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Chapter 85

Characterization of a New Compound Eluting in an Ergovaline HPLC Assay Associated with Reproductive Problems in Dairy Cows

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Introduction

Tall fescue (*Festuca arundinacea*) and perennial ryegrass (*Lolium perenne*) are perennial cool-season grasses which are infected with the endophytic fungi, *Neotyphodium coenophialum* and *N. lolii*, respectively. These endophytes have been increasingly promoted in fescue and ryegrass because they confer benefits such as pest resistance and drought tolerance to the plant (Adcock 1997), decreasing the use of pesticides, fertilizers and irrigation. However, livestock grazing endophyte-infected (E+) grasses are negatively impacted by the production of ergot and lolitrem alkaloids from the fungi which are responsible for a variety of insect and mammalian diseases (Joost 1995; Tor-Agbidye 2001). Endophyte toxicosis affects cattle production by decreasing average daily weight gain and reproductive efficiency, resulting in approximately $1 billion in livestock damage each year in the United States alone (Comis 2000).

Ergot alkaloids act as α-adrenergic agonists which stimulate smooth muscle cell contraction, resulting in vasoconstriction of the extremities (Thompson 1993). This can result in tissue ischemia, necrosis and sloughing of hooves, tails and ears during exposure to cold ambient temperatures (fescue foot) and reduced average daily weight gain with elevated temperatures (summer slump). Cows consuming E+ fescue straw exhibit altered reproductive hormone levels, including decreased prolactin (Emile 2000) which is used as a diagnostic

indicator of endophyte toxicosis. Additional reproductive problems include reduced pregnancy rates (Schmidt 1983) and increased rates of early embryonic death (Schmidt 1986).

Ergovaline is the ergot alkaloid found in highest abundance in E+ tall fescue (85-97% of total ergopeptine alkaloid content) (Lyons 1986) and is detected along with lolitrem B in E+ perennial ryegrass (Hovermale 2001). A high-performance liquid chromatography (HPLC) method was developed to quantify ergovaline in forage samples submitted by farmers and/or veterinarians (Craig *et al.* 1994). Using this assay, threshold levels correlating concentration of ergovaline in feed to the appearance of clinical disease were established for horses, sheep and cattle (Tor-Agbidye 2001), namely 300-500 ppb for horses (except for mares in the last 60 to 90 days of gestation when the recommended threshold is 0 ppb), 400-750 ppb for cattle and 500-800 ppb for sheep. However, it is likely that not all of the ergot alkaloids involved in endophyte toxicosis have been identified.

During sample processing for the diagnostic analysis of ergovaline, unidentified peaks occasionally elute which appear to coincide with clinical disease. In the winter of 2003-2004, such a situation occurred when numerous dairies in the Willamette Valley, Oregon encountered reproductive problems, specifically decreased cycling and conception rates. Mycotoxins, viral and bacterial pathogens were ruled out as a cause, while prolactin concentrations showed no obvious trend. Hay and silage samples (processed from the same given field for each dairy) were sent in for endophyte toxin analysis. While all samples had low concentrations of ergovaline, haylage (ensiled perennial ryegrass) samples showed the appearance of a large peak eluting at five minutes in the ergovaline HPLC chromatogram.

The objective of this research was to characterize this peak using mass spectroscopy to determine its chemical structure and identity. We believe this peak represents an ergot alkaloid toxin not previously reported in correlation with clinical signs of fescue toxicosis and should be investigated further as to its association with development of reproductive problems in livestock.

Materials and Methods

Collection and preparation of hay and silage samples

Hay and silage samples were collected from four dairies in the Willamette Valley, Oregon, from July 2003 through April 2005. Samples were dried and submitted to the College of Veterinary Medicine for ergovaline analysis. Dried samples were ground to pass through a 0.5 mm screen (Cyclotec 1093 sample mill, Tecator, Hönganäs, Sweden).

Ergovaline HPLC analysis and collection of five minute peak

Ergovaline concentration was analyzed according to a previously reported method (Craig *et al.* 1994). Briefly, 1.0 g of ground sample was extracted over 24 hours with ergotamine tartrate (internal standard), chloroform and sodium hydroxide and then centrifuged at 2000 rpm for 5 minutes. Solid phase extraction (SPE) columns containing Ergosil were conditioned with chloroform before the sample supernatant was applied and drawn through the column. The column was then washed with a chloroform:acetone mixture followed by methanol. The ergot alkaloids were eluted with methanol and concentrated before analysis by HPLC. A polymeric divinyl benzene column (Jordi RP SM-500A, 5μ (150mm x 4.6mm)) was eluted with acetonitrile/2.5 mM ammonium carbonate (70/30 v/v) and run at a flow rate of 1.0 mL min^{-1}. Detection was by fluorescence with excitation and emission wavelengths of 250 and 420 nm, respectively.

A new peak, eluting at approximately five minutes in the ergovaline HPLC assay, appeared in the perennial and annual ryegrass silage samples at varying concentrations (Figure 1). To concentrate and purify this peak, samples with a large concentration of this compound were extracted using the ergovaline methodology, pooled, dried under nitrogen stream at 50°C and reconstituted in 0.5 ml methanol. The pooled sample was then vortexed, sonicated and centrifuged for five minutes at 2000 rpm. The supernatant was injected onto the HPLC system and the peak eluting at five minutes was captured manually. This fraction was dried under a nitrogen stream at 50°C, reconstituted with 1 ml methanol, vortexed, sonicated and centrifuged for five minutes at 2000 rpm. The final supernatant was stored in an amber vial in the freezer (-20°C) until mass spectral analysis was performed.

