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Effects of Ergot Alkaloids and Bovine Bodily Fluids on Cytochrome P450 Activity

Nicholas Scott Ezell
University of Arkansas, Fayetteville

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EFFECTS OF ERGOT ALKALOIDS AND BOVINE BODILY FLUIDS ON
CYTOCHROME P450 ACTIVITY

EFFECTS OF ERGOT ALKALOIDS AND BOVINE BODILY FLUIDS ON
CYTOCHROME P450 ACTIVITY

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Animal Science

By

Nicholas Ezell
University of Arkansas
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University of Arkansas

ABSTRACT

This thesis evaluates the Promega™ P450-Glo assay (Promega™ V9800) as a tool for quantifying ergot alkaloid concentration. Current techniques used for detection of ergot alkaloids are slow and expensive, do not detect all ergot alkaloids, or are not effective on bovine bodily fluids. The first study was conducted to determine effects of commercial ergot alkaloids (n = 6; 0 - 400 μM) on the Promega™ P450-Glo assay. Cytochrome P450 (CYP450) activity in assay had a differential response to each ergot alkaloid and concentration. As concentrations of ergotamine, dihydroergotamine, ergocornine, and ergocryptine increased CYP450 activity was inhibited (P < 0.05). Increases in ergonovine and pergolide concentration did not affect (P > 0.1) CYP450 activity. These results verify that the Promega™ P450-Glo assay is able to detect presence of ergot alkaloids. The second study was conducted to determine ability of the Promega™ P450-Glo assay to detect ergot alkaloids in biological samples. Bovine urine and serum were analyzed with the Promega™ P450-Glo assay to test for effects of forage and genotype. The single nucleotide polymorphism tested was designated by alu1 cleaving enzyme. Crossbred Angus-sired steers (n = 39; 216 ± 2.6 days; 203 ± 1.7 kg) were blocked by weight and assigned to graze toxic tall fescue (E+; n = 4) or non-toxic fescue (HM4; n = 4) pastures. After grazing 105 days animals were weighed, and blood and urine samples were collected. Samples were analyzed using the Promega™ P450-Glo assay. Enzyme linked immunosorbent assay (ELISA) was used to test urine for total ergot alkaloids. Animals were genotyped for CYP450 single nucleotide polymorphism. Data were analyzed for correlations and one way analysis of variance. Genotype affected (P < 0.05) ability of urine to inhibit CYP450 activity, while forage did not. Genotype and forage did not affect ability

of serum to inhibit CYP450 activity. Inhibition of CYP450 activity by urine was strongly correlated ($r = -0.50$; $P < 0.025$) with total ergot alkaloids in urine as determined by ELISA. Results indicate the Promega™ P450-Glo assay is able to detect ergot alkaloid presence in urine.

This thesis is approved for recommendation
to the Graduate Council

Thesis Director:

Dr. Charles F. Rosenkrans, Jr.

Thesis Committee:

Dr. Ali S. Moubarak, Ex Officio

Dr. Michael L. Looper

Dr. Nicholas B. Anthony

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INTRODUCTION

Lolium arundinaceum, Darbyshire, or tall fescue, is a cool season perennial that is often used as forage (Stuedemann and Hoveland, 1988). Consumption of *Neotyphodium coenophialum* infected tall fescue causes fescue toxicosis in cattle, which results in significant economic loss for producers (Paterson et al., 1995). Fescue toxicosis is responsible for decreased reproductive performance, feed intake, and weight gain (Strickland et al., 1993). Ergot alkaloids, a significant contributor to this condition, are produced by the ergot fungus. Metabolism of ergot alkaloids by cytochrome P450 enzymes occurs primarily in the liver (Oliver, 1997). Ergot alkaloid concentration also is known to have a significant negative effect on hepatic function (Settivari et al., 2006).

Detection of ergot alkaloids in biological samples is very difficult. There are three main methods of detection currently in use. High performance liquid chromatography (HPLC; Hill et al., 1993; Rottinghaus et al., 1991; Spiering et al., 2002; Kallenbach et al., 2003), mass spectrometry (Yates et al., 1985), and enzyme-linked immunosorbent assay (ELISA) which detects total ergot alkaloids (Reddick, 1988; Gwinn et al., 1991).

The Promega™ P450-Glo assay directly measures activity of the cytochrome P450 enzyme. In this assay CYP450 converts a luminogenic substrate, Luciferin 6-benzyl ether (Luciferin-BE), into luciferin, which binds to luciferase to produce luminescence. Luminescence produced is directly proportional to activity of the CYP450 enzyme. Ergot alkaloid concentration influences CYP450 activity in vitro (Settivari et al., 2006; Moubarak et al., 1998), so any alkaloids present will affect the ability of CYP450 to convert the Luciferin-BE into luciferin, inhibiting luminescence of the assay. The objective of these studies was to evaluate the Promega™ P450-Glo Assay as a tool for detecting ergot alkaloid presence in a sample.

CHAPTER 1

LITERATURE REVIEW

Tall Fescue. Tall fescue, *Lolium arundinaceum* Darbyshire, formerly *Festuca arundinacea*, is the most prominent forage pasture grass in the United States of America, currently covering approximately 15 million hectares (Buckner et al., 1979). While originally native to Europe, tall fescue is not the forage of choice there. Rather, the favored forage is perennial ryegrass (*L. perenne*) due to its ability to thrive in the milder climates and heavier rainfall present in Europe. It is speculated that tall fescue was accidentally included in seed thought to contain only meadow fescue (Hoveland et al., 2009), which was planted across the humid areas of North America before 1800 (Hoveland et al., 2009). It didn't take long until tall fescue, originally seeded by accident, was considered a valuable grass for grazing and lawns (Hoveland et al., 2009) due to its superior growth, height, and drought tolerance as compared to meadow fescue according to Kentucky grass trials (Hoveland et al., 2009).

The early 1940's saw the release of Kentucky 31 (K31) fescue, which led to tall fescue seed being planted extensively across the country. Kentucky 31 originated from a strain of tall fescue found on a steep mountain pasture of eastern Kentucky by Dr. E.N. Fergus, a professor at the University of Kentucky, in 1931. It was noted that the grass remained green throughout the winter, so testing on the seed began. In 1943, K31 was released (Fergus and Buckner, 1979). This new strain was described as being dependable, adaptable to many types of soil, and able to be grazed for the majority of the year. At this time, no other cool season perennial grasses were able to persist throughout the year in United States pastures, so K31 became the cool season forage of choice. Along with its use as forage, tall fescue also became a popular roadside cover, which led to the transformation of the United States landscape.

Livestock growers soon began noticing poor animal performance while grazing on tall fescue (Pratt and Haynes, 1950) which we can now attribute to tall fescue toxicosis, a

combination of three syndromes (Stuedemann and Hoveland, 1988). The first syndrome, known as fescue foot, resulted in cattle with elevated respiration rates and gangrene, which caused the loss of hooves, tails, and ears, and was first described in New Zealand (Hoveland et al., 2009). The second syndrome, called fat necrosis, occurs primarily on tall fescue pasture with high levels of nitrogen fertilizer. It consists of hard fat accumulating along the intestinal tract, which results in upset digestion and difficult births (Bush et al., 1979; Stuedemann et al., 1975). The final syndrome, referred to as summer syndrome, involves failure to shed the winter hair coat, high respiration rates, heat intolerance, poor animal gain, reduced milk production, depressed feed intake, and low conception rates (Hoveland et al., 1983; Stuedemann and Hoveland, 1988). Annual losses due to tall fescue toxicosis in the United States beef industry have been estimated at well over \$600 million from reduced calf births and lower weaning weights (Hoveland, 1993).

When these symptoms first began presenting in livestock, ergotism was suspected; however, it was initially ruled out when there was no ergot sclerotia present in seed heads of tall fescue. Research was then conducted on external plant fungi, plant alkaloids, toxins produced in rumen, and anions (Bush et al., 1979). Perlolone, an alkaloid found in tall fescue, was thought to be the issue, but a perloline free strain developed in Kentucky showed the same symptoms as normal tall fescue.

Because the symptoms of consumption of tall fescue closely resemble those of ergot toxicity, some scientists were convinced that the fescue toxicity involved a fungus (Robbins, 1983). In 1973, three species of *Balansia* fungi were isolated from a toxic tall fescue plant (Bacon et al., 1975). All three fungi were endophytic, any organism that lives inside a plant, with an epiphyte living on the plant exterior (Clay and Schardl, 2002), and caused no harm to the grass itself, but were shown to produce grass toxicosis in cattle and goats (Clay and Schardl,

2002). Being endophytic, the fungi spend their life cycle within the plant, and symbiotically improve the fitness of the host (Bacon and Siegel, 1988; Malinowki and Belesky, 2000). This is believed to be where tall fescue gains its heat and drought tolerance, along with pest resistance, that make it a desirable forage (West et al., 1990; Latch, 1997; Clay and Schardl, 2002).

