sealed and stored at −15 °C until it was analyzed by GC (2.0-μL injections).

Bovine Blood Plasma. NML (50 μg) was added to 1000 μL of bovine blood plasma in a 10-mL screw-cap centrifuge tube, and the sample was tightly sealed with a Teflon-lined screw-cap and shaken. After 15 min, CH_3CN (1000 μL) was added to precipitate the plasma proteins. The tube was again sealed and shaken, and the precipitate was allowed to settle. After 15 min, the solvent was pipetted into another 10-mL screw-cap centrifuge tube, the tube sealed, and the sample centrifuged at 2500 rpm for 20 min. Centrifugation was repeated until all interfering particulates were removed from the sample. After the final centrifugation, the sample was transferred to a screw-cap vial (1.5 dram), sealed, and stored at −15 °C until it was analyzed by GC (2.0-μL injections).

Bovine Urine Samples. A 100-μL sample of bovine urine was added to 900 μL of MeOH and 50 μg of NML in a 1.5-dram vial. The vial was sealed with a Teflon-lined screw-cap, and the sample was vortexed for 30 s and stored at −15 °C until it was analyzed (2.0-μL injections) by GC.

Calculation of Response Factors and Alkaloid Concentrations. *Forage Samples.* PM (Yates et al., 1990) was used as the internal standard for forage samples. Response factors were determined by chromatographing mixtures of pure alkaloids in ratios of 1:1:1:1:1 and 5:1:1:1:1 of PM/L/NML/NFL/NAL (where [L] = 100 ng/μL). Response curves were determined from injections of 1.0, 2.0, and 3.0 μL with a minimum of six replicates. Response factors (standard deviation) were determined to be 1.47 (0.08) for L, 1.36 (0.03) for NML, 1.85 (0.15) for NFL, and 1.82 (0.14) for NAL. The lower limit of detection for all analyses was about 1.0 ng of alkaloid in a GC injection.

Urine and Plasma Samples. NML was used as the internal standard for urine and plasma samples. Stock solutions of alkaloid ratios of 1:1:1:1 and 5:1:1:1 of NML/L/NFL/NAL (where [L] = 100 ng/μL) were prepared, and response curves were determined from injections of 1.0, 2.0, and 3.0 μL with a minimum of five replicates. Response factors (standard deviation) with NML as the internal standard were determined to be 1.03 (0.05) for L, 1.20 (0.11) for NFL, and 1.18 (0.10) for NAL.

Recovery Experiments. *Urine Samples.* Analysis of urine from cattle fed a diet not supplemented with EI tall fescue revealed the absence of interfering materials. Blank urine samples were prepared as described above, and a mixture of pure alkaloids in a ratio of 1:1:1:1 (three samples) and 5:1:1:1 (one sample) of NML/L/NFL/NAL (where [L] = 10 ng/μL) was added to each sample. The spiked samples were analyzed (2.0-μL injections), and the percentage of each alkaloid recovered was calculated (a minimum of five replicates per sample). Percent recoveries (percent) were calculated as the ratio between the amount of alkaloid recovered and alkaloid added.

Plasma Samples. Analysis of plasma samples from cattle fed a diet not supplemented with EI tall fescue revealed the absence of interfering materials. Three blank plasma samples were spiked with pure alkaloids in a ratio of 5:2:2:2 of NML/L/NFL/NAL (where [L] = 20 ng/μL) and were prepared for analysis as described above. The spiked samples were analyzed (2.0-μL injections) and the percent recoveries were determined as described for the urine samples (a minimum of five replicates per sample).

Table I. Loline Alkaloid Recoveries from Bovine Urine

trial	n	L	NML	NFL	NAL
		Recoveries (Percent)			
1	5	68.5	66.0	56.9	54.6
2	6	73.0	66.6	60.0	59.0
3	5	72.7	70.8	60.8	56.7
4	6	67.0	69.1	65.0	62.5
av (SD)	5.5	70.3 (3.0)	68.1 (2.2)	60.7 (3.3)	58.2 (3.4)
		Relative Recoveries (NML Internal Standard, Percent)			
4	5.5	103		89	85

Table II. Loline Alkaloid Recoveries from Bovine Blood Plasma

trial	n	L	NML	NFL	NAL
		Overall Recoveries (Percent)			
1	6	71.3	71.9	78.4	72.9
2	6	68.3	66.9	71.2	63.2
3	7	67.3	78.7	72.2	65.5
av (SD)	6.3	69.0 (2.1)	72.5 (5.9)	73.9 (3.9)	67.2 (5.1)
		Relative Recoveries (NML Internal Standard, Percent)			
3	6.3	95		102	93

RESULTS AND DISCUSSION

The loline alkaloid recoveries from blank bovine urine and blood plasma samples are presented in Tables I and II, respectively. Glassware was used for this work (and all of the other, as well) since plasticware contains materials that interfere with loline analysis. The loline alkaloid recoveries (Table I) from blank bovine urine were around 65%. Forage sample loline alkaloid concentrations are presented in Table III. The identities of the loline alkaloids in all samples (except recovery samples) were verified by GC–MS.