Mass spectral analysis of "five minute peak"

Spectra were obtained on a LCQ Classic ion trap mass spectrometer (Thermo Finnigan) equipped with a custom-designed electrospray inlet consisting of a 30 micron i.d. steel capillary heated at 170°C and operated at 2.7 kV. Solvent flow was controlled by a HPLC system consisting of a Waters Automated Gradient Controller with two Waters 515 HPLC pumps and a Rheodyne 8125 injector. The solvents used were water and acetonitrile each containing 0.1% acetic acid and 0.01% trifluoroacetic acid eluting under isocratic conditions in a 50:50 ratio. Samples were introduced to the spectrometer by loop injection with data acquisition in the positive ion mode. MS/MS analysis was selected for the peak with the highest relative intensity by MS.

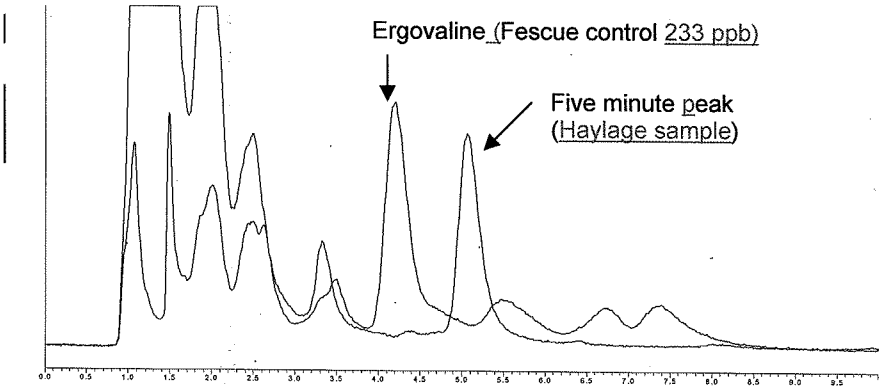

Figure 1. Chromatogram from reverse-phase HPLC of ergovaline showing the appearance of a new peak eluting around five minutes.

Results

Characterization of the "five minute peak" by LC-MS gave a pseudomolecular ion at m/z ratio 593.5 [M+H]+ (Figure 2A). LC-MS/MS analysis of ions with a m/z ratio of 593.5 produced daughter ions with a m/z ratio of 533.3 and 534.3 with little other fragmentation occurring (Figure 2B). Further mass spectrometry (MS^7) produced a scan showing peaks with a m/z ratio of 197, 223 and 268.

Conclusion

The LC-MS scan produced a peak with a m/z ratio of 593.5 which is not consistent with the molecular weight of any known ergot alkaloid. The LC-MS/MS scan produced a spectrum with two main peaks, one at 533.3 and another at 534.3. It was interesting to note the close association with ergovaline, whose molecular weight is 533.63. However, it was evident that this molecule had not completely fragmented, as the characteristic lysergic acid ring structure peaks typically present from ergot alkaloids fragmented in a mass spectrometer (*i.e.*, 197, 208, 223, 268, etc.) were absent (Lehner 2004). Higher collision energies at MS^7 finally fragmented the molecule into many peaks, including those with a m/z ratio of 197, 223 and 268. Those at 197, 223 and 268 are consistent with molecules having the lysergic acid ring as its base; those at 235 and 342 are unknown compounds.

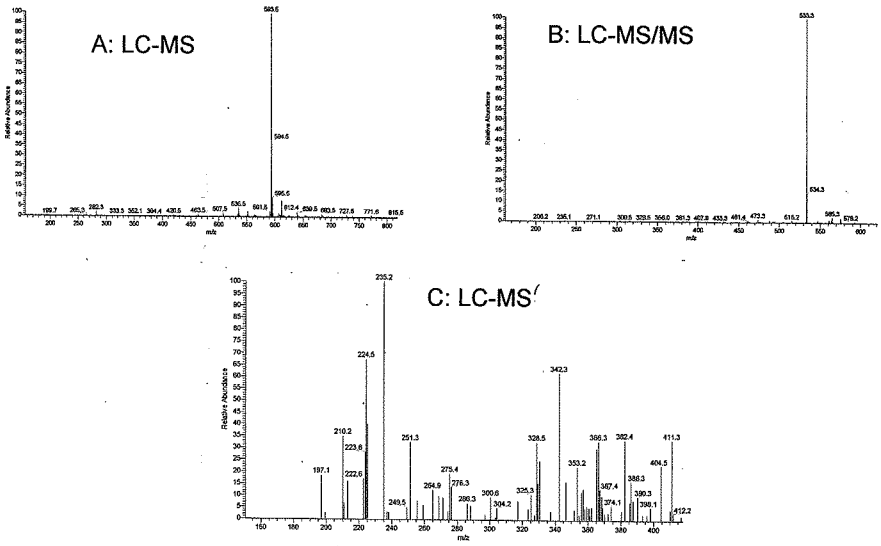

Figure 2. Mass spectroscopy of the five minute peak by LC-MS (A), LC-MS/MS (B) and LC-MS^7.

Further characterization of this compound is ongoing, including analysis by LC-TOF/MS and 1H-NMR. Once this compound is identified, attempts to correlate the appearance of it in silage samples with reproductive problems in dairy herds will be made. In addition, the endophyte testing service is now aware of this unusual peak and its potential toxicity; thus, it will be able to identify samples containing this compound and caution farmers and veterinarians in using feed containing this molecule.

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