Endophyte growth occurs within the intercellular region of the plant, as there is an abundance of both sugars and amino acids (Clay and Schardl, 2002). While it is known that many forages commonly grazed by livestock are hosts to endophytes, endophyte infected tall fescue remains to carry the greatest economic impact (Oliver et al., 2000).

Grass endophyte was first reported in 1898 by Vogl in Germany (White et al., 1993), and fungal endophyte was first confirmed to be present in tall fescue in New Zealand (Neil, 1941). It was later demonstrated that the *Balansia* species of fungi had the potential to produce ergot alkaloids (Porter et al., 1979). This was the initial step in forming the hypothesis that the endophyte was responsible for the toxicosis, and led to the first research into endophyte infected fescue toxicosis (Bacon, 1995). The primary endophyte responsible for toxicosis is *Neotyphodium coenophialum* (Bacon et al., 1977; Hoveland et al., 1980; Hoveland et al., 1983). It has been shown that this fungus is responsible for the heat and drought resistance of tall fescue (Schardl, 1996). It also produces many ergot alkaloids that are believed to be responsible for the insect and pest resistance, along with the toxicosis of tall fescue (Panaccione et al., 2001).

Cattle that are grazed on endophyte infected tall fescue can be expected to perform much poorer than those grazing non-infected or endophyte free fescue (Read and Camp, 1986). The animals grazing tall fescue may only gain half as much as animals grazing non-infected forages (Read and Camp, 1986). There is also a strong association of endophyte infected fescue with fescue foot (Read and Camp, 1986). Many management practices for reducing the effects of

fescue toxicosis have been explored. One technique for reducing the effects of fescue toxicosis is to graze animals on other forages along with the infected fescue. Pastures can be seeded with other forages such as bermudagrass or clover, or cattle can be rotated to pastures that contain no endophyte infected fescue. This reduces the amount of ergot toxin consumed, and also the effects of toxicosis. However, other grasses are much more expensive than tall fescue, they may not be as heat or drought tolerant, and rotating pastures is much more management intensive.

The discovery of a non-toxic strain of endophyte (Bouton et al., 1997), known as the “AR-547” strain, allowed producers to take advantage of the benefits of infected fescue, with many of the compounds known to affect cattle removed. Completely developing new pastures and reseeding with non-toxic tall fescue is a very expensive process. This is much preferred to endophyte free tall fescue, which no longer has the heat and drought tolerance from the endophyte presence, which significantly reduces the usefulness of tall fescue as forage (Watson et al., 1995).

A new strain of *Neotyphodium coenophialum* has been developed by the University of Arkansas and the Missouri Agricultural Experiment Station (Nihsen et al., 2004). This strain is compatible with tall fescue and does not produce many of the ergot alkaloids found in traditional endophyte strains (Vough, 2003).

Another method for reducing the effects of endophyte infected tall fescue on animals is genetic selection. This involves culling animals that underperform when grazing tall fescue pastures, and promoting phenotypes that show resistance to the toxin. This method has proven beneficial in mice, but genetic markers have yet to be isolated for the effect (Wagner et al., 2000). The drugs domperidone and sulpiride also may reduce the effects of endophyte toxicosis (Strickland et al., 1994).

We see the effects of fescue toxicosis in herds across the country, but we still understand very little about what causes the disease and its mechanisms. There is still uncertainty about the role ergot alkaloids play, and which ones are involved, in tall fescue toxicosis. Research is needed to determine which alkaloids cause toxicosis, and the mechanisms used in clearing them from the animal's system.

Ergot Alkaloids. It has been established that ergot alkaloids, produced by the endophytic fungi present in infected tall fescue are the source of fescue toxicosis (Bacon et al., 1997; Cornell et al., 1990). There are four main groups of ergot alkaloids: the ergopeptines, the clavines, lysergic acids, and the lysergic acid amides (Berde, 1980). Many of these alkaloids have been found within endophyte infected tall fescue including ergotamine (an ergopeptine), ergonovine (a lysergic acid amide), and peramine (Jones, 1981; Bush et al., 1982; Siegel et al., 1990; Porter, 1995), but it is ergovaline that makes up the majority, and the most active, of alkaloids isolated from infected fescue (Porter et al., 1979; Lyons et al., 1986). It has been shown that the endophytic fungus produces these alkaloids when separated from fescue, so it follows that the fungus produces the toxins, and not the fescue (Porter et al., 1979).

Ergot alkaloids play a small part in pharmacology today, but in the past were used prevalently as vasoconstrictors, uterine stimulators, and alpha adrenoceptor agonists (Silberstein, 1997; Lipton, 1997). They have recently been investigated for treatment of postural hypotension (Oliver et al., 1993; Lipton, 1997; Villalon et al., 2002). Bromocriptine was used in the past to treat Parkinson's disease in humans (Hoehn and Elton, 1985; Shiraishi et al., 2004), and in obstetrics for its effects on the uterus (Albert-Puleo, 1979; de Groot et al., 1998). Because we use these alkaloids in treating certain medical conditions, it is important that we understand their structures. We can then manipulate these compounds to better utilize their strengths. There are

similarities between the ergot alkaloids and certain neurotransmitters such as serotonin, dopamine, and norepinephrine, as all have structures based on the ergoline ring, which is central to all ergopeptines (Berde, 1980). These molecules vary in side chains and radical groups leading off of the ergoline ring (Berde, 1980). Synthetic alkaloids have been developed based on research done with natural ergot alkaloids (Madlom, 2002). These synthetic molecules take advantage of the benefits of traditional alkaloids, while minimizing the side effects, and reducing the potency, allowing for more controlled dosing options (Madlom, 2002).

The endophyte produces many compounds, but it has been shown that the ergopeptines are to blame for most toxicosis problems (West, 2000). Symptoms of fescue toxicosis have been found in animals grazing on tall fescue containing as little as 50 ng/g ergovaline, an ergopeptine produced by the endophyte. Ergovaline concentrations can vary from two to six µg/g (Porter, 1995). While it has not been shown which symptoms are caused by which alkaloids, the two most common alkaloids found in common tall fescue are ergovaline and ergotamine.

Ergopeptines appear to be absorbed in the omasum, but it has been suggested that absorption through mucosal membranes in the mouth may be a significant contributor to alkaloid ingestion (Hill et al., 2001). While alkaloid detection is possible with certain methods, no method has been developed to accurately measure alkaloid content of bodily fluids of animals grazing tall fescue, but alkaloids can be detected after injection (Cox et al., 1992; Moubarak et al., 1996).

While ergotamine has been detected in tall fescue extracts, it has been suggested that its presence is due to infection with *Claviceps purpurea*, which has been shown to produce this alkaloid (Riederer et al., 1996; Shelby et al., 1997). This would mean that *Neotyphodium* is not the cause of ergotamine presence in tall fescue. Ergot alkaloids produce symptoms by inhibiting a number of biological systems. Ergotamine and ergonovine have been shown to influence liver

mitochondrial calcium ATPases (Moubarak et al., 1998). Similar effects have been seen in the renal system with sodium/potassium ATPases that effect kidney function (Moubarak et al., 1993).

Cytochrome P450. Degradation and clearance from the body of many therapeutic drugs, including the ergot alkaloids, begins in the liver and involves the cytochrome P450 (CYP450) group of enzymes (Elkins et al., 1999). It has been shown that ergocryptine, along with other alkaloids, interacts metabolically with CYP450 isoforms (Althaus et al., 2000). Cytochrome P450 proteins are grouped because they are known to metabolize steroids, fatty acids, and related compounds, and because they have a similar structure. All CYP450 proteins have molecular weights of approximately 50,000 daltons (Sono, 1996). Cytochrome P450 enzymes may take part in both anabolism and catabolism (Omura, 1999). The CYP450 enzymes can be found throughout the cell endoplasmic reticulum (ER) and within the inner membrane of the mitochondria. It has been theorized that their presence is connected to the formation of large protein molecules in the ER (Szczesna-Skorupa et al., 1998). Because all CYP450 enzymes have a similar structure, they all share a similar active site that is compatible with lipid soluble compounds (Omura, 1999).