PM recoveries from both bovine urine and blood plasma samples were significantly different from those of the loline alkaloids. Therefore, it was not a suitable internal standard for these analyses. However, NML behaved chemically like the other loline alkaloids (in detector response and recovery from these matrices), and it was readily prepared from loline (Petroski et al., 1989). Importantly, NML was not observed to be present in the

Table III. Loline Alkaloid Concentrations in Feed Materials

sample	saturated pyrrolizidine alkaloids, ppm					
	NL	L	NML	NANL	NFL	NAL
urine-related materials	-	+	+	+	370	84
serum-related materials						
EI seed	-	126	196	107	1590	330
EI feed	-	31	50	48	460	102
EF seed	-	-	-	-	62	10
EF feed	-	-	-	-	8	-

Table IV. Loline Alkaloid Analysis of Bovine Urine[a] (Concentrations in Micrograms per Milliliter)

sample	n	L	NFL	NAL
1	5	71.7	10.8	5.6
2	6	68.0	6.1	2.8
3	6	70.4	8.9	4.8
av (SD)		70.0 (1.9)	8.6 (2.4)	4.4 (1.4)

[a] Total loline concentration: 83.0 μg/mL of urine.

urine or the plasma samples. Overall, NML served as an excellent internal standard for this work.

Fortunately, there were no volatile materials that interfered with the loline analyses of blank urine samples. Direct analysis of the urine samples at the animal pH eliminated concern for incomplete alkaloid extraction or deacylation of NFL or NAL.

Blank bovine plasma samples were also free of volatile materials that might interfere with GC analysis but contained substantial amounts of protein which prevented direct GC analysis. Attempts to extract the loline alkaloids from basified plasma with organic solvents resulted in either deacylation of NFL and NAL or incomplete alkaloid extraction. Protein precipitation by the addition of acetonitrile at the original sample pH avoided these problems and afforded much higher overall alkaloid recoveries (39.4% vs 73.5% for NFL). Unfortunately, subsequent sample filtration resulted in filter-related contaminants which interfered with GC analysis. However, multiple centrifugations of the samples effectively removed the protein precipitates and eliminated this source of contamination, but they were time-consuming.

The overall loline alkaloid recoveries from bovine blood plasma were all around 70% (Table II). It should be noted, however, that we were unable to detect measurable levels of loline alkaloids in any of the bovine plasma samples tested. Perhaps loline alkaloids are rapidly cleared from the body following ingestion. Plasma levels of unsaturated pyrrolizidine alkaloids drop by nearly 90% within 2 h after intravenous administration (Ames and Powis, 1978).

The urine sample was collected from a cow exhibiting symptoms of fescue toxicosis (e.g., split hooves and a rough coat). Table IV contains the results of the bovine urine sample analyses. The concentration of the loline alkaloids in this animal's urine was found to be 70.0 μg/mL of L, 8.6 μg/mL of NFL, and 4.4 μg/mL of NAL, and the total loline alkaloid concentration was 83.0 μg/mL (Table IV). No other lolines were observed in this sample. Both NFL and NAL appear to be substantially converted to loline within cattle. Since loline alkaloids have been reported in horse urine (Takeda et al., 1991), it was not surprising to also find them in bovine urine. It has recently been shown that NFL is converterd to loline by rumen fluid, but NAL (as the sole substrate) is mostly unchanged (Westendorf et al., 1993).

The loline alkaloid concentration in the forage available to this animal was at least 450 ppm (Table III). Unfortunately, the levels presented in Table III may not reflect the animal's actual dietary loline levels since substantial quantities of loline alkaloids may be lost when forage samples are air-dried and cattle select their diet when they graze. Furthermore, the feces was not analyzed to quantify any unadsorbed lolines. Due to these unknown variables, the mass transfer of loline alkaloids through this animal cannot be determined accurately with these data. However, these results suggest that loline alkaloids are absorbed in cattle and, therefore, may play a role in fescue toxicosis.

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