The CYP in all CYP450 enzymes is an abbreviation for the word **CY**tochrome **P**450. The name P450 represents the electromagnetic absorption spectra, 450 nanometers, determined when they were first isolated in 1964 (Omura et al., 1964). This defines a group of membrane bound hemoproteins that contain heme groups and carry out electron transport. After the name CYP the specific enzyme is named. An example would be the enzyme CYP3A4. The first number after CYP, in this case 3, represents the family. Members of the same family must share greater than 40 percent amino acid identity. Next comes the substrate, A. Enzymes that have the

same substrate share a greater than 59 percent amino acid identity. Followed by the specific isoform, in this case 4 (Cupp et al., 1998). So, CYP3A4 and CYP3A23 are in the same family and act on the same substrate, but are different isoforms, and are found in different organisms, the human and the rat respectively (Echizen et al., 2000; Komori et al., 1994). Now that we have other methods to describe the proteins found in this group, the absorption seems like an odd way to describe this microsomal fraction. Specific enzymes; however, were named based on their function, and the species they were isolated in as they were discovered (Renaud, 1990). The cytochrome name is actually inaccurate as the proteins found in this group do not use electron transfer as their mechanism of action. Rather these proteins should be called heme-thiolate proteins (Hofmann et al., 1999).

Human 3A4. All physiological testing done for these experiments was completed using the human enzyme CYP3A4. In humans it is this enzyme that is responsible for the breakdown of ergot alkaloids (Althaus et al., 2000). The corresponding enzymes in cattle and rats are CYP3A28 and CYP3A23 (Sales et al., 2011; Poole, 2004) respectively. These enzymes degrade ergotamine into four metabolites and their isomers (Moubarak et al., 2000). In humans CYP3A4 is the most highly produced enzyme in the CYP3A group of enzymes and is found in the liver and small intestine. This enzyme was previously thought to have a mechanism of electron transfer, but it was shown that it will bind to substrates that cannot transfer electrons; therefore activity is the result of substrate stimulation and not electron transfer (Yamazaki et al., 1996).

Promega™ P450-Glo Assay. The assay used as the basis of this thesis is the P450-Glo assay from Promega™. This assay was designed to test the effects of drugs and other substances on CYP450 activity. It uses luminogenic CYP450 probe substrates, compatible with luciferase, to measure CYP450 activity. The derivatives used are not substrates for luciferase, but can be

converted to luciferin by CYP450. They are then able to bind with luciferase and produce light. The light produced is directly proportional to the activity of CYP450. If the agent being tested has an effect on CYP450 activity, the amount of light produced will reflect that change.

The assay begins by incubating the luminogenic substrate with active CYP3A4 and a sample to be tested. In our case the substrate was Luciferin 6-benzyl ether (Luciferin-BE). The CYP3A4 converts the substrate into luciferin. The activity of CYP3A4 is then halted and luciferase enzyme is added. Any luciferin present would bind to the luciferase in the indicator and produce luminescence. If the sample being tested inhibits CYP450 activity, there will be proportionally less luciferin after initial incubation, and this would be reflected by a lower luminescence read.

The Promega™ P450-Glo assay was designed to be superior to other CYP450 assays in many ways. The indicator used, Ultra-Glo Luciferase, is stabilized in a proprietary buffer system. The buffer system reduces false positives by stabilizing CYP450 in the assay to any inhibition except by the sample. Ultra-Glo Luciferase has a half-life of greater than two hours, which allows the test to be read without significant loss of luminescence over time, which allows batch samples to be run. It is possible to run an entire 96 well plate without significant loss of luminescence. The assay generates luminescence as opposed to fluorescence, which reduces error from fluorescent reactions between NADPH, CYP450, and any analytes. It is possible to completely test a sample and generate an inhibition curve in less than two hours. This reduces the need to run more time consuming tests such as HPLC, which require up to 30 minutes per sample. The protocol for testing a sample is very consistent. It is not difficult to run hundreds of samples manually. There is little to no luminescence in the background of the assay, which

allows it to be much more sensitive to any variation in CYP450 activity. Finally, all substrates used are easily soluble in aqueous solutions, which allows for easy preparation. (Cali, 2003)

The P450-Glo assay is available in many different human isoforms: CYP1A1, 1A2, 2C8, 2C9, 3A4, 3A7, and 1B1. There are also many rat isoforms available. The primary reason for CYP450 assays is to detect any inhibition by a sample. In order to be valid the assay must show similar inhibition for samples to other CYP450 assays. This assay was shown to produce similar results as other CYP450 assays that used conventional probe substrates (Sai et al., 2000). It was also shown to be more sensitive than florescent CYP450 assays, and to show lower background ratios. Overall, this assay appears to be the optimal method for testing CYP450 inhibitors as it reduces the limitations found in most luminescent and non-optical CYP450 assays (Cali, 2003).

Summary. There are several methods for the detection of ergot alkaloids in non biological samples. However, those methods can be expensive, time consuming, or require intricate knowledge of alkaloid chemistry and enzyme function. There is very little information on the detection of ergot alkaloids within biological samples. The objective of this study was to evaluate the Promega™ P450-Glo assay as a tool for measuring ergot alkaloid concentration both in known concentrations and in bovine biological samples of serum and urine.

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CHAPTER 2

EFFECTS OF ERGOT ALKALOIDS ON IN VITRO CYTOCHROME P450 ACTIVITY

ABSTRACT

This study was conducted to evaluate the ability of the Promega™ P450-Glo assay to detect commercial ergot alkaloids. Effects of ergot alkaloids (n = 6; 0 - 100 μM) [ergotamine (ET), dihydroergotamine (DHET), ergonovine (EN), ergocryptine (ECO), ergocornine (ECR), and pergolide (PER)] were tested on human CYP3A4 using the P450-Glo assay (Promega™ V9800). The experimental design was a randomized complete block, with replicate serving as the block. Each alkaloid by concentration combination was tested in triplicate within three independent replicates. Alkaloids were dissolved in methanol (MeOH), serially diluted, and dried. Alkaloids were incubated with cytochrome P450 (CYP450) and a luminogenic substrate (Luciferin-BE). Cytochrome P450 converted Luciferin-BE to luciferin, a direct substrate for luciferase. Luciferase was added and luminescence of each well in the plate was recorded. With this assay, luminescence is directly proportional to CYP450 activity within the well. Luminescence data was analyzed using mixed procedure to test for the influence of alkaloid and concentration. If F-tests were significant ($P < 0.05$), multiple t-tests were used to separate means. Linear, quadratic, and cubic polynomial contrasts also were tested. As concentrations of ET, DHET, ECO, and ECR increased, P450 activity decreased ($P < 0.05$). Pergolide and ergonovine showed no concentration effect ($P > 0.10$) on enzyme activity. The effects of concentration of ET, DHET, ECO, and ECR on CYP450 activity above zero were not different. Concentration of PER and EN showed no significant polynomial effects. This study demonstrated that the Promega™ P450-Glo assay can quickly and easily measure the effects of commercially available ergot alkaloids on CYP450 activity. Future research is necessary to determine ability of assay to detect alkaloid presence in biological samples.

INTRODUCTION

Lolium arundinaceum, Darbyshire, formerly *Festuca arundinacea*, or tall fescue, is a cool season perennial that is a popular forage (Stuedemann and Hoveland, 1988). It has been documented that consumption of tall fescue infected with the *Neotyphodium coenophialum* fungus leads to many physiological effects, which result in significant economic loss for livestock producers (Paterson et al., 1995). Consumption of endophyte-infected tall fescue is the primary cause of fescue toxicosis in cattle, which is responsible for decreased reproductive performance, feed intake, and weight gain (Strickland et al., 1993). Ergot alkaloids, mycotoxins produced by the ergot fungus, are a significant contributor to this condition. Metabolism of ergot alkaloids is primarily in the liver and gastrointestinal tract by cytochrome P450 enzymes (Oliver, 1997). Likewise, ergot alkaloid concentration has a significant negative impact on hepatic and renal function (Settivari et al., 2006).

Detection of ergot alkaloids in biological samples is difficult, expensive, and slow. One method of measuring alkaloids in cattle is by analyzing grass sample extracts with high performance liquid chromatography (HPLC) for presence of ergovaline (Hill et al., 1993; Rottinghaus et al., 1991; Spiering et al., 2002; Kallenbach et al., 2003). Ergovaline is an ergopeptine and is thought to be a good representation of total ergot alkaloid content. Concentrations of ergovaline exceeding 150 μ g/kg are considered potentially toxic to cattle (Stamm et al., 1994). Additionally alkaloids have been measured using mass spectrometry (Yates et al., 1985). Currently quantification of total ergot alkaloids is done using enzyme-linked immunosorbent assay (ELISA) which has an advantage over HPLC as it can detect total ergot alkaloids, not just ergovaline (Reddick, 1988; Gwinn et al., 1991).

The Promega™ P450-Glo assay directly measures activity of the cytochrome P450 enzyme. In this assay CYP450 converts a luminogenic substrate, Luciferin 6-benzyl ether (Luciferin-BE), into luciferin, which binds to luciferase to produce luminescence. Luminescence produced is directly proportional to activity of the CYP450 enzyme. Ergot alkaloid concentration influences CYP450 activity (Settivari et al., 2006; Moubarak et al., 1998), so any alkaloid present will affect the conversion of Luciferin-BE to luciferin, resulting in a change in luminescence. The objective of this study was to evaluate the Promega™ P450-Glo Assay as a tool for detecting ergot alkaloid presence in a non-biological sample.

MATERIALS AND METHODS

Sample Preparation

Six ergot alkaloids - ergotamine (ET), ergonovine (EN), dihydroergotamine (DHET), ergocornine (ECO), ergocryptine, (ECR), and pergolide (PER) - were dissolved in methanol (100%) resulting in final assay concentrations of 0-100 μ M (Yates and Powell, 1988).

Assay Procedure

Assay was conducted in a 96 well plate. Sample (12.5 μ L) was added in triplicate to the plate and allowed to dry. Wells were rehydrated with potassium phosphate buffer (1 M; 12.5 μ L) and recombinant CYP3A4 solution (12.5 μ L). Reactions were incubated at 20° C for 10 min. Conversion of luciferin-BE was initiated by adding NADPH regeneration system (25 μ L), and reactions were incubated at 20° C for 30 min. Luciferin detection reagent (50 μ L) was added and allowed to incubate for 20 min at 20° C to stabilize luminescent signal. Luminescence (arbitrary units) was determined with a luminometer (Perkin-Elmer, Victor 1420 multilabel counter) with

no filters and an integration time of one sec/well. Method was repeated three times on independent days for each alkaloid by concentration combination.

Statistical Analysis

Luminescence data for each alkaloid were analyzed independently using mixed procedures. Main effects were replicate and alkaloid concentration; response variable was luminescence, as determined by CYP450 activity. If F-tests were significant ($P < 0.05$), multiple t-tests were used to separate means. Linear, quadratic, and cubic polynomial contrasts also were tested. Results were calculated as a percent of CYP450 activity with zero alkaloid present.

RESULTS

Concentration of ET, DHET, ECO, and ECR affected ($P < 0.05$) CYP450 activity. Ergonovine and pergolide showed no significant concentration effect on enzyme activity. Cytochrome P450 activity was reduced with the introduction of ET ($P < 0.01$), but there was no difference in the effect of varying concentration (Figure 2.1). Cytochrome P450 activity was not affected by ergonovine concentration ($P > 0.05$; Figure 2.2). There was a depression in P450 activity when treated with DHET ($P < 0.01$), but there was no concentration effect (Figure 2.3). Presence of ECO caused reduction in P450 activity ($P < 0.01$), but increased concentration had no effect (Figure 2.4). Ergocryptine reduced CYP450 activity ($P < 0.01$), but increased concentration had no effect (Figure 2.5). Pergolide showed no ($P > 0.05$) polynomial effects on P450 activity (Figure 2.6). In a pair wise comparison ET, ECO, and ECR all powerfully inhibited CYP450 activity. Dihydroergotamine moderately inhibited CYP450 with increased concentration, which was unique in our alkaloid sample. Pergolide and ergonovine demonstrated no effect on P450 activity with increased concentration.

DISCUSSION

While there has been much research conducted on detecting ergot alkaloid concentration in a biological sample, the methods yielded are time consuming and expensive (Rottinghaus et al., 1993; Moubarak et al., 1996; Craig et al., 1994; Rottinghaus et al., 1991). Some research has been done using HPLC to detect the presence of ergotoxin in ground and pelleted feeds (Rottinghaus et al., 1993). This method uses a chloroform extraction into methanol and analysis by HPLC to detect the presence of ergot contamination (Rottinghaus et al., 1993; Moubarak et al., 1996). Most work done to quantify ergot alkaloid content is done using endophyte infected plant tissue. This method is similar to above method, but analyzes sample for ergovaline, believed to be a good indicator of total ergot alkaloid contamination. This method is very time consuming. It requires the use of an HPLC machine and highly trained personnel (Craig et al., 1994; Rottinghaus et al., 1991).

In 2001 Schnitzius et al. studied the use of an ELISA method using the 15F3.E5 antibody. This antibody binds well to ergonovine, which had no effect in our study, due to its similarity to lysergic acid and lack of large side groups, but it failed to detect other alkaloids, which makes it less accurate than the HPLC method, but much faster (Schnitzius et al., 2001).

In 2003 Moubarak et al. used liver microsomes prepared from rats treated with dihydroergotamine and ergonovine to convert ergotamine and its isomers to their metabolites. The conversion rate was measured and compared with that of the control group. They found that CYP450 activity was inhibited by both ergonovine and dihydroergotamine treatment groups when compared with the control (Moubarak et al., 2003). Our findings concur with that research with respect to DHET, but we saw no effect by EN. We saw a significant inhibition of CYP450

enzyme activity when treated with dihydroergotamine. Ergonovine did not inhibit CYP450 activity in a non-biological sample.

Settivari et al., in 2007 exposed primary rat hepatocellular cultures to ergovaline and measured hepatic antioxidant enzymes using a spectrophotometric assay. Those rats treated with ergovaline had reduced hepatic CYP450 activity and expression. They concluded that such results could indicate that animals exposed to ergot alkaloids would have increased oxidative stress, which might be responsible for the decrease in hepatocellular proliferation (Settivar et al., 2007). The results of our experiment fit with their findings. The majority of alkaloids tested by the CYP450-Glo assay displayed inhibition of CYP450 activity with increased concentrations. While ergovaline is not a perfect representation of all alkaloids, it is often used as an indicator of alkaloid presence in a sample, and does share many structural similarities to the alkaloids tested in our study. It is based on the ergoline ring structure, is classified as an indole, and is synthetically derived from L-tryptophan, all of which is true of the tested alkaloids. The primary difference in our chosen alkaloids is in their side chains, which affect the level of inhibition of CYP450 activity. So it follows that the samples used in this study would react similarly, but with different levels of effect on CYP450 enzymes.

Detection of ergot alkaloids in cattle grazing tall fescue is time consuming and expensive. Currently the antibody used for the enzyme-linked immunosorbent assay is not commercially available (Cox et al., 1992). Moubarak et al. (1996) showed that it is possible to determine alkaloid concentrations in biological samples after injection of alkaloids, but basal concentrations on fescue grazing individuals were not detected (Moubarak et al., 1996).

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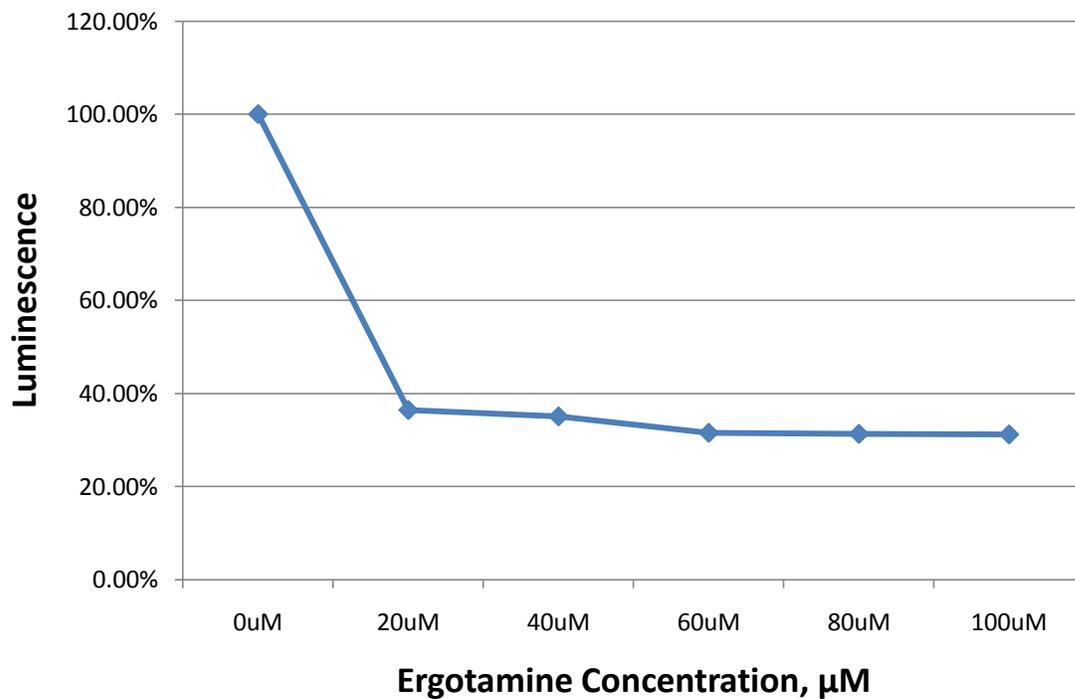


Figure 2.1 Effects of ergotamine concentration on CYP450 activity. CYP450 activity was expressed as a percent of activity with no treatment. Ergotamine (0-100 μM) was incubated with CYP450 in a luminogenic assay. Luminescence was directly proportional to CYP450 activity ($P < 0.01$).

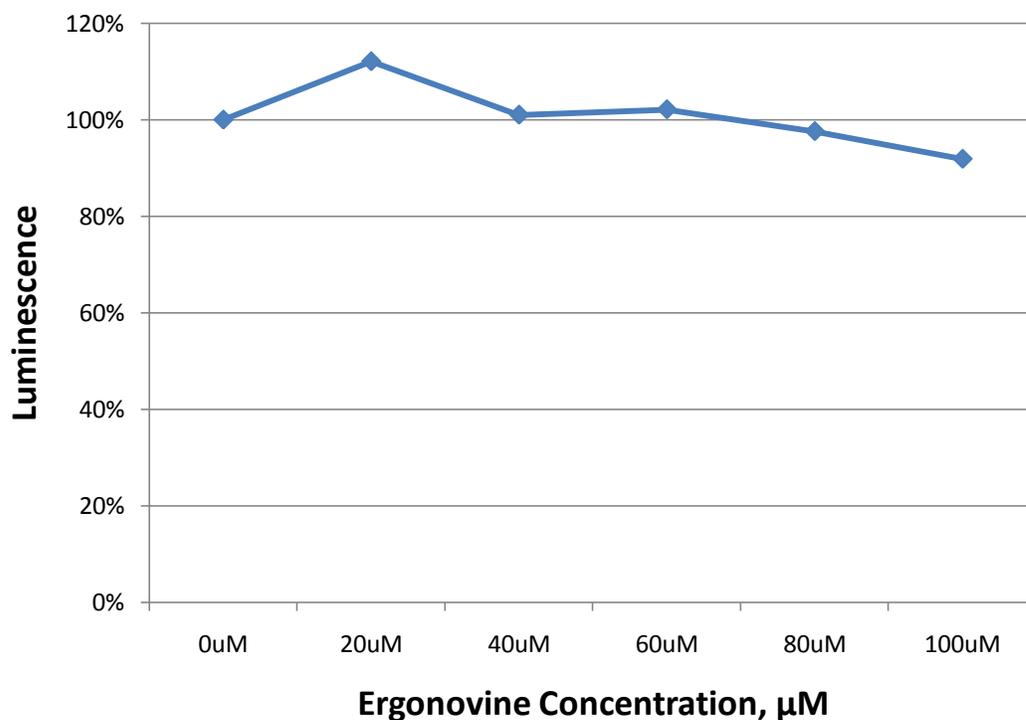


Figure 2.2 Effects of ergonovine concentration on CYP450 activity. CYP450 activity was expressed as a percent of activity with no treatment. Ergonovine (0-100 μM) was incubated with CYP450 in a luminogenic assay. Luminescence was directly proportional to CYP450 activity. Concentration of ergonovine did not affect ($P > 0.05$) CYP450 activity.

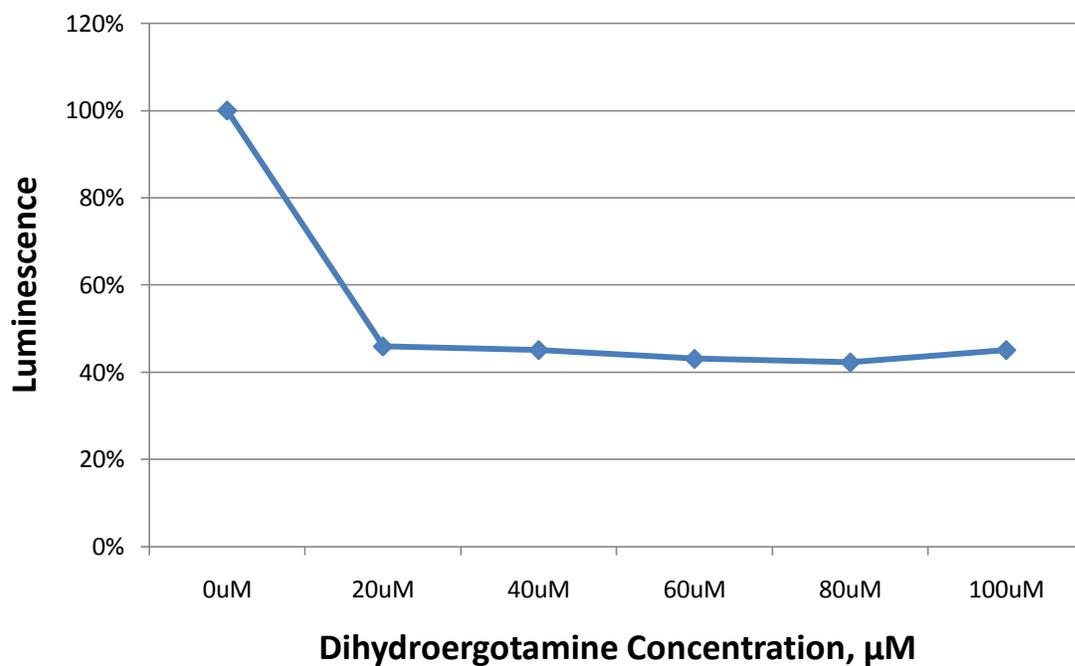


Figure 2.3 Effects of dihydroergotamine concentration on CYP450 activity. CYP450 activity was expressed as a percent of activity with no treatment. Dihydroergotamine (0-100 μM) was incubated with CYP450 in a luminogenic assay. Luminescence was directly proportional to CYP450 activity ($P < 0.01$).

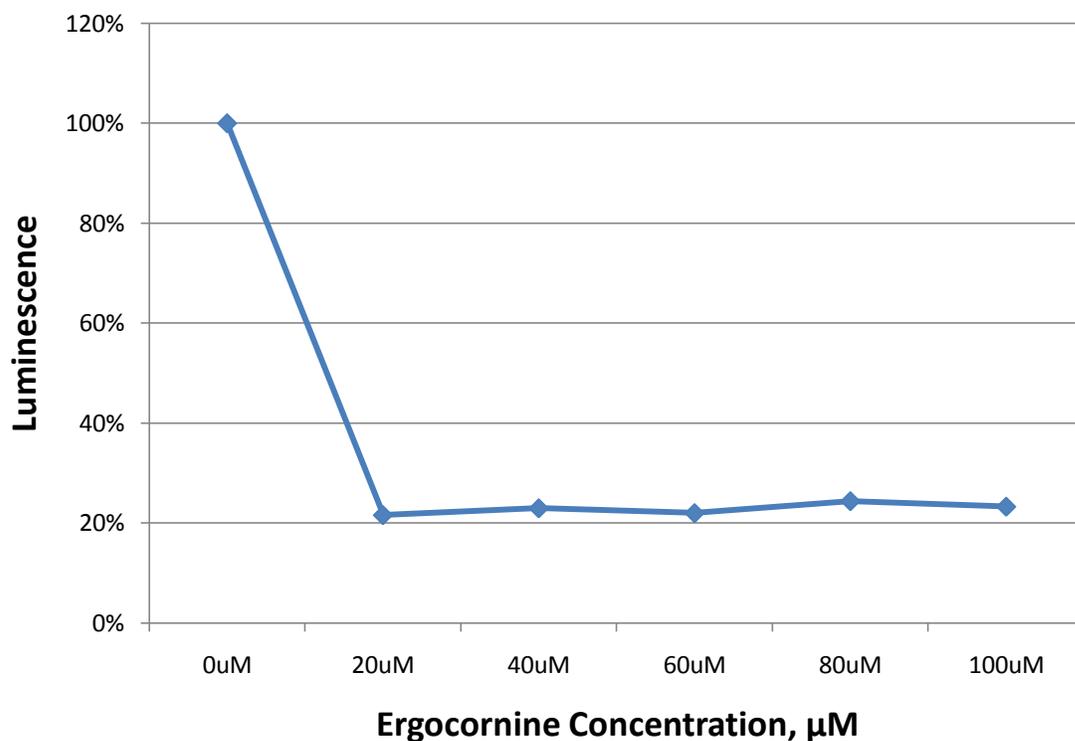


Figure 2.4 Effects of ergocornine concentration on CYP450 activity. CYP450 activity was expressed as a percent of activity with no treatment. Ergocornine (0-100 μM) was incubated with CYP450 in a luminogenic assay. Luminescence was directly proportional to CYP450 activity ($P < 0.01$).

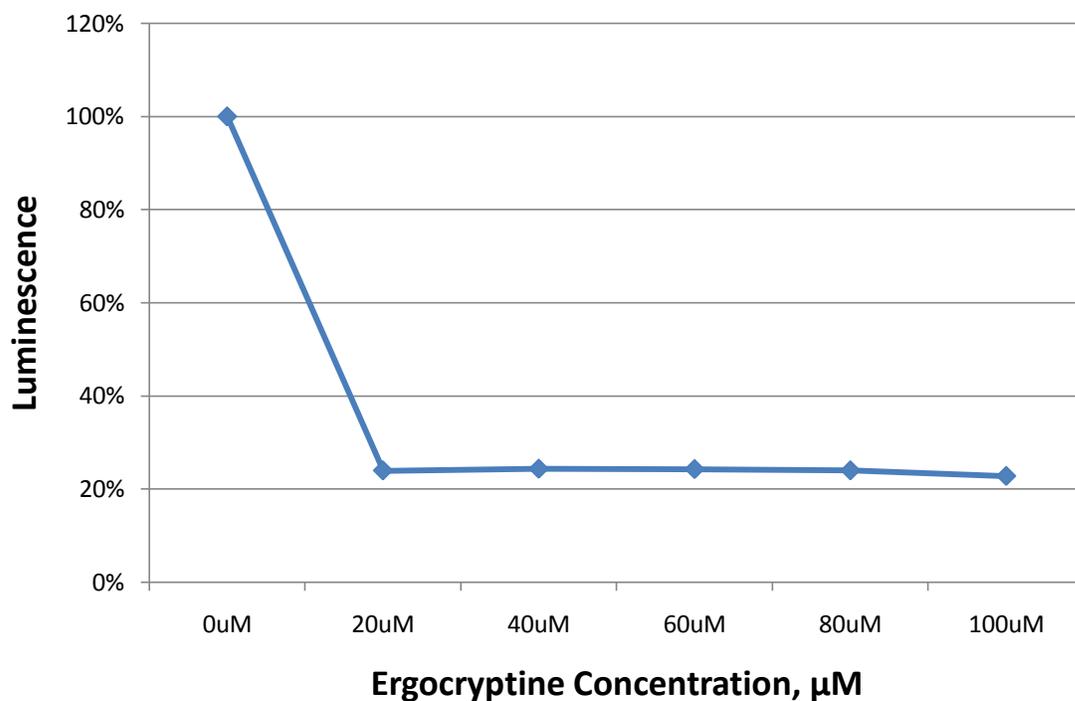


Figure 2.5 Effects of ergocryptine concentration on CYP450 activity. CYP450 activity was expressed as a percent of activity with no treatment. Ergocryptine (0-100 μM) was incubated with CYP450 in a luminogenic assay. Luminescence was directly proportional to CYP450 activity ($P < 0.01$).

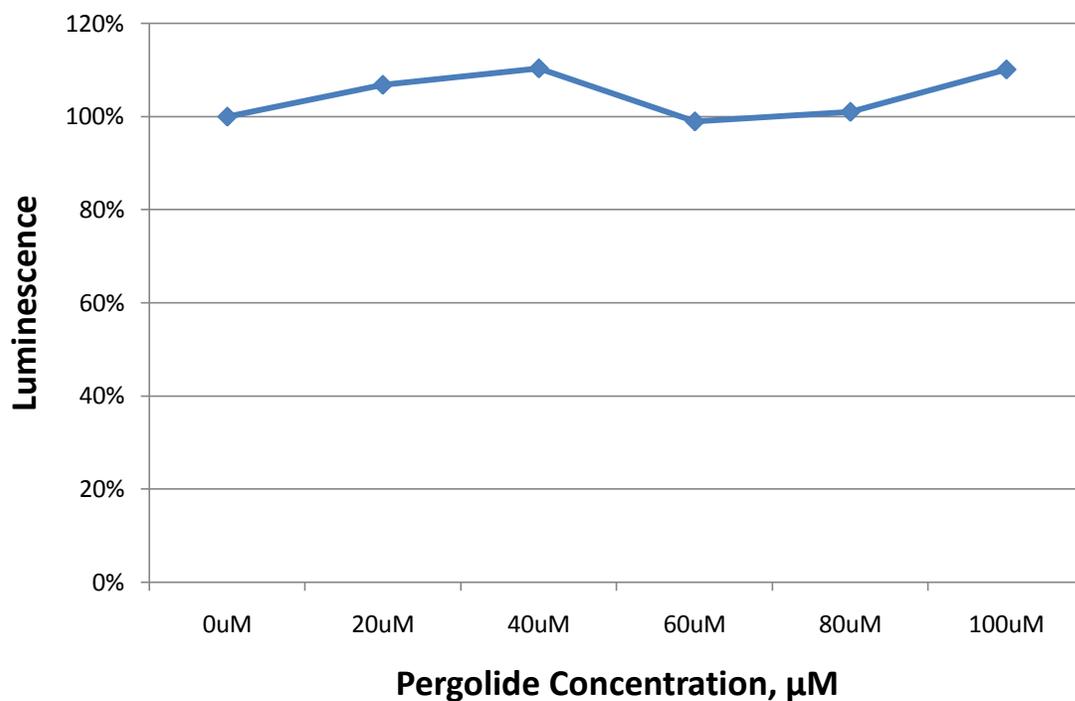


Figure 2.6 Effects of pergolide concentration on CYP450 activity. CYP450 activity was expressed as a percent of activity with no treatment. Pergolide (0-100 μM) was incubated with CYP450 in a luminogenic assay. Luminescence was directly proportional to CYP450 activity. Concentration of pergolide did not affect ($P > 0.05$) CYP450 activity.

CHAPTER 3

EFFECTS OF BOVINE BODILY FLUIDS ON CYTOCHROME P450 ACTIVITY

ABSTRACT

Objective of this study was to quantify the concentration of ergot alkaloids in biological samples. Serum and urine were evaluated in crossbred Angus-sired steers ($n = 39$; 216 ± 2.6 d of age; 203 ± 1.7 kg). Animals were immediately blocked by weight and assigned to graze toxic tall fescue (E+; $n = 4$) or non-toxic tall fescue (HM4; $n = 4$) pastures. Steers grazed tall fescue for 105 d at which time blood and urine samples were collected. Samples were analyzed using the Promega™ P450-Glo assay. Urine was analyzed using enzyme linked immunosorbent assay (ELISA) for total ergot alkaloids. Polymerase chain reaction (PCR) method was used to amplify and genotype DNA at the single nucleotide polymorphism known to code for CYP450. Experimental design was a randomized complete block with main effects of pasture, forage, and genotype with dependent variables of weight, ADG, total ergot alkaloids, and luminescence. Genotype affected ($P < 0.03$) inhibition of CYP450 activity by urine, but forage had no effect on inhibition of CYP450 activity ($P > 0.1$). Inhibition of CYP450 activity by urine when corrected for creatinine concentration was correlated ($r = -0.50$; $P < 0.025$) with total ergot alkaloid concentration. Inhibition of CYP450 activity by serum was not significantly correlated with alkaloid concentration as determined by ELISA, but it was negatively correlated with average daily gain ($r = -0.43$; $P < 0.05$) and starting weight ($r = 0.41$; $P < 0.05$). This study demonstrated that it is possible to detect presence of ergot alkaloids in biological samples quickly and efficiently using the Promega™ P450-Glo assay. In the future it may be possible to utilize this assay to quantify many substances that inhibit CYP450 within biological samples.

INTRODUCTION

Tall fescue (*Lolium arundinaceum*, Darbyshire) infected with the endophytic fungus (*Neotyphodium coenophialum*) is responsible for the production of ergot alkaloids which have a significant economic impact on beef cattle (Porter, 1995; West, 2000). Consumption of these mycotoxins is known to lower average daily gain, negatively impact reproductive traits, decrease parasite resistance, and reduce heat tolerance, all of which drive up the cost of production (Filipov, 1999). These ergot alkaloids are degraded through hydroxylation performed by the cytochrome P450 (CYP) enzymes (Althaus et al., 2000). Ergot alkaloids are likewise known to inhibit enzyme activity (Settivari et al., 2006).

This study used the Promega™ P450-Glo assay. It employs human CYP3A4 to convert a luminogenic substrate into luciferin, which binds with luciferase to produce luminescence. The amount of light produced by the assay is directly proportional to the activity of CYP3A4. If the sample contains anything that inhibits enzyme activity, the conversion of the luminogenic substrate to luciferin by CYP3A4 will not be complete, yielding less luminescence. It is then possible to quantify the presence of enzyme inhibiting substance in the sample, believed to be caused by the presence of ergot alkaloids.

Current methods for detecting ergot alkaloid concentrations in bodily fluids are limited. High pressure liquid chromatography (HPLC), which requires a highly trained technician to operate the equipment (Yates and Powell, 1988; Rottinghaus et al., 1991; Moubarak et al., 1996). Enzyme linked immunosorbent assay is a faster method with proprietary reagents that is limited in selectivity (Shelby and Kelley, 1990; Hill and Agee, 1994). The Promega™ P450-Glo assay is quick and efficient and follows an easy to understand protocol. This makes it a much more convenient tool for data collection.

It has been difficult to develop strategies to improve symptoms of fescue toxicosis because many of the mechanisms of action are still not proven. However, the ability to measure concentrations of alkaloids in biological samples will help to explain these systems. For example, ELISA was used to test bovine urine for ergot alkaloid concentration on steers (Stuedemann et al., 1998) that had grazed either endophyte infected or endophyte free tall fescue. They found that 96% of the ergot alkaloids consumed were excreted in the urine. The objective of this study was to quantify ergot alkaloids in bovine urine and serum and validate results with those found using the ELISA method.

MATERIALS AND METHODS

Sample collection

The University of Arkansas Animal Care and Use Committee approved all animal procedures (protocol # 04024). Angus-sired crossbreed steers ($n = 39$; 203 ± 1.7 kg; 216 ± 2.6 d) were assigned a random pasture of either Kentucky 31 (K31) or HiMag4 (HM4) and supplemented with 1.8 kg/d of a corn:soybean meal (80% ground corn: 20% soy-bean meal; 12% CP) ration for 105 d. At d 105, each animal was weighed, a blood sample was taken from the jugular vein, and a urine sample was collected in chute. Approximately 10 mL of blood was extracted from the animal for serum (Vacutainer™, BD, Franklin Lakes, NJ). All blood samples were stored at 5° C until centrifuged at 1200 x g, serum decanted, and stored at -20° C. Serum was diluted to 10% with biological saline (0.85% NaCl).

CYP450 Analysis

Assay was conducted using methods previously described (Moubarak et al., 2012). Briefly, sample (12.5 μ L) was added in triplicate to plate, followed by CYP3A4 solution (12.5

μL), and incubated at 20° C for 10 min. Reaction was initiated by addition of NADPH regeneration system (25 μL) and plate was incubated 30 min at 20° C. Luciferin Detection Reagent (50 μL) was added and plate was incubated at 20° C for 20 min. Luminescence was recorded with a luminometer (Perkin-Elmer, Victor 1420 multilabel counter) using no filters and an integration time of 1 sec/well.

Statistical Analysis

Results of enzyme assay were reported in luminescence (arbitrary units). Pearson correlation coefficients were determined between dependent variables. Data were analyzed using mixed procedure of SAS (SAS Institute Inc., 2000-2004) with pasture as the experimental unit, genotype within pasture as random term, fescue cultivar as repeated, and creatinine as a covariate. If F-test for main or interactive effects were significant ($P < 0.05$) then means were separated using multiple t-tests.

RESULTS

Table 3.1 presents the mean, standard deviation, minimum, and maximum for each variable associated with the steers. Steers grew at approximately 0.63 kg/d. Table 3.2 contains Pearson correlation coefficients of all variables tested. Cytochrome P450 activity, creatinine, and alkaloid concentration were analyzed at d 105, before the steers were moved from pasture. Inhibition of CYP450 activity by urine was correlated with total ergot alkaloid concentration ($r = -0.50$; $P < 0.025$). Inhibition of CYP450 activity by urine also tended to be correlated ($r = -0.26$; $P < 0.10$) with ADG. Inhibition of CYP450 activity by serum was correlated ($r = -0.43$; $P < 0.03$) with ADG. There was no correlation between inhibition of CYP450 activity when treated with serum and alkaloid concentration found by ELISA.

Figure 3.1 displays the mean inhibition of CYP450 activity by urine, by genotype of the animal, single nucleotide polymorphism CYP450 coding sequence. Genotype affected ($P < 0.03$) inhibition of CYP450 activity by urine. Figure 3.2 presents the means of inhibition of CYP450 activity by urine sorted by both genotype and forage. The effect of urine on CYP450 activity tended ($P < 0.055$) toward a forage by genotype interaction. Serum tested in the Promega™ P450-Glo assay was not affected by forage type or genotype.

DISCUSSION

The Promega™ P450-Glo assay was able to detect inhibition of CYP450 activity due to treatment with both bovine urine and serum. Genotype of the animal at a single nucleotide polymorphism known to be within the CYP450 coding sequence affected inhibition of CYP450 activity by urine. Inhibition of CYP450 activity by urine was highly correlated with the concentration of ergot alkaloids when creatinine was used to correct for urine volume. Creatinine is a direct indicator of urine volume, so correcting for creatinine removes error caused by dehydration. Inhibition of CYP450 activity by serum was not affected by either forage or genotype, and was not correlated with ergot alkaloid concentration. This may be because alkaloid concentration was tested using an ELISA of the urine. Alkaloid concentration may vary between bodily fluids by some mechanisms not yet understood. It is possible the alkaloids exist in the serum in a bound state, which would limit their bioavailability to inhibit the CYP450 enzyme.

Little is known about the bioavailability of ergot alkaloids in biological fluids of animals grazing on ergot infected tall fescue. This reduces the ability to discover new methods to ameliorate the toxicosis. In 1998, Stuedemann et al tested urine and biliary samples using

competitive ELISA of cattle grazing E+ and E- tall fescue. They discovered that 96% of ergot alkaloids were excreted through the urine. The animals were then moved to new pastures and samples were collected and tested again at days 0, 2, 5, and 7. Animals were finally returned to their original pastures and samples were again collected on days 0, 2, 5, and 7. Those animal switched from E+ to E- had similar alkaloid levels as those grazing only E- after two days. This means that it takes more than two days to clear ergot alkaloids from the body (Stuedemann et al., 1998).

Forage had no significant effect on inhibition of CYP450 activity by urine. This is surprising because animals grazing K31 pastures likely ingested a much higher concentration of ergot alkaloids than those grazing HM4 pastures. Animals were supplemented with 1.8 kg a day, which diluted the effect of forage. This would reduce total alkaloids available to inhibit the enzyme system. However, the results of the ELISA indicate alkaloid presence.

There are few methods available for quantifying ergot alkaloids in biological samples. The method used as comparison for this study is the ELISA method for detection of total ergot alkaloids (Shelby and Kelley, 1990, 1992; Hill and Agee, 1994; Schnitzius et al., 2001). Stuedemann et al. (1998) successfully quantified ergot alkaloid concentration in urine using the ELISA method. They found that 96% of all ergot alkaloids consumed were excreted through the urine. We analyzed urine from steers in our study using the same methods and found correlation ($r = -0.50$; $P < 0.03$) between the ELISA method and our glo assay. The ELISA method is currently considered a rapid analysis of total ergot alkaloids. The Promega™ P450-Glo assay can analyze samples in a fraction of the time and cost. Enzyme linked immunosorbent assay quantification of total ergot alkaloids lacks selectivity as it does not differentiate between ergot alkaloids. The Promega™ P450-Glo assay also lacks selectivity as any alkaloids that will inhibit

CYP450 enzymes will be detected. However, the antibody used for ELISA is specific for the lysergic acid moiety (Hill and Agee, 1994), meaning any precursor molecules present, such as any clavines, will not be detected using ELISA, but they will be detected in the Promega™ P450-Glo assay if they inhibit CYP450.

Another method used for detection of ergot alkaloids in biological samples is HPLC separation followed by fluorescent detection (Yates and Powell, 1988). This method is slower than ELISA, but is much more selective as it is possible to differentiate between individual alkaloids and their metabolites. However, this method is limited to detection of known ergot alkaloids, rather than the discovery of new and unknown molecules because it requires the use of a known sample as a standard to compare results.

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Table 3.1 Least-squares means (SE) of traits for crossbred steers.

VARIABLE ¹	N	MEAN	STD DEV	MIN	MAX
CYP450 Activity when Treated with Serum (%)	39	24	5.5	15	39
CYP450 Activity when Treated with Urine (%)	39	4	2.7	0.8	11
Total Alkaloids (ng/L)	39	98	76	1.6	214
Creatinine (mg/dL)	39	38	21	4.4	78
Alkaloids/CRT (ng/mg)	39	331	326	23	1363
ADG (kg)	39	4.4	0.8	1.7	5.9
Starting Weight (kg)	39	203	27	150	249
Ending Weight (kg)	39	258	34	188	324

¹Measurements are effects of serum on CYP450 expressed as percent of control (CYP450 Activity when Treated with Serum), effects of urine on CYP450 expressed as percent of control (CYP450 Activity when Treated with Urine), total ergot alkaloids (Total Alkaloids), creatinine (Creatinine), total ergot alkaloids corrected for creatinine (Alkaloids/CRT), average daily gain (ADG), weight at day 0 (Starting Weight), and weight at day 105 (Ending Weight).

Table 3.2 Pearson correlation coefficients among traits of crossbred steers.

Variable ¹	CYP450 Activity when Treated with Serum (%)	CYP450 Activity when Treated with Urine (%)	Total Alkaloids (ng/g)	Creatinine (mg/dL)	Alkaloids/CRT (ng/mg)	ADG (kg)	SW (kg)
CYP450 Activity when Treated with Serum	-	-0.22	0.15	0.13	0.1	-0.43*	0.41*
CYP450 Activity when Treated with Urine		-	0.05	0.29†	-0.50*	-0.26†	0.03
Total Alkaloids			-	0.21	0.65**	-0.22	0.15
Creatinine				-	-0.40**	-0.18	0.18
Alkaloids/CRT					-	-0.15	0.04
ADG						-	-0.54**
SW							-

¹Measurements are effects of serum on CYP450 expressed as percent of control (CYP450 Activity when Treated with Serum), effects of urine on CYP450 expressed as percent of control (CYP450 Activity when Treated with Urine), total ergot alkaloids (Total Alkaloids), creatinine, total ergot alkaloids corrected for creatinine (Alkaloids/CRT), average daily gain (ADG), weight at day 0 (SW).

**P < 0.01

*P < 0.05

†P < 0.1

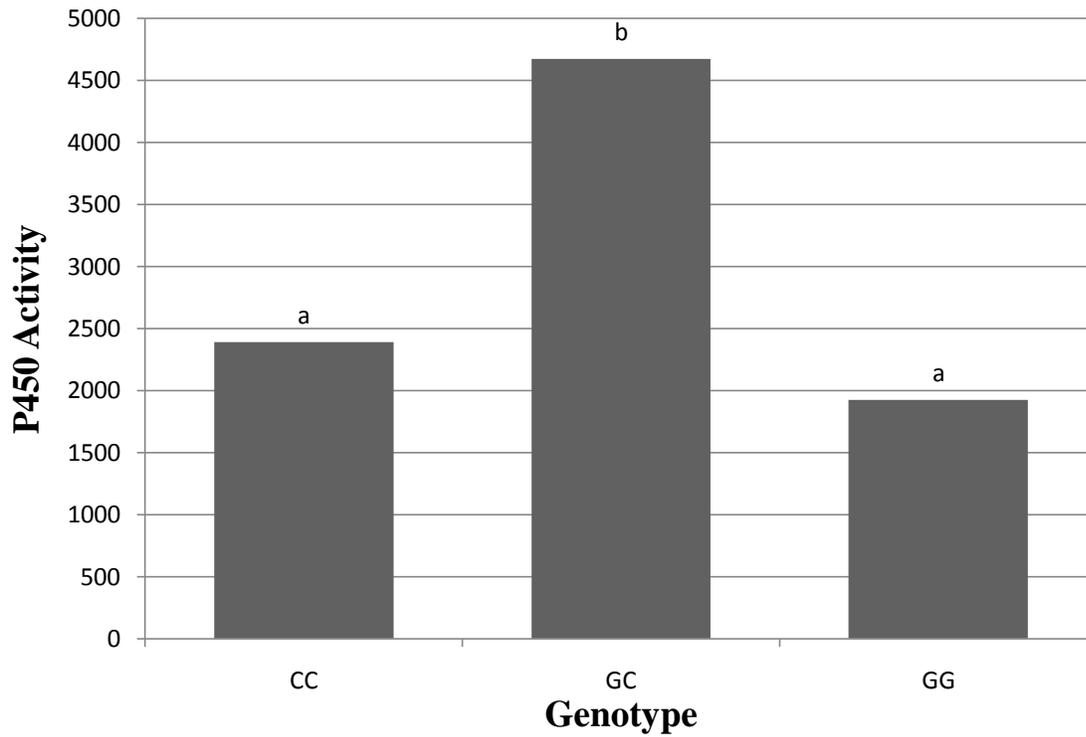


Figure 3.1 Effects of bovine urine on CYP450 activity sorted by CYP450 genotype. Urine was incubated with CYP450 in a luminogenic assay. Luminescence was directly proportional to CYP450 activity. Genotype affected ($P < 0.03$) inhibition of CYP450 activity by urine.

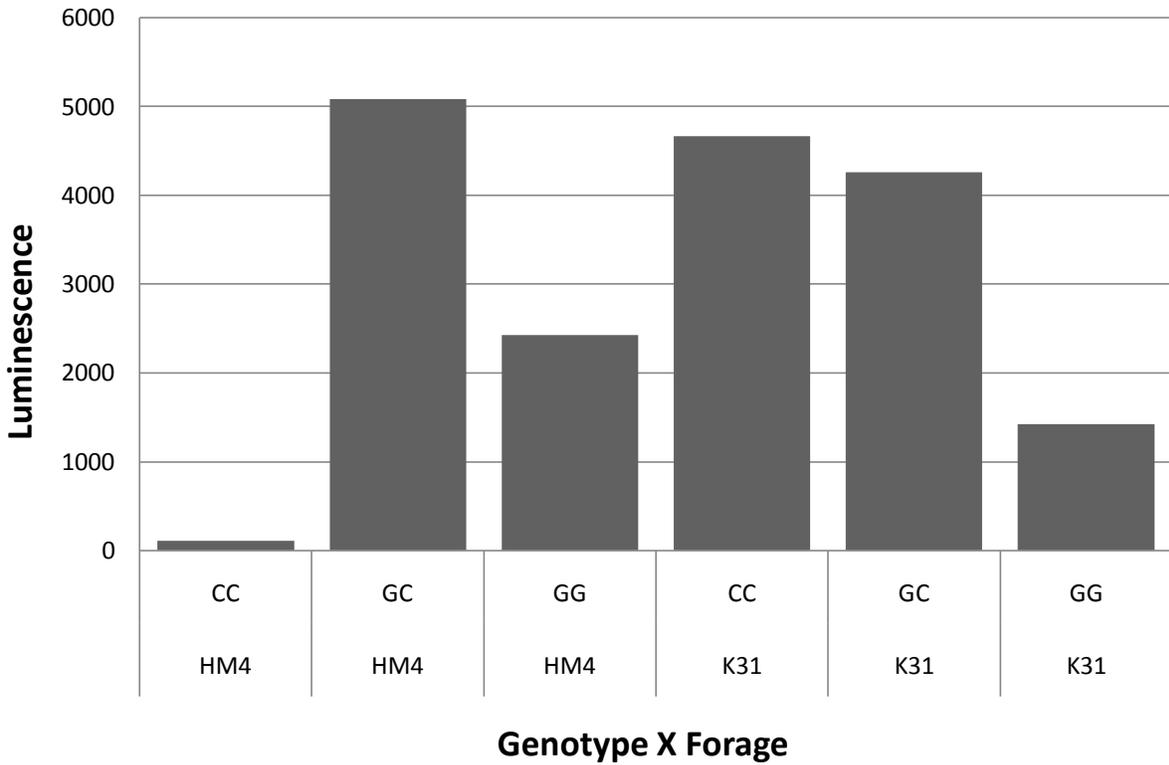


Figure 3.2 Effects of bovine urine on CYP450 activity sorted by forage and CYP450 genotype. Distribution was GG: n=13, GC: n=20, CC: n=6. Urine was incubated with CYP450 in a luminogenic assay. Luminescence was directly proportional to CYP450 activity. There was a trend of an interaction ($P < 0.055$) between forage and genotype.

CONCLUSION

The studies found in this thesis have all been conducted to evaluate the ability of the Promega™ P450-Glo assay to detect the presence of ergot alkaloids in both commercial and biological samples. We have shown that this assay can detect commercial ergot alkaloids, and exhibit a differential response to different alkaloids and concentrations. Ergotamine, ergonovine, dihydroergotamine, ergocornine, and ergocryptine were all shown to inhibit activity of CYP450 with increasing concentrations. Ergonovine had a unique pattern and reduction rate. Pergolide showed no concentration effect on enzyme activity. We have also demonstrated that the Promega™ P450-Glo assay can be used to detect ergot alkaloids within bovine bodily fluids. Both urine and blood had inhibiting effects on CYP450 activity. Inhibition of CYP450 by urine was highly correlated with total ergot alkaloid content found by enzyme linked immunosorbent assay. It may be possible to utilize the Promega™ P450-Glo assay as a tool for quantifying ergot alkaloid concentration in biological samples, providing new possibilities for research in this